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RESEARCH ARTICLE

Genotoxic assessment and toxicity evaluation of peginesatide in CByB6F1 hybrid mice

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Abstract

Peginesatide is a PEGylated, investigational, peptide-based erythropoiesis-stimulating agent (ESA) that was designed and engineered to stimulate specifically the erythropoietin receptor dimer that governs erythropoiesis. Clinical use of peginesatide is anticipated to result in chronic dosing in chronic kidney disease (CKD) patients, and the nonclinical data to support development should include an evaluation of carcinogenic potential evaluation. Peginesatide was not mutagenic or clastogenic in a standard genotoxicity battery of tests. Doses for a rasH2 transgenic mouse carcinogenicity assay were defined in a 28-day study in the wild-type littermates of the rasH2 transgenic mouse strain, using intravenous doses of 1–25 mg/kg on days 1 and 22. The findings were consistent with exaggerated pharmacology, including polycythemia, with associated increases in hemoglobin level and extramedullary hematopoiesis and bone marrow hypercellularity.

Keywords: Erythropoiesis-stimulating agents, genotoxicity, toxicology, pharmacokinetics, safety

Introduction

Anemia, due to inadequate renal erythropoietin (EPO) production, is a common complication in patients with chronic kidney disease (CKD) (Weiss and Goodnough, 2005). EPO is a glycoprotein hormone, which regulates erythropoiesis (Fisher, 2003) by binding to specific receptors on the cell surface of red blood cell (RBC) precursors in the bone marrow, thereby promoting their proliferation, differentiation, and survival. Recombinant human EPO and EPO-related drugs, termed erythropoiesis-stimulating agents (ESAs), have become the standard care for the treatment of anemia in CKD patients (MacDougall, 2008). Commercially available ESAs in the United States require frequent administration (i.e., three times weekly to once every 2 weeks). An ESA that can be administered less frequently (e.g., once-monthly) may reduce treatment practice costs (Schiller et al., 2008).

Hematide™/peginesatide, a synthetic, peptidic ESA attached to polyethylene glycol, is currently in phase III clinical trials for the treatment of anemia associated with CKD in both dialysis and nondialysis patients. Peginesatide binds to and stimulates the EPO receptor, resulting in erythroid progenitor cell proliferation and differentiation (Fan et al., 2006). Although peginesatide has the erythropoietic activity characteristic of naturally occurring EPO, its amino acid sequence is unrelated to EPO, and it does not possess structural alerts. Peginesatide has a prolonged duration of action that may allow for once-monthly dosing in patients (Macdougall et al., 2008). Because anemia in CKD patients is a chronic disorder, the duration of patient treatment with peginesatide would be chronic. Regulatory agency requirements for pharmaceuticals that are administered continuously for 6 months or longer include assessment of the carcinogenic potential of the compound (ICH S1B: *Testing for Carcinogenicity of Pharmaceuticals*). An integral component of the evaluation of the carcinogenic potential of a drug is genotoxicity testing (ICH S2A: *Specific Aspects of*

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Regulatory Genotoxicity Tests for Pharmaceuticals; ICH S2B: *Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals*). In contrast, genetic toxicology studies are not warranted for biologic pharmaceuticals, as they are thought not internalized by cells, thereby avoiding mutagenic/DNA damage.

Historically, the strategy for defining the potential for drug-induced carcinogenicity comprised a long-term (e.g., lifetime) study in two rodent species, typically the rat and the mouse. Increased understanding of the mechanisms leading to carcinogenesis, coupled with the ability to generate transgenic animals carrying human proto-oncogenes, has provided an alternative to the conduct of two long-term carcinogenicity studies. Specifically, a 6-month murine transgenic bioassay to complement the conventional 2-year rat bioassay is now considered an acceptable approach to the assessment of a drug's carcinogenic potential (ICH S1B: *Testing for Carcinogenicity of Pharmaceuticals*; Morton et al., 2002).

Proto-oncogenes are normal genes that, upon activation (e.g., become an oncogene), are associated with the development of many human and rodent cancers and are highly conserved across species. The rasH2 transgenic mouse is hemizygous for the human c-Ha-ras proto-oncogene carrying both the human c-Ha-ras proto-oncogene, in addition to the endogenous murine ras proto-oncogene (Tamaoki, 2001). The gene is involved in the regulation of cell growth, proliferation, and malignant transformation and is expressed in over 50% of all cancers (Pritchard et al., 2003). The rasH2 transgenic mouse assay is an acceptable alternative to the traditional 2-year mouse carcinogenicity assay, as described in the ICH S1B document, *Testing for Carcinogenicity of Pharmaceuticals*.

The aims of the studies described below were to evaluate the genotoxicity of peginesatide and to provide data for the subsequent design of a pivotal 6-month rasH2 transgenic study, based on a safety and PK study in the wild-type littermates of the rasH2 transgenic mouse strain, CByB6F1.

Methods

This study was conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and all animals received care recommended in *The Guide for the Care and Use of Laboratory Animals* (1996).

Genotoxicity studies

Bacterial reverse-mutation assay

Salmonella typhimurium tester strains TA98, TA100, TA1535, and TA1537 (Discovery Partners International, San Diego, California, USA) and *Escherichia coli* tester strain WP2 *uvr*A (National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland), in the presence and absence of Aroclor-induced rat-liver S9, were used. The assay was performed by using the plate-incorporation method (Maron and Ames, 1983). As mutagenic activity, formulation precipitation, and appreciable cytotoxicity were not observed with peginesatide in a preliminary experiment (data not included in this article), the maximum concentration level tested in the definitive trial was 5,000 µg/plate. The maximum concentration is consistent with recommendations outlined in ICH S2A, *Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals*. The plates were incubated with peginesatide at concentration levels of 0, 75, 200, 600, 1,800, and 5,000 µg/plate at 37°C for 48–72 hours. Positive controls were included with and without metabolic activation, as detailed in Table 1.

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated. For the test article to be evaluated as positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test article. Tester strains TA1535 and TA1537 were judged positive if the increase in mean revertants was equal to or greater than 3.0 times the mean vehicle control value. Tester strains TA98, TA100, and WP2 *uvr*A were judged positive if the increase in mean revertants was equal to or greater than 2.0 times the mean vehicle control value. In addition, plates were visually assessed for the presence of drug precipitation and cytotoxicity. Cytotoxicity was defined based on reductions in the bacterial lawn.

In vitro *mammalian chromosome aberration test*

Chinese hamster ovary (CHO) cells were incubated (i.e., duplicate cultures) with peginesatide, in both the absence and presence of an Aroclor-induced S9 metabolic activation system, using an established procedure (Evans, 1976; Swierenga et al., 1991). The vehicle used to formulate peginesatide was water. Positive and solvent (water) controls were included in each treatment condition in duplicate cultures. Mitomycin C (1 and 2 µg/mL) and cyclophosphamide (10 µg/mL) were used as the positive controls in the nonactivated and the S9-activated test systems, respectively. The cells were treated for 4 and 20 hours in the nonactivated test system and for 4 hours in the S9 metabolic-activated test system. All cells were harvested 20 hours after initiation of the treatment. Due to the absence of peginesatide precipitation in the treatment medium and minimal cytotoxicity to the CHO cells, the highest concentration level evaluated for chromosome aberrations (5 mg/mL) was selected to be consistent with ICH guidance S2A *Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals*. Two additional lower concentration levels (1,250 and 2,500 µg/mL) were also included in the evaluation. Statistical analysis of the percent aberrant cells in the peginesatide-treated cells, compared to respective water control cells, was performed using Fisher's exact test. For the test article to

Table 1. Number of revertants in *Salmonella typhimurium* and *Escherichia coli* following *in vitro* exposure to peginesatide in the absence and presence of a metabolic-activating enzyme (S9).

Dose (μg/plate)	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
Liver microsomes: none					
Vehicle	18 ± 3	136 ± 13	20 ± 1	5 ± 3	20 ± 3
75	16 ± 3	123 ± 15	18 ± 3	5 ± 2	19 ± 6
200	23 ± 6	149 ± 6	21 ± 6	8 ± 4	21 ± 2
600	22 ± 3	131 ± 19	17 ± 3	6 ± 1	20 ± 3
1,800	18 ± 5	141 ± 7	21 ± 7	6 ± 2	21 ± 5
5,000	18 ± 1	130 ± 4	19 ± 1	8 ± 2	18 ± 1
2-nitrofluorene (1)	101 ± 7				
sodium azide (1)		594 ± 8	204 ± 24		
9-aminoacridine (75)				406 ± 48	
methyl methanesulfonate (1,000)					72 ± 5
Liver microsomes: rat-liver S9					
Vehicle	33 ± 3	140 ± 4	11 ± 4	8 ± 2	24 ± 5
75	29 ± 2	173 ± 13	14 ± 2	5 ± 1	24 ± 4
200	31 ± 4	165 ± 14	13 ± 5	3 ± 0	20 ± 2
600	29 ± 3	144 ± 15	15 ± 2	5 ± 2	23 ± 4
1,800	32 ± 3	171 ± 15	17 ± 3	6 ± 0	20 ± 3
5,000	34 ± 5	161 ± 26	21 ± 3	5 ± 3	20 ± 2
2-aminoanthracene (1)	1,363 ± 27	1,381 ± 143	122 ± 7	140 ± 46	
2-aminoanthracene (10)					704 ± 71

Average number of revertants per plate ± standard deviation ($n = 3$).

be evaluated as positive, it must cause a dose-related increase in the percentage of cells with aberrations, with one or more concentrations being statistically significant, and must exceed the range of historic solvent controls.

Bone marrow erythrocyte micronucleus assay

The micronucleus study was conducted from using an established procedure that allows the identification of drug-induced micronucleated polychromatic erythrocytes in the bone marrow of ICR (imprinting control region) mice (Hayashi et al., 1994). Peginesatide does not exhibit any acute toxicity up to a maximum dose of 2,000 mg/kg, with drug-related effects apparently limited to activity at the EPO receptor (unpublished data). Consequently, the maximum dose tested was 2,000 mg/kg, which is in accord with OECD guidelines (OECD Guideline 474: *Genetic Toxicology: Mammalian Erythrocytes Micronucleus Test*). Mice were assigned to five treatment groups. Five male and 5 female mice each received either a low (500 mg/kg) or mid-peginesatide dose (1,000 mg/kg), 10 males and 10 females received a high peginesatide dose (2,000 mg/kg), 10 males and 10 females received the negative control article (water), and 5 males and 5 females received the positive control article (50 mg/kg of cyclophosphamide). The peginesatide doses were achieved via two subsequent intraperitoneal (i.p.) administrations, separated by approximately 3 hours. Total peginesatide doses were achieved using solutions at concentrations of 12.5, 25, and 50 mg/mL and a dose volume of 20 mL/kg/treatment. Five male and 5 female mice treated with the negative or positive control article or peginesatide at the low, mid, or high doses were euthanized 24 hours after dosing, and 5 remaining animals per sex in the negative control and high-peginesatide-dose groups were euthanized 48 hours after dosing. At the time of euthanasia, femoral bone marrow was collected, then smears (i.e., slides) were prepared and stained with May-Gruenwald-Giemsa stain.

Bone marrow cells, 2,000 polychromatic erythrocytes (PCEs) per each animal, were examined microscopically for the presence of micronucleated polychromatic erythrocytes (MPCEs). In addition, the ratio of PCE to total erythrocyte (PCE:EC ratio) was calculated and used as a parameter of bone marrow bioavailability and cytotoxicity. The statistical analysis of data was performed from using the Kastenbaum-Bowman tables, which are based on the binomial distribution for a significance level of $P < 0.05$ (Kastenbaum and Bowman, 1970). The test article was judged negative if no statistically significant increase in the incidence of MPCEs above the concurrent vehicle control and no evidence of dose-related increase were observed at any sampling time.

28-day i.v. toxicity study

Wild-type CByB6F1 hybrid littermate mice of the transgenic CB6F1/Jic-TgN(RasH2)@Tac strain were obtained from Taconic Farms (Germantown, New York, USA). The CByB6F1 mouse strain was used for defining the doses and dosing regimen for the subsequent 6-month carcinogenicity study in the rasH2 transgenic mouse because it is the genetic background for the CB6F1/Jic-TgN(RasH2)@Tac mouse. The CB6F1/Jic-TgN(RasH2)@Tac mouse is a hemizygous knock-in mouse carrying the human prototype c-Ha-ras gene, with its own promoter/enhancer (C57BL/6J-Tg.rasH2 × BALB/cByJ; Tamaoki,

2001). At initiation of dosing, the mice were 7–8 weeks of age, weighing 20.4–29.5 and 16.0–23.3 g for males and females, respectively.

Peginesatide or vehicle was administered to CByB6F1 hybrid mice (N=10/sex/group) by intravenous (i.v.) injection into the tail vein once every three weeks (days 1 and 22), for a total of two doses (Table 2). Mice were sacrificed on day 29. Peginesatide was administered at 0, 0.1, 1.0, 10, or 25 mg/kg at a dose volume of 5 mL/kg, formulated in vehicle comprising 20 mM of phosphate and 0.003% Tween 20 in 4.7% sorbitol (pH 6.0). A separate cohort of animals was assigned to the pharmacokinetic (PK) groups (Table 2) and administered a single i.v. dose of either vehicle or peginesatide at 0.1, 1, or 10 mg/kg (N=3/time point).

Toxicity was assessed in the safety-group animals based on mortality and clinical observations evaluated twice-daily, body weights and food consumption recorded weekly, and clinical pathology (i.e., hematology and serum chemistry), gross necropsy, organ weights, and histopathology evaluated on day 29. Data are expressed as the mean with standard deviation (SD). Comparisons of parameters were performed using a one-way analysis of variance (ANOVA), followed by a post-hoc Dunnett's test. A P-value of <0.05 was considered statistically significant.

PK parameters were derived from drug-plasma concentrations obtained at six time points on day 1. Different blood-sampling time points were used for the dosing groups (Table 2) in order to collect sufficient data to accurately define peginesatide exposure while using a minimum number of animals. Data for males only are presented here, as there were no gender PK differences. The time points were determined based on data obtained in a separate safety/pharmacokinetic study in mice (Woodburn et al., 2010). Plasma drug-concentration levels were determined from using a competition enzyme-linked immunosorbent assay (ELISA), as previously described (Fan et al., 2006; Stead et al., 2006). The lower quantification limit was 0.025 µg/mL. Noncompartmental PK parameters were calculated from plasma concentration-time profiles of peginesatide, using WinNonlin® software (version 5.1, Pharsight, Mountain View, California, USA).

Table 2. Experimental design.

	Number of animals		
	Safety group		PK group
Treatment groups	Male	Female	Male
Group 1: vehicle-control	10	10	3
Group 2: low dose (0.1 mg/kg)	10	10	18
Group 3: mid-low dose (1 mg/kg)	10	10	18
Group 4: mid-high dose (10 mg/kg)	10	10	18
Group 5: high dose (25 mg/kg)	10	10	NA

PK bleeds (3 mice/time point): group 1: at 1 hour postdose; group 2: predose and at 0.25, 1, 8, 24, and 48 hours postdose; group 3: predose and at 0.25, 8, 24, 48, and 72 hours postdose; group 4: predose and at 1, 24, 48, 72, and 96 hours postdose.
PK, pharmacokinetic; NA, not assessed.

Results

Genotoxicity studies

In vitro *mutagenicity*

The mutagenic potential of peginesatide was evaluated in an *in vitro* bacterial reverse-mutation (Ames) assay with four strains of *S. typhimurium* and an *E. coli* strain. Assays were conducted with each of the strains in the absence and presence of metabolic Aroclor-induced rat-liver S9 at concentrations ranging from 75 to 5,000 µg/plate. The results of the mutagenicity assay are listed in Table 1. The positive control agents yielded the expected mutagenic activity, and the solvent control values are within the ranges of the historic control data for the conducting laboratory (data not shown). Neither test article precipitate nor appreciable toxicity were observed. Peginesatide did not increase the number of revertants, with or without metabolic activation, and, therefore, was nonmutagenic in the bacterial reverse mutation assay.

In vitro *chromosomal aberrations*

The *in vitro* effect of peginesatide on chromosomes was studied in CHO cells at concentrations up to 5,000 µg/mL in both the absence and presence of an Aroclor-induced rodent S9 metabolic activation system. The results of the chromosomal analysis are provided in Table 3. The mitotic index at the highest dose level evaluated for chromosome aberrations (5,000 µg/mL) was 33% reduced and not reduced relative to the solvent control following a 4-hour treatment time in the absence and presence of S9 activation, respectively; therefore, dose levels selected for microscopic analysis were 1,250, 2,500, and 5,000 µg/ mL. No statistically significant increases in aberrations in the CHO studies were observed at any of the peginesatide concentrations tested up to 5 mg/mL following 4 or 20 hours of treatment without metabolic activation. There was a nonsignificant and, possibly, nonbiologically relevant moderate rise (with large ± SD) in numeric aberrations per CHO cell following 4 hours of treatment with metabolic activation. Peginesatide was negative for clastogenicity in CHO cells. Following a 4-hour incubation, the percentage of structurally damaged cells in the positive control groups, mitomycin C (22%) and cyclophosphamide (16.5%), was significantly increased, compared to the vehicle control (0%). Following a 20-hour incubation, the increased percentage of structurally damaged cells in the mitomycin C (positive control) treatment group (21.0%), compared to the vehicle control (0%), was statistically significant.

In vivo *micronuclei assay*

The bone marrow *in vivo* micronucleus assay results are provided in Table 4. No mortalities were observed at any of the doses tested. Clinical observations were limited to transient piloerection after each dose of 1,000 or 2,000 mg/kg. Reductions of 2–45% in PCE:EC ratio were observed in the peginesatide-treated groups relative to the negative control groups. The change in PCE:EC ratio demonstrates the bioavailability of the test article to the bone marrow and reflects the pharmacologic activity of

Table 3. Chromosome aberrations of cultured Chinese hamster ovary cells treated with peginesatide.

Treatment(μg/mL)	S9 activation	Treatment time (hours)	Mitotic index (mean)	Aberrations per cell (mean ± SD)	Numeric (%)	Structural (%)
Water	–	4	13.3	0 ± 0	3.0	0.0
Peginesatide						
1,250	–	4	12.7	0.0 ± 0.0	5.0	0.0
2,500	–	4	12.8	0.0 ± 0.0	4.0	0.0
5,000	–	4	8.9	0.0 ± 0.0	5.5	0.0
MMC (0.2)	–	4	10.9	0.270 ± 0.548	2.0	22.0**
Water	+	4	12.3	0.005 ± 0.071	10.0	0.5
Peginesatide						
1,250	+	4	11.9	0 ± 0	8.5	0.0
2,500	+	4	13.5	0.005 ± 0.071	9.0	0.5
5,000	+	4	14.6	0.010 ± 0.100	9.5	1.0
CP (10)	–	4	9.2	0.225 ± 0.562	4.5	16.5**
Water	–	20	15.4	0 ± 0	3.5	0.0
Peginesatide						
1,250	–	20	12.9	0.0 ± 0.0	4.5	0.0
2,500	–	20	13.0	0.015 ± 0.122	5.5	1.5
5,000	–	20	12.4	0.010 ± 0.100	5.5	1.0
MMC (0.1)	–	20	9.6	0.230 ± 0.468	2.5	21.0**

Mitomycin C (MMC) and cyclophosphamide (CP) were the positive controls. Mitotic index equals the number of mitotic figures × 100/500 cells counted. Aberration frequency was based on scoring 200 cells, with the exception of 100 cells, for the structural characterization of MMC. Aberrations per cell: severely damaged cells were counted as 10 aberrations. Percent aberrant cells: *$P \leq 0.05$; **$P \leq 0.01$, using Fisher's exact test.

Table 4. *In vivo* bone marrow micronucleus analysis following administration of peginesatide to ICR mice.

Treatment	Sex	Time (hours)	PCE/total erythrocytes	Change in PCE:EC ratio (from negative control)	Number of MPCEs/10,000 PCEs
Water					
	M	24	0.476 ± 0.04	—	3/10,000
	F	24	0.478 ± 0.04	—	5/10,000
Peginesatide (i.p.)					
500 mg/kg	M	24	0.450 ± 0.04	–5	5/10,000
	F	24	0.440 ± 0.02	–8	4/10,000
1,000 mg/kg	M	24	0.437 ± 0.08	–8	7/10,000
	F	24	0.414 ± 0.05	–13	3/10,000
2,000 mg/kg	M	24	0.452 ± 0.09	–5	6/10,000
	F	24	0.262 ± 0.05	–45	7/10,000
Cyclophosphamide (i.p.)					
50 mg/kg	M	24	0.344 ± 0.02	–28	241/10,000*
	F	24	0.325 ± 0.03	–32	255/10,000*
Water					
	M	48	0.493 ± 0.03	—	7/10,000
	F	48	0.490 ± 0.03	—	7/10,000
Peginesatide (i.p.)					
2,000 mg/kg	M	48	0.483 ± 0.04	–2	4/10,000
	F	48	0.418 ± 0.02	–15	7/10,000

Values presented are the mean ± SD of 5 animals.
EC, erythrocyte; MPCEs, micronucleated polychromatic erythrocytes; PCEs, polychromatic erythrocytes; ICR, imprinting control region; i.p., intraperitoneal.
*Statistically significant at $P \leq 0.05$ (Kastenbaum-Bowman tables).

the drug. The incidence of MPCE per 10,000 PCEs in the peginesatide groups was not significantly increased relative to the respective negative controls in either male or female mice, regardless of dose level or bone marrow collection time. Peginesatide, therefore, was nonclastogenic (i.e., not genotoxic) in the mouse micronucleus assay.

28-day i.v. CByB6F1 hybrid study

Pharmacokinetic group

PK parameters, based on the measured plasma concentrations of peginesatide, are depicted in Table 5. There were no quantifiable plasma levels of peginesatide in the vehicle group samples. Maximum concentration (C_{max})

was dose proportional across all doses tested; however, the increase in AUC was greater than dose proportional. With a 10-fold increment in dose, there was approximately a 15–20-fold increase in AUC. The greater than dose proportion increase in AUC is consistent with the trend toward reduced plasma clearance and a longer half-life at the higher doses. Volume of distribution was similar for all male groups, ranging from 38 to 44 mL/kg. Nonlinear PK of peginesatide has also been observed in monkeys (Woodburn et. al., 2008). The nonlinear kinetics suggest that receptor-mediated clearance may play a role in the overall disposition of peginesatide at these doses. When receptor-mediated clearance is not saturated, a concentration-dependent elimination is expected, resulting in faster clearance and shorter half-life at lower doses. Once receptor-mediated clearance is saturated, clearance would approach the rate associated with nonspecific clearance, such as renal filtration, and linear PK and dose-proportional exposure would be expected.

Safety group: in-life observations

All main study animals survived until scheduled sacrifice on day 29. Clinical observations included a dose- and time-dependent increase in limb erythema in mice at ≥10 mg/kg of peginesatide. The limb erythema, which was noted on day 29 and days 22 and 29 in the 10- and 25-mg/kg dose groups, respectively, was considered to be due to pronounced test-article–induced polycythemia.

Statistical analysis of mean nonfasted body weight revealed no differences in the peginesatide-dosed groups of either sex, when compared to the vehicle controls. A statistically significant decrease in total body-weight gain was observed in males only at 25 mg/kg; 0.92 ≠ 0.63 g vs. 2.46 ± 0.78 g for vehicle controls. Statistical analysis of group mean daily food-consumption data, which were calculated based on food consumption measured at weekly intervals, revealed a significant (6.33 ± 1.04 g; $P < 0.05$) increase in the 25-mg/kg group males between days 22 and 28, when compared to the vehicle control (3.96 ± 0.59 g). The relationship of a statistically significant decrease in body weight, and a concurrent increase in food consumption to test article in male mice at 25 mg/ kg of peginesatide, is unclear.

Clinical pathology

The primary pharmacology of peginesatide following administration on days 1 and 22 resulted in polycythemia, based on blood samples collected on day 29. Findings included a generally dose-dependent increase in RBC count, hematocrit (Hct), and hemoglobin (Hgb) levels for both males and females, with responses comparable between genders. Peginesatide increased RBCs at all dose levels, reaching statistical significance at ≥1 mg/kg (Figure 1). RBCs increased up to 44% over control values, reaching 15.96 ± 1.53 10^6/μL in the 25-mg/kg female group, compared to 11.06 ± 1.08 × 10^6/μL for the concurrent vehicle control group ($P < 0.01$).

Hemoglobin (Hgb) was increased at all dose levels, reaching statistical significance at ≥10 mg/kg in males and at 25 mg/kg in females (Figure 2). Hgb values in males at 0, 0.1, 1, 10, and 25 mg/kg were 17.7, 18.2, 19.9, 21.1, and 20.6 g/dL, which represented an increase of 0.5, 2.2, 3.4, and 2.9 g/dL, respectively, compared to vehicle controls. Hgb values in females at 0, 0.1, 1, 10, and 25 mg/kg were 17.4, 18.2, 19.1, 19.3, and 21.3 g/ dL, which represented an increase of 0.8, 1.7, 1.9, and 3.9 g/dL, respectively, compared to vehicle controls.

Serum iron was increased at the lower doses and decreased at the higher doses (Figure 2). Serum iron was increased at 0.1 mg/kg for males and females and 1 mg/kg for females and decreased at 1 mg/kg for males and at the

Table 5. Selected peginesatide pharmacokinetic parameters in CByB6F1 hybrid mice following a single intravenous dose.

Dose (mg/kg)	C_{max} (μg/mL)	$AUC_{0-\infty}$ (μg•h/mL)	$t_{1/2}$ (hours)	CL (mL/h•kg)
0.1	2.1	52.0	14.3	1.92
1.0	24.6	821	24.5	1.22
10	281.3	16,669	50.8	0.60

Data are expressed as the mean.
C_{max}, maximum concentration; $AUC_{0-\infty}$, area under the concentration-time curve from time zero to infinity; $t_{1/2}$, half-life; CL, plasma clearance.

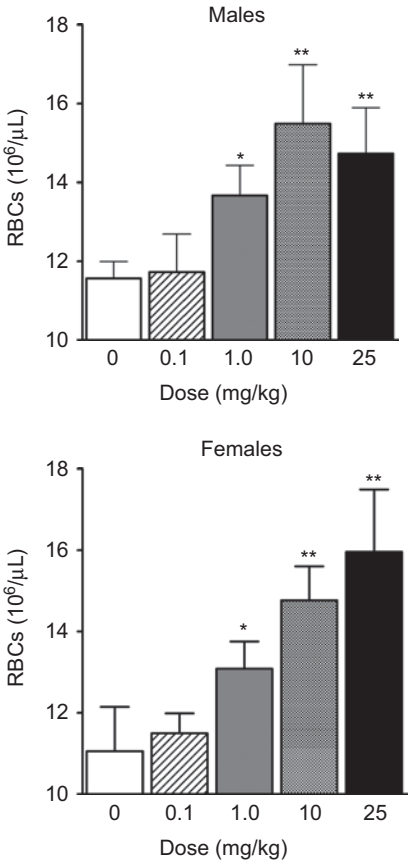

Figure 1. The primary pharmacology of peginesatide resulted in excessive erythropoiesis as evidenced by significant increases in red blood cells (RBCs). Data represent day 29 mean RBC values ($n = 5$) ± SD following i.v. dosing on days 1 and 22. Statistically significant ($^*P < 0.05$; $^{**}P < 0.01$), using Dunnett's t-test, when compared to concurrent vehicle controls (0 mg/kg).

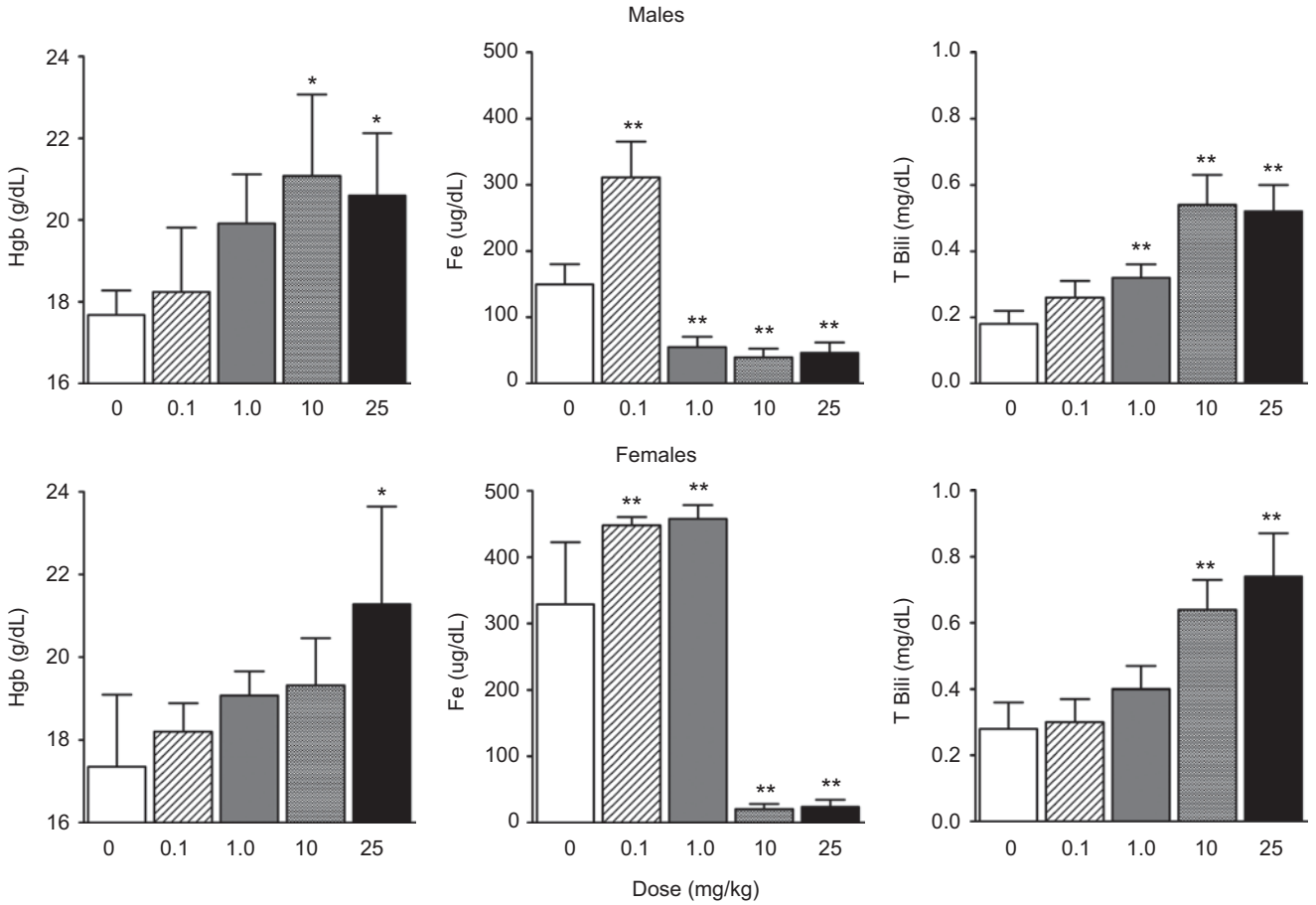

Figure 2. Serum-free iron (Fe) and bilirubin (T Bili) profiles were consistent with excessive erythropoiesis following peginesatide administration. Data represent mean values ± SD ($n = 5$ per group) on day 29 following i.v. dosing on days 1 and 22. Statistically significant ($^{*}P < 0.05$; $^{**}P < 0.05$), using Dunnett's t-test, when compared to 0 mg/kg.

higher doses for both genders. The increase was considered secondary to an increase in mobilization following the increase in erythropoiesis. The decrease was considered to be secondary to excessive consumption of iron following a more pronounced, sustained erythropoiesis at the higher doses. Concomitantly, a dose-dependent increase in bilirubin was observed, reaching statistical significance at ≥1 and ≥10 mg/kg in males and females, respectively. The increase in bilirubin was likely due to an increase in extravascular hemolysis secondary to the increased erythropoiesis and sustained polycythemia.

Peginesatide administration induced increases in hematocrit (Hct) levels and associated changes in RBC indices reflective of pronounced erythropoiesis (Figure 3). Alterations in RBC indices [e.g., mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) and RBC morphology (i.e., anisocytosis, polychromasia, and macrocytosis) were consistent with accelerated erythropoiesis. The accelerated erythropoiesis, when high ESA doses are administered to normocythemic animals, is associated with pronounced alterations in RBC parameters and indices as well as serum iron levels. The relative iron deficiency observed in normal animals administered high doses of peginesatide translates into impaired heme synthesis, resulting in the generation of smaller RBCs (decreased MCV) containing less Hgb (lower MCH) and Hgb concentration (lower MCHC) per cell (Duncan & Prasse's Veterinary Laboratory Medicine, 2003).

Clinical chemistry

Changes in serum chemistry related to peginesatide administration were considered to reflect excessive erythropoiesis, increased RBC destruction (i.e., extravascular hemolysis and damage or lysis during sample collection) and/or hemoconcentration and was, therefore, secondary to the exaggerated pharmacology. Changes included an apparent dose-dependent increase in potassium (males only), phosphorous, and aspartate aminotransferase (AST). Changes reached statistical significance for potassium at ≥1 mg/kg, for phosphorous at ≥0.1 mg/kg in females and at 10 mg/kg in males, and for AST at ≥1 mg/kg in females. A significant decrease in glucose at ≥1 mg/kg for males and ≥10 mg/kg for females was observed.

Gross necropsy and organ weights

Splenic enlargement was the only gross necropsy finding noted at necropsy. Spleen weights ranged from approximately 3.2 to 11.8 times the control values (Figure 4).

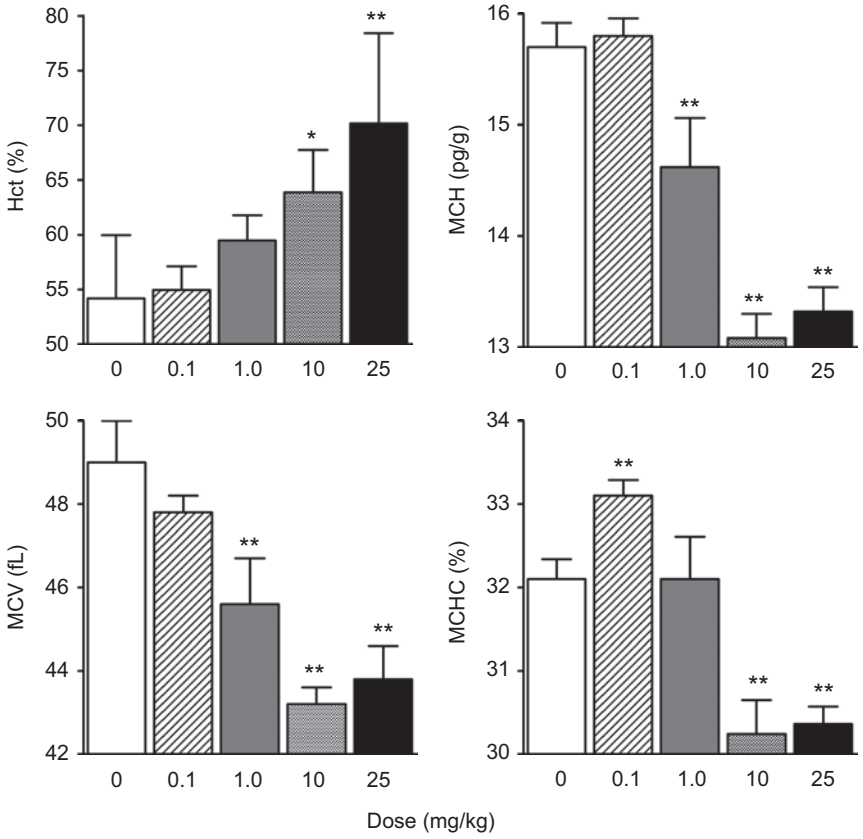

Figure 3. Peginesatide-induced increases in hematocrit (Hct) and associated changes in secondary hematologic indices [e.g., mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC)] reflective of accelerated erythropoiesis. Day 29 results (mean ± SD; $n = 5$ per group) are depicted for female mice following i.v. administration on days 1 and 22. Similar results were obtained for male mice. Statistically significant ($^{*}P < 0.05$; $^{**}P < 0.01$), using Dunnett's *t*-test, when compared to 0 mg/kg.

There was a statistically significant increase in liver weight in males at 10 and 25 mg/kg, with 26 and 42.5% increases over concurrent vehicle controls.

Microscopy

Mild to marked bone marrow hypercellularity was characterized by a pronounced increase in the erythroid series. Minimal to mild extramedullary hematopoiesis (EMH) was observed in the liver at ≥10 mg/kg. Minimal to marked EMH was noted in the spleen at ≥1 and ≥10 mg/kg for males and females, respectively. The histopathologic finding of EMH correlated with the increases in spleen and liver weights.

Discussion and conclusion

Peginesatide is a synthetic, PEGylated, peptide-based ESA. Peginesatide specifically and selectively activates the EPO receptor (Fan et al., 2006). Peginesatide is being developed for the treatment of anemia in patients with CKD, including patients on dialysis and patients not on dialysis. The clinical course of anemia in CKD patients is protracted, and, therefore, peginesatide administration is expected to be chronic. For chronically administered drugs, carcinogenicity evaluation, including an assessment of genotoxic potential, is warranted.

A standard battery of genotoxicity studies was performed with peginesatide, which was consistent with the ICH guidances (S2A: *Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals*; S2B: *Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals*) and OECD guideline 474 (*Genetic Toxicology: Mammalian Erythrocytes Micronucleus Test*) available at the time the studies were conducted. Peginesatide was nonmutagenic in the bacterial reverse-mutation assay and was nonclastogenic in the CHO cell chromosomal aberration assay and the *in vivo* mouse bone marrow micronucleus assay. The highest concentration evaluated in the *in vitro* chromosomal assay was 5 mg/mL, equating to a 1,724-fold increase over the C_{max} (2.9 µg/mL) obtained following the administration of 0.1 mg/kg to normal healthy volunteers (NHVs; Duliege et al., 2005). A single dose of 0.1 mg/kg of peginesatide administered i.v. to NHVs caused hemoglobin levels to rise by a clinically meaningful 1 g/dL over a 1-month period (Stead et al., 2006).

The nonclinical data for peginesatide obtained, to date, do not suggest a carcinogenic potential. Peginesatide binding is highly specific for the EPO receptor, since no pharmacologically relevant binding was detected against a panel of 66 diverse receptors (Fan et al., 2006). Also, peginesatide did not stimulate tumor

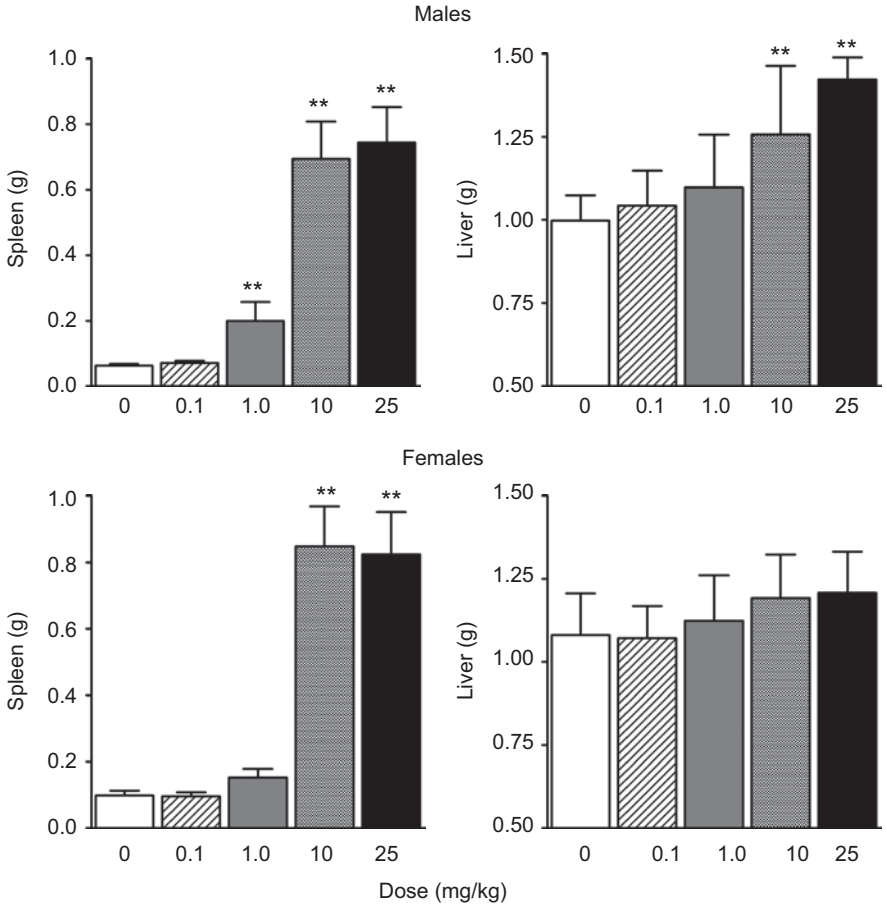

Figure 4. Peginesatide-induced significant increases in spleen weights and increases in liver weights correlating with extramedullary hematopoiesis. Mean (± SD) spleen ($n = 10$) and liver ($n = 10$) weights following i.v. administration to mice on days 1 and 22. Weights were evaluated on day 29. Statistically significant ($^{*}P < 0.01$), using Dunnett's t-test, when compared to 0 mg/kg.

cell proliferation when tested in seven human tumor cell lines, but did stimulate the proliferation of aberrant EPO-responsive hematopoietically derived TF-1 erythroleukemia cells (Fan et al., 2006). Additionally, the data include an absence of a proliferative response in nonerythroid tissues or tumor development in the 6-month rat (Woodburn et al., 2009) and 9-month monkey (Woodburn et al., 2008) chronic toxicity studies as well as no apparent genotoxic potential. Standard carcinogenicity studies have not routinely been conducted for biotechnology products because of the development of neutralizing antibodies in rodents following repeated dosing of proteins, especially human proteins (ICH S6: *Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals*). Because neutralizing antibodies have been demonstrated following repeated dosing of recombinant human protein ESAs to rodents, the currently available ESAs have not been evaluated in either 2-year rodent or transgenic mouse assays (Serabian, 2001; Ouyang, 2007). Peginesatide, however, is nonimmunogenic in rodents (Woodburn et al., 2010); thus, it is feasible to conduct long-term toxicity studies in rats and mice, including carcinogenicity studies. As a result, the toxicologic assessment of peginesatide has been more rigorous than the assessment for recombinant protein ESAs. The CByB6F1 hybrid mouse study, therefore, was undertaken to support the dose selection for a 6-month transgenic murine carcinogenicity study in rasH2 mice.

PK analyses revealed a prolonged half-life for peginesatide. Exposure of CByB6F1 hybrid mice to peginesatide increased with increasing dose. The PK of peginesatide was not linear over an i.v. dose range of 0.1–10 mg/kg, with exposure, based on AUC, increasing in a greater than dose-proportional relationship. The prolonged half-life of peginesatide resulted in a sustained pharmacodynamic response characterized by a pronounced, sustained polycythemia. A no-observed effect level (NOEL) was not identified in the safety study, as exaggerated pharmacologic responses were noted at all dose levels. The no-observed adverse effect level (NOAEL) was ≥25 mg/kg of peginesatide, based on an absence of mortality or significant toxicity. In general, the peginesatide-related effects in mice were associated with the primary pharmacology of the drug, which is exaggerated when administered to normocythemic animals. The findings in the mice are consistent to what has been observed in rats and cynomolgus monkeys administered peginesatide (Woodburn et al., 2008, 2009). In addition, the findings with peginesatide were consistent with findings observed with other ESAs in animals

not affected by neutralizing antibodies (Serabian, 2001; Ouyang, 2007). The peginesatide-related findings included an increase in RBC parameters (i.e., RBC count, Hgb, and Hct), altered RBC morphology and indices, increased serum bilirubin, dose-dependent effects on serum iron (i.e., increased at the low dose and decreased at the higher doses), splenomegaly, hepatic and splenic extramedullary hematopoeisis, and bone marrow hypercellularity.

In conclusion, peginesatide is nongenotoxic. Following two i.v. injections on days 1 and 22 in the wild-type CByB6F1 hybrid littermate mice of the transgenic CB6F1/jic-TgN(RasH2)@Tac mouse strain, peginesatide induced erythropoiesis, and findings were related to the exaggerated pharmacology (i.e., polycythemia) that occurs with the administration of an ESA to a normocythemic animal.

Declaration of interest

KWW and PJS are employees of Affymax Inc. Affymax Inc is developing peginesatide for the treatment of anemia associated with chronic kidney disease.

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