Peginesatide Clearance, Distribution, Metabolism, and Excretion in Monkeys following Intravenous Administration

Kathryn W. Woodburn,1 Kei-Lai Fong, Susan D. Wilson, Steven Sloneker, Paul Strzemienski, Eric Solon, Yuu Moriya, and Yoshihiko Tagawa

Affymax, Inc., Palo Alto, California (K.W.W.); Accellient Partners LLC, Berkeley, California (K.-L.F.); Aclairo Pharmaceutical Development Group, Inc., Vienna, Virginia (S.D.W.); Calvert Laboratories Inc., Scott Township, Pennsylvania (S.S.); QPS LLC, Newark, Delaware (P.S., E.S.); Takeda Pharmaceutical Company, Ltd., Jusohonmachi, Yodogawa-ku, Osaka, Japan (Y.M., Y.T.)

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ABSTRACT

Peginesatide, a polyethylene glycol (PEG)ylated peptide-based erythropoiesis-stimulating agent, stimulates the erythropoietin receptor dimer that governs erythropoiesis. Studies were designed to determine the erythropoietic response, pharmacokinetics (PK), tissue distribution, metabolism, and excretion of peginesatide in nonhuman primates following a single i.v. dose. The PK profile of peginesatide (0.1–5 mg/kg) is characterized by low, dose-dependent plasma clearance; small volume of distribution; and long half-life. The peginesatide PK profile following a single i.v. dose is consistent with the sustained erythropoiesis. Biodistribution quantitative whole-body autoradiography demonstrated high peginesatide levels in bone marrow (i.e., primary hematopoietic site) as well as other known hematopoietic sites persisting through at least 3 weeks at 2.1 mg/kg. Microautoradiography analysis at 48 hours postdose revealed uniform and high distribution of radioactivity in the bone marrow and splenic red pulp with less extensive distribution in the renal cortex (glomeruli, associated ducts, interstitial cells). Radioactivity in the kidney was most prominent in the outer medullary and papillary interstitium. At 2 weeks after dosing, cumulative radioactivity recovery in the urine and feces was 60 and 7% of the administered dose, respectively, with most of the radioactivity associated with the parent molecule. In conclusion, the PK characteristics are consistent with a PEGylated peptide of a 45-kDa molecular mass, specifically low volume of distribution and long half-life. Drug was localized principally to hematopoietic sites, and nonspecific tissue retention was not observed. The nonhuman primate data indicate that peginesatide is metabolically stable and primarily excreted in the urine.

Introduction

Erythropoietin is a glycoprotein hormone synthesized by the kidney in response to hypoxia. The hormone activates the erythropoietin (EPO) receptor (EPOr) on bone marrow erythroid-cell precursors supporting their proliferation, growth, and differentiation, which leads to reticulocytosis and subsequent red blood cell maturation. Anemia in chronic kidney disease (CKD) patients occurs as a result of damage to the renal cells that are responsible for EPO production (Maxwell et al., 1997). Benefits of anemia treatment in CKD patients include decreased morbidity, hospitalization, mortality, and transfusion avoidance (Vanrenterghem et al., 2002; Silverberg, 2003; Li et al., 2004). Currently in the United States, management of anemia secondary to CKD relies on recombinant human erythropoietin (rHuEPO, epoetin alfa, Procrit, Epogen) or darbepoetin alfa (Aranesp), which are typically administered up to three times weekly or every 1–2 weeks, respectively.

Peginesatide is a polyethylene glycol (PEG)ylated, peptide-based erythropoiesis-stimulating agent (ESA) that is approved in the United States for the treatment of anemia due to CKD in adult patients on dialysis. Peginesatide stimulates the EPOr, but has no sequence homology to rHuEPO (Woodburn et al., 2011). Peginesatide, by virtue of its lack of immunologic crossreactivity (Fan et al., 2006), has been shown to correct anemia in rats with anti-EPO antibody-mediated pure red cell aplasia (Woodburn et al., 2007) as well as increase hemoglobin levels and reduce the need for transfusions in CKD patients with pure red cell aplasia caused by anti-EPO antibodies (Macdougall et al., 2009).

The peginesatide peptide sequence was identified by peptide screening against the human EPOr, followed by synthetic chemical optimization (Fan et al., 2006). The screening methodology allows the generation of peptide alternatives to endogenous proteins with amino acid sequences unrelated to the natural ligand or any other human sequence (Cwirla et al., 1997). The peginesatide peptide was PEGylated in an effort to prolong systemic circulation, increase solubility, and decrease the peptide’s immunogenic potential by masking epitopes (Calicieti and Veronese, 2003; Harris and Chess 2003).

ABBREVIATIONS: ADME, absorption, distribution, metabolism, and excretion; AUC, area under the plasma concentration-time curve; CBC, complete blood counts; CKD, chronic kidney disease; CL, clearance; EHH, extramedullary hematopoiesis; EPO, erythropoietin; EPOr, erythropoietin receptor; ESA, erythropoiesis-stimulating agent; GFR, glomerular filtration rate; HPLC, high-performance liquid chromatography; LSC, liquid scintillation counting; MARG, microautoradiography; PEG, polyethylene glycol; PK, pharmacokinetic; QWBA, quantitative whole-body autoradiography; RBC, red blood cell; rHuEPO, recombinant human erythropoietin; RME, saturable receptor-mediated endocytosis; TFA, trifluoroacetic acid; Vss, volume of distribution at steady state.
Studies conducted in the rat characterizing the absorption, distribution, metabolism, and excretion (ADME) of peginesatide after i.v. administration and correlating the ADME properties with the pharmacological activity have been reported (Woodburn et al., 2012). The toxicologic assessment of peginesatide was also performed in both the rat (Woodburn et al., 2009) and the monkey (Woodburn et al., 2008a,b). The monkey and the rat are appropriate species for the nonclinical assessment of peginesatide based on pharmacological, toxicological, and pharmacokinetic (PK) considerations. Peginesatide has been shown to be pharmacologically active in both rats and monkeys. In addition, the red blood cell (RBC) life span is 52–128 days in the monkey and 45–60 days in the rat (Moore, 2000), compared with 120 days in normal adult humans and 45–85 days in patients with renal failure (Kruse et al., 2008). Unlike the rat, humans and monkeys undergo little or no extramedullary hematopoiesis (EMH) in the spleen and liver under normal conditions, whereas rodents commonly exhibit a fairly robust EMH response throughout their lives (Greaves and Faccini, 1992). The erythropoietic response in monkeys, therefore, more closely approximates the response in humans when compared with the rats, which allows a more direct translation of the data obtained in monkeys to human clinical use.

Extensive nonclinical studies have been conducted in nonhuman primates to support clinical development and approval of peginesatide. The results of studies designed to determine the erythropoietic response, pharmacokinetics, tissue distribution, and major routes of elimination of peginesatide following a single i.v. dose in cynomolgus monkeys are described.

### Materials and Methods

#### Chemicals

Peginesatide is a synthetic, dimeric peptide (approximate molecular mass 4,900 Da). The molecule is comprised of two identical 21-amino acid chains covalently bonded via a linker to a single lysine-branched bis-PEG chain (approximate molecular mass 40,000 Da), yielding a total molecular mass of approximately 45,000 Da (Fig. 1). The linker is derived from iminodiacetic and β-alanine.

Both unlabeled and radiolabeled peginesatide were used in the studies. Radiolabeled peginesatide, with the 14C-radiolabel on the lysine linker between the peptide dimer and the two 20-kDa PEG chains (Fig. 1) was used for the quantitative whole-body autoradiography (QWBA) biodistribution and for the excretion/metabolism studies. Radiolabeled [14C] peginesatide, with activity ranging from 5.5 to 5.7 μCi/mg and a radiochemical purity of approximately 90%, was synthesized by GE Healthcare (Buckinghamshire, UK). The process to prepare the labeled material used a synthetic route that resulted in a mono-PEGylated 14C-labeled species at a level of approximately 10%, in addition to the radiolabeled parent compound. The mono-PEG is a specific impurity formed during radiolabel chemical synthesis and has not been identified in any unlabeled lots. The mono-PEG species results from incomplete PEGylation (i.e., attachment of only one of the two 20-kDa PEG chains) and likely represents PEGylation of either the alpha or epsilon amine of lysine. The level of mono-PEG present in the formulation was not deducted from the total radioactivity measured in plasma and tissues.

#### Animal Studies

Animals were maintained at each of the study facilities in accordance with the Guidelines for Care and Use of Laboratory Animals.

#### Pharmacology

**Dimeric Peptide.** Male non-naïve cynomolgus monkeys (n = 3 per group; 3 kg; Covance, Denver, PA) were administered an i.v. injection of dimeric peptide (i.e., peginesatide minus PEGylation) at 0.1 or 1 mg/kg (1 ml/kg) on day 1. Blood samples for complete blood counts (CBCs), including reticulocyte counts, were collected prior to dosing and on days 4, 6, 10, 14, 19, and 29 postdose.

**Peginesatide.** Male non-naïve cynomolgus monkeys (n = 4 per group; 3–5 years old) were administered an i.v. injection of vehicle (10 mmol/l acetic acid in isotonic saline, pH 5.5) or 0.02, 0.1, or 0.5 mg/kg peginesatide at 0.5 ml/kg on day 1. Blood samples for CBCs, including reticulocyte counts, were collected prior to and on 4, 7, 14, 21, 28, 35, 42, 49, 56, and 63 days following administration.

The RBC and reticulocyte data following administration of the dimeric peptide or peginesatide are expressed as mean ± standard deviation. Comparisons of hematologic parameters were performed using a one-way analysis of variance followed by a post hoc Dunnett’s test. A P value less than 0.05 was considered statistically significant.

#### Pharmacokinetics

The PK and linearity of the PK profile were evaluated following single i.v. doses of peginesatide. Male non-naïve cynomolgus monkeys (n = 4 per group; 4 years old, 4.2–4.4 kg) were administered single escalating i.v. doses of 0.1, 0.5, and 5 mg/kg peginesatide at 0.5 ml/kg with a 42-day washout period between doses. The vehicle was isotonic saline. Blood samples for PK analysis were collected from animals predose and at 0.25, 1, 6, 24, 48, 72, 120, 168, 240, 336, 408, and 504 hours postdose. Plasma was obtained and analyzed for peginesatide by a validated competition enzyme-linked immunosorbent assay in monkey plasma with a lower limit of quantification of 40 ng/ml (Fan et al., 2006; Woodburn et al., 2007).

The PK parameters were calculated using WinNonlin software (version 5.0.1; Pharsight, Mountain View, CA). Area under the plasma concentration-time curve (AUC) and area under the first-moment curve of the plasma concentration-time curve (AUMC) were calculated by the linear trapezoidal rule. Clearance (CL) was calculated as dose/AUC, mean residence time as area under the first-moment curve of the plasma concentration-time curve/AUC, and volume of distribution at steady state (Vss) as CL*mean residence time. The Cmax was designated as the measured value at the earliest sampling time point of 0.25 hours postdose.

#### Tissue Distribution

Tissue distribution and localization were investigated using QWBA and microautoradiography (MARG). Male naïve cynomolgus monkeys (n = 4; 2 years old, 1.9–2.0 kg) were given an i.v. bolus dose of [14C] peginesatide at 2.1 mg/kg (7.14 μCi/mg). Three monkeys were sacrificed for QWBA analysis 48 hours or 1 and 3 weeks after dosing. One monkey was sacrificed for MARG analysis at 48 hours after dosing. Blood samples for CBCs, including reticulocyte counts, were collected predose; 48 and 120 hours postdose; and at 1, 2, and 3 weeks after dosing. Blood samples for PK determination were collected predose; at 1, 24, 48, 72, and 120 hours postdose; and at 1, 2, and 3 weeks after dosing.

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**Fig. 1.** Sites of radiolabel for [14C] peginesatide.
The RBC and reticulocyte data were expressed and analyzed as previously described. Total radioactivity in whole blood samples was determined by combustion then liquid scintillation counting (LSC) analyses and plasma samples by LSC analyses. The PK parameters were calculated as previously described.

**Quantitative Whole-Body Autoradiography Preparation.** Carcasses from animals euthanized at 48 hours, 1 week, and 3 weeks after dosing were immediately frozen in a hexane-dry ice bath for approximately 1 hour. Following embedding, the carcasses were cut into sagittal sections (40 μm thick) using a CM3600 cryomicrotome (Leica, Nussloch, Germany) and then mounted on cardboard backing. A set of sections from each animal were exposed to 14C-sensitive phosphorimaging plates (Fuji Biomedical, Stamford, CT) for a 4-day exposure period. Tissue radioactivity concentrations were quantified from the whole-body autoradiograms using a validated image analysis system [Typhoon 9410 image acquisition system (GE Healthcare/ Molecular Dynamics, Sunnyvale, CA) and MCID image analysis software (v. 6.0 and 7.0; GE Healthcare/Imaging Research, Inc., St. Catharines, Ontario, Canada)]. A set of 14C-calibration standards (range was approximately 0.0007–6.5 μCi/g) applied to 14C-sensitive phosphorimaging plates (Fuji Biomedical) was used as the calibration standard.

**Microautoradiography Preparation.** Liver, spleen, thymus, mesenteric lymph nodes, axillary lymph nodes, sternum, bone marrow (femur), kidney cortex, kidney medulla, and kidney papilla were immediately removed from one of the animals euthanized at 48 hours postdose and were prepared for MARG analysis. Tissues were cut into blocks of approximately 0.5 cm2. Each block was mounted on a cryosectioning stage and quick-frozen in isopentane cooled to the temperature of liquid nitrogen. Cryosections were collected (5 μm in thickness, chamber temperature approximately −25°C) and thaw-mounted under darkroom conditions onto slides previously coated with nuclear photographic emulsion (NTB; Kodak, Rochester, NY). Slides were exposed under darkroom conditions onto slides previously coated with nuclear photographic emulsion (NTB; Kodak, Rochester, NY). Slides were exposed and then developed in Kodak D19 developer and fixed with Kodak fixer. Slides were co-stained with hematoxylin and eosin for geographic orientation.

**Metabolism and Excretion**

Metabolism and excretion of peginesatide was evaluated in male non-native cynomolgus monkeys (n = 4; 4 years old, 3.6–4.3 kg) following a single i.v. administration of 5 mg/kg radiolabeled [14C]peginesatide (28 Metabolite profiling were also obtained at 0.25, 1, 6, 24, 48, 72, 96, 120, 144, 168, 240, and 336 hours postdose.

The total plasma concentration of radioactivity was measured by a direct method (LSC). Quantitative metabolite profiling was performed using pooled plasma samples prepared from combining each respective time point. Samples were extracted and subjected to high-performance liquid chromatography (HPLC) separation and fractionation as subsequently described. Radioactivity concentrations in the various fractions were measured by LSC. Unlabeled metabolites. The unidentified minor peaks were combined as peptide dimer and a mono-PEG, were used for identification of potential standards, including the non-PEGylated peptide monomer, non-PEGylated concentrations in the various fractions were measured by LSC. Unlabeled standards, including the non-PEGylated peptide monomer, non-PEGylated peptide dimer and a mono-PEG, were used for identification of potential metabolites. The unidentified minor peaks were combined as “Others.” The PK parameters were analyzed for peginesatide and metabolites as follows. The Cmax was obtained from the actual values measured at the earliest time point (i.e., 0.25 hours). The t1/2 and AUC were calculated by the WinNonlin noncompartmental model (Ver. 4.1; Pharsight Corporation). Clearance was calculated as dose/AUC. The AUC of metabolites designated as “Others” was calculated by the subtraction of the AUC of the identified components from the AUC of total radioactivity in the plasma.

Urine and fecal samples were collected every 24 hours up to 14 days after dosing. Radioactivity in the urine samples was measured using the direct method. Radioactivity in fecal homogenates was measured using the combustion followed by direct LSC methods. Cumulative excretion ratio was calculated and expressed as percent of administered dose.

For quantitative metabolite profiling, two aliquots (i.e., 0–168 and 168–336 hours postdose) of each biologic matrix were obtained by combining pooled samples from the 4 animals at respective 24-hour collection intervals. The samples were subsequently extracted and analyzed by HPLC fractionation and radioactivity was measured by a direct method (LSC) for plasma and urine and by a combustion method for the fecal homogenate.

**High-Performance Liquid Chromatography for Metabolite Profiling**

The radioactive HPLC fraction eluents were identified as peginesatide or mono-PEG (peginesatide with one PEG chain), with the remaining radioactivity designated as “Other.” The classification of Other is considered likely to encompass smaller PEGylated-peptides and peptide/amino acid components.

**Sample Preparation.** Plasma, urine, and fecal homogenates were extracted with 5 volumes of methanol containing 0.1% of trifluoroacetic acid (TFA). The homogenate samples were then mixed and centrifuged at 1,500 × g for 10 minutes to obtain supernatant. A portion of the supernatant was counted by LSC to estimate the extraction ratio of [14C]peginesatide and its related compounds in the biologic samples. Extraction efficiencies for plasma and urine were above 95 and 97%, respectively.

The rest of the supernatant was evaporated to dryness under a nitrogen gas stream. The residue was dissolved in a small volume of mobile phase A and B [MP(A):MP(B) = 1:1 by volume]. A mixture of 0.1% TFA aqueous solution and 0.1% TFA in methanol solution were used for MP(A) and MP(B), respectively. The samples were injected into the HPLC, which consisted of an LC-10A/Dvp pump, a CTO-10ACvp column oven, an SPD-10Avp UV detector, and an SCL-10Avp system controller. The HPLC separation was achieved at 40°C with a Zorbax 300SB-C8 column (150 mm × 2.1 mm i.d., 5 μm; Agilent Technologies Japan, Ltd., Kyoto, Japan). The flow rate was 0.5 mL/min, and the peaks were monitored by a UV absorption detector at 215 nm and a radioisotope detector. The gradient elution involving increasing MP(B) from 50 to 80% and from 80 to 83% over a period of 2–10 minutes and 10–20 minutes, respectively. The concentration of MP(B) was continuously increased to 90% by 20.1 minutes and was held at 90% for 10 minutes, then cycled back to the initial condition (50%) yielding a total run time of 40 minutes. Under these conditions uncharged peginesatide and mono-PEG eluted at 18 and 17 minutes, respectively.

**Results**

**Pharmacology.** The pharmacological activity of the dimeric peptide is enhanced by PEGylation. Dimeric peptide doses of 0.1 and 1 mg/kg did not induce increases in RBCs in male cynomolgus monkeys following a single i.v. administration (not shown). The lowest peginesatide dose tested of 0.02 mg/kg, which contains approximately 0.0022 mg/kg of the dimeric peptide, on a weight basis, produced a statistically significant (P < 0.05) increase in RBCs, compared with the concurrent vehicle control (Fig. 2).

Peginesatide induced an initial, generally dose-dependent increase in percent reticulocytes. At peak response on day 5, however, a notable difference between the response at 0.1 and 0.5 mg/kg was not observed, suggesting that the magnitude of the peak response plateaus at a given dose. The increase in reticulocytes subsequently resulted in a dose-dependent increase in RBC parameters. By day 21, reticulocyte values were similar or lower than baseline values across all doses. A decrease below baseline values likely represents negative feedback mechanisms secondary to the peginesatide-induced polycythemia. The duration of the increase in both reticulocytes and RBC parameters was also dose-dependent.

On day 14, mean RBC counts were 5.83 ± 0.513, 6.17 ± 0.533, and 6.72 ± 0.316 × 10^12/μL at 0.02, 0.1, and 0.5 mg/kg, respectively, compared with concurrent vehicle control levels of 5.48 ± 0.384 × 10^12/μL. The increase in RBCs, which reached statistical significance at doses of 0.1 and 0.5 mg/kg, corresponded to hemoglobin increases of 0.50 ± 0.424, 1.40 ± 0.408, and 2.95 ± 1.25 g/dL at 0.02, 0.1, and 0.5 mg/kg, respectively, compared with predose levels. The increases from predose levels in RBCs on day 21 were 0.025 ± 0.114, 0.330 ± 0.075, and 0.855 ± 0.166 × 10^12/μL for 0.02, 0.1, and 0.5 mg peginesatide/kg, respectively, reaching statistical significance at all dose levels. By day 35, RBC counts in the 0.1 and 0.5 mg/kg groups remained elevated. The increase, however, was statistically significant compared with the concurrent vehicle control group at only the high
dose of 0.5 mg/kg. A significant elevation in RBC counts persisted through day 42 in the 0.5 mg/kg group, with increases over concurrent controls of 0.453 ± 0.389 \times 10^6/\mu l. By day 63, values across all dose groups were similar to or approached the levels of the concurrent control group.

The \([14C]\)peginesatide-induced erythropoiesis following an i.v. dose of 2.1 mg/kg was characterized by an initial increase in reticulocytes and a subsequent time-dependent increase in RBCs (Fig. 3). Twenty-one days following administration of radiolabeled peginesatide, there was an increase in RBCs of 1.73 \times 10^6/\mu l. The kinetics of the erythropoietic response, including magnitude of response, following administration of unlabeled 2 mg/kg peginesatide was similar to that obtained with radiolabeled material. Specifically, there was an increase in RBCs of 1.67 \times 10^6/\mu l 21 days after the administration of unlabeled peginesatide (Woodburn et al., 2008b). The data support, therefore, that radiolabeling does not interfere with the pharmacologic activity of peginesatide.

Pharmacokinetics. Plasma concentrations of peginesatide were sustained for at least 72 hours postdose following a single i.v. dose at 0.1 mg/kg, at least 168 hours postdose at 0.5 mg/kg, and for at least 504 hours postdose at 5 mg/kg (Fig. 4). The PK parameters are provided in Table 1. The \(C_{\text{max}}\) values increased in a roughly dose-proportional manner between the doses of 0.1 and 5 mg/kg, while the increase in AUC\(_{0\text{-inf}}\) was greater than dose-proportional. Following a 5-fold increase in dose from 0.1 to 0.5 mg/kg, there was an approximately 8- to 9-fold increase in AUC. Following a 10-fold
increase in dose from 0.5 to 5 mg/kg, there was an approximately 19- to 21-fold increase in AUC.

The CL was typically low, decreasing from 1.22 to 0.435 ml/h per kg across the dose range of 0.1–5 mg/kg, and was only a fraction of the estimated monkey glomerular filtration rate (GFR) of 125 ml/h per kg (Davies and Morris, 1993). The half-life (t1/2) of peginesatide, consequently, increased as a function of dose. The Vss, which was low and apparently independent of dose, ranged from 32.0 to 41.4 ml/kg over 0.1–5 mg/kg and approximated the plasma volume of 44.8 ml/kg described for monkeys (Davies and Morris, 1993). The volume of distribution, therefore, suggests that peginesatide is generally confined to the vascular compartment. Because the kinetics of peginesatide are nonlinear, in the absence of any further data, CL values may have been in flux over the time course of the study (i.e., CL low at earlier time points characterized by high plasma concentrations and higher at later time points characterized by lower plasma concentrations of peginesatide). The CL values, therefore, may represent time- and concentration-dependent averaged values.

The plasma PK of [14C]peginesatide was evaluated in an excretion study and a QWBA study in male cynomolgus monkeys following a single i.v. administration of 5 or 2.1 mg/kg, respectively. The PK parameters, including measured Cmax (i.e., measured at the first sampling time point), AUC, t1/2, CL, and Vss for unlabeled peginesatide as determined by enzyme-linked immunosorbent assay analysis, were similar to the kinetics derived via quantitative radiometric (HPLC fractionation) profiling at a dose of 5 mg/kg (Table 1). The measured Cmax, AUC, CL, and t1/2 obtained in the QWBA study at 2.1 mg/kg (unpublished data) were similar to the toxicokinetic values obtained at a dose of 2 mg/kg administered in a 9-month toxicology study in monkeys (Woodburn et al., 2008b). The data indicate that radiolabeling peginesatide does not alter the PK profile of the molecule and that the analytical methods yield comparable results at a given dose level.

The PK in the QWBA monkeys was characterized in both blood and plasma. Regardless of the biologic matrix, CL was low (0.885 and 0.520 ml/kg per hour for blood and plasma, respectively) and half-life was prolonged (approximately 70 hours in blood or plasma). At all PK time points, radioactivity concentration was greater in plasma than blood. The blood-to-plasma ratios ranged from 0.466 to 0.622, indicating minimal association with blood cells and demonstrating that the majority of [14C]peginesatide was confined to the plasma compartment.

Quantitative Whole-Body Autoradiography Study—Tissue Distribution. Representative whole-body autoradiograms from male cynomolgus monkeys at 48 hours, 1 week, and 3 weeks postdose following a single i.v. administration of [14C]peginesatide at 2.1 mg/kg are depicted in Fig. 5. Drug-derived radioactivity was widely distributed at 48 hours postdose throughout the tissues with the exception of the central nervous system separated from the vasculature by the blood-brain barrier. Tissue distribution, however, was slow as evidenced by tissue/plasma ratios generally <1 through at least 48–72 hours postdose. Based on the sustained blood and plasma levels of [14C]peginesatide (t1/2 of approximately 70 hours), the tissue distribution demonstrated by QWBA analysis at 48 hours is considered likely to reflect the presence of drug within the vascular/lymphatic space of the individual tissues (e.g., confinement of radiolabel to the vascular compartment). Accumulation of radioactivity (tissue concentrations higher than blood) in spleen, lymph node, bone marrow, adrenal gland, and pituitary gland was observed 1 week following administration. Persistently high levels of radioactivity were also observed in the urine through 1 week following administration, supporting renal clearance as a major route of excretion (Table 2).

The highest concentrations of tissue radioactivity or measured Cmax (i.e., measured values at ≥48 hours ranging from approximately 6.5–29 μg equiv/g) were found, based on rank order from highest to lowest, in the red pulp of the spleen, blood, lung, renal medulla, adrenal medulla and capsule, and the highly vascularized nasal turbinates. Tissues with the lowest exposure (i.e., Cmax at 48 hours <1.0 μg equiv/g) included bone, skeletal muscle, small intestine contents, brain, and spinal cord. Concentrations in urine/bladder were notably higher than bile or intestinal contents at each time point throughout the study, supporting renal excretion as a primary route of elimination.

At 1 week postdose, the concentrations of radioactivity were decreasing in the majority of the tissues except in the spleen, lymph node, bone marrow, and adrenal gland. In these tissues, radioactivity concentrations were increasing, which suggested partitioning of peginesatide. Concentrations of radioactivity increased through 3 weeks for the spleen and lymph node and pronounced levels persisted.

### Table 1

Plasma pharmacokinetic parameters of peginesatide in male cynomolgus monkeys after i.v. administration

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Cmax (μg/ml)</th>
<th>t1/2 (h)</th>
<th>AUC0→∞ (μg*h/ml)</th>
<th>CL (ml/h per kg)</th>
<th>Vss (ml/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>2.94 ± 0.30</td>
<td>19.0 ± 2.0</td>
<td>82.9 ± 11.8</td>
<td>1.22 ± 0.16</td>
<td>32.0 ± 2.3</td>
</tr>
<tr>
<td>0.5</td>
<td>13.2 ± 1.04</td>
<td>34.0 ± 5.9</td>
<td>646.2 ± 104</td>
<td>0.79 ± 0.13</td>
<td>37.9 ± 4.1</td>
</tr>
<tr>
<td>5</td>
<td>121 ± 15.6</td>
<td>99.0 ± 37.1</td>
<td>11892 ± 2372</td>
<td>0.435 ± 0.097</td>
<td>41.4 ± 5.7</td>
</tr>
<tr>
<td>5 [14C]</td>
<td>140 ± 10.2</td>
<td>84.0 ± 5.6</td>
<td>11438 ± 1322</td>
<td>0.441 ± 0.46</td>
<td>47.6 ± 3.17</td>
</tr>
</tbody>
</table>

AUC, area under the plasma concentration-time curve; CL, clearance; Vss, volume of distribution at steady state.
in the bone marrow and adrenal gland. The bone marrow had sustained levels that remained fairly constant throughout the study, ranging from 2.32 to 4.93 μg equiv/g, although values appeared to be decreasing by the final time point in the study at 3 weeks postdose.

Elimination of the radioactivity was not complete by the end of the study, but tissue concentrations had generally declined at 3 weeks with the levels in most tissues <1 μg equiv/g of tissue. The exceptions were the spleen, lymph node, bone marrow, adrenal gland, and pituitary gland. The radioactivity levels in the spleen and lymph nodes remained increased compared with 48 hours but were similar for week 1 versus week 3. The radioactivity levels in the bone marrow, adrenal gland, and pituitary gland were approximately 53, 36, and 56% lower, respectively, at week 3 compared with week 1.
Microautoradiography. Microautoradiography analyses at 48 hours postdose revealed uniform distribution of radioactivity in the bone marrow and liver. Radioactivity within the bone marrow extracted from the femur was uniform and relatively high (Fig. 6A). There was no obvious cell-associated accumulation of radioactivity. Bone matrix did not contain radioactivity (not shown). Moderate distribution of radioactivity was seen throughout the marrow cells of the sternum. Similarly, the radioactivity in the liver (Fig. 6D) and thymus (not shown) was scattered and light.

Microautoradiography demonstrated differential distribution in the spleen and, to a lesser extent, the lymph nodes. A notable differential distribution was observed between red and white pulp of the spleen (Fig. 6B). The MARG results are consistent with the QWBA data (Table 2) that yielded red pulp/white pulp ratios of 2.56- to 7.8-, and 4.94-to-1 at 48 hours and 1 and 3 weeks, respectively. Radioactivity was diffusely distributed in the axillary and mesenteric lymph nodes, with only a slight accumulation at the blood/lymphatic vessels and no cell-specific localization (Fig. 6C).

In the kidney, comparable distribution was noted across the glomeruli, proximal and distal convoluted tubules, and collecting ducts, blood vessels, and interstitial cells of the renal cortex (Fig. 7A). In the medulla, however, radioactivity was more obvious in the interstitium. There was less association of radioactivity with the lumen of the ducts compared with cells in the interstitium (Fig. 7B). Similarly, radioactivity in the papilla was associated primarily with cells in the interstitium, while little was seen in the collecting ducts (Fig. 7C).

Excretion. The major route of drug excretion following i.v. administration of 5 mg/kg [14C]peginesatide was in the urine with lesser amounts excreted in the feces. By 24 hours after administration of [14C]peginesatide, the excretion ratios for urine and feces were 12.4 ± 2.4% and 0.2 ± 0.2% of the radioactive dose, respectively. The ratios of the radioactive dose excreted into the urine and feces at 96 hours after dosing were 34.3 ± 4.6% and 2.2 ± 1.6%, respectively. Excretion was not complete by 14 days postdose with approximately 67% of the total radioactive dose excreted by the end of the collection period. Of the total radioactivity excreted by the end of the collections, approximately 60% was excreted in the urine and approximately 7% in the feces. Of the excreted radioactivity, a large proportion (approximately 34% of the radioactive dose) was excreted in the urine during the first 96 hours postdose. During the remainder of the study...
(i.e., 96–366 hours postdose), the total daily radioactivity excretion ranged from 1.5 to 5.6% of the administered dose. The extent of excretion and recovery of the dose administered are depicted in Fig. 8 and Table 3.

**Plasma, Urine, and Fecal Metabolite Profiles.** Quantitative metabolite profiling was performed using pooled plasma, urine, or fecal samples. The plasma concentration-versus-time profile is depicted in Fig. 9. Peginesatide was the major component in plasma. At 0.25 hours postdose, the plasma content of the parent and mono-PEG was essentially the same as the dose formulation. Specifically, peginesatide and mono-PEG accounted for approximately 90 and 10% of the total radioactivity, respectively, in both plasma and dose formulation. By 168 hours (day 7) postdose, [14C]peginesatide represented over 90% of the total radiolabel in the plasma. At 336 hours (day 14), [14C]peginesatide remained the only major circulating drug-related component in plasma, with a 14C-AUC for the parent molecule accounting for 87.1% of the total 14C-AUC (Table 4). The disappearance of mono-PEG from plasma appeared to be faster than peginesatide, resulting in a relative exposure of 5.4% of total 14C-AUC and a half-life of 34.7 hours (Table 4). The rate of elimination of mono-PEG versus peginesatide provides further support that the mono-PEG in plasma comes from the impurity in the radiolabeled preparation, rather than being formed in vivo.

The major moiety in urine was also the parent molecule, peginesatide (Table 3). The cumulative recovery of radioactivity (i.e., at 336 hours postdose) in the urine associated with peginesatide, mono-PEG, and unidentified components was 42.6, 15.9, and 1.2%, respectively, of the total radioactive dose. The urinary radioactivity of peginesatide, mono-PEG, and unidentified components comprised 71.4, 26.6, and 2.0%, respectively, of the cumulative excreted radioactive dose. The recovery of peginesatide and mono-PEG in the feces was below the limit of quantification. Unidentified components (Other) represented 7% of the administered radioactive dose or 100% of the fecal radioactivity.

**Discussion**

Erythropoietic activity of the dimeric peptide portion of peginesatide, discovered by screening against human EPOr (Fan et al., 2006), was enhanced by PEGylation with a 40-kDa branched PEG. PEGylation improves the PK of protein and peptide therapeutics by reducing renal clearance and protecting the molecule from enzymatic proteolysis or antibody neutralization (Caliceti and Veronese, 2003; Harris and Chess, 2003). Enhanced erythropoietic activity of PEGylated dimeric peptide (i.e., peginesatide) is considered largely a function of prolonged peginesatide plasma levels (Green et al., 2011).

Peginesatide plasma PK in monkeys demonstrated a profile consistent with concentration-dependent processes, exhibiting a Michaelis-Menten type saturable elimination. Population PK modeling, based on doses of 0.02–0.21 mg/kg, indicated that peginesatide kinetics in humans is also characterized by Michaelis-Menten elimination. The near dose-proportional increase in \( C_{\text{max}} \) in monkeys suggests a dose-independent initial volume of distribution. A greater than dose-proportional increase in AUC (i.e., 143-fold increase) was related...
to reduced plasma clearance and increased half-life with increasing dose from 0.1 to 5 mg/kg (i.e., a 50-fold increase).

A $V_{ss}$ in the monkey approximating plasma volume (44.8 ml/kg; Davies and Morris, 1993) suggests peginesatide is generally confined to the vascular compartment. The initial widespread tissue radioactivity in the biodistribution study is considered primarily due to peginesatide-associated radiolabel confined to the vascular space. Based on size, peginesatide would not readily cross the tight interendothelial junctions and complete basement membrane of the vasculature that constitutes a relatively effective barrier to molecules $\geq 2$ kDa (Porter et al., 2001).

A similar peginesatide PK profile was observed across species (i.e., mouse, rat, and monkey) following a single i.v. dose (Woodburn et al., 2011, 2012). Regardless of species, peginesatide plasma elimination was generally biphasic with a relatively rapid initial phase and prolonged elimination phase; CL was typically slow and less than the GFR; and $V_{ss}$ was low often approximating plasma volume. A generally dose-related decrease in clearance and increase in half-life generally translated into nonlinear kinetics. Half-life in the mouse at 0.1–10 mg/kg ranged from approximately 14 to 51 hours (Woodburn et al., 2011) and in rats at 0.1–5 mg/kg from approximately 18 to 32 hours (Woodburn et al., 2012).

A dose-dependent effect on clearance has also been reported for rHuEPO (Woo et al., 2007), with ESA clearance attributed to multiple mechanisms, both saturable and nonsaturable (Agoram et al., 2009). Nonsaturable clearance mechanisms may involve renal glomerular filtration (Kato et al., 1997). Peginesatide clearance via renal glomerular filtration is consistent with the excretion data in normal animals and the decrease in clearance of approximately 57% for 5/6 nephrectomized rats (Fan et al., 2006). Most of the excreted urine radioactivity in the monkey was associated with parent compound. Although branched PEG clearance may be facilitated by slow release of one of the two PEG lysine-linked chains on the carbamate bond between the PEG chains and the linker (Guiotto et al., 2004), the monkey data do not support that there is a similar degradation of peginesatide.

The nonlinear kinetics of rHuEPO has been attributed to saturable receptor-mediated endocytosis (RME) (Woo et al., 2007). Following ESA binding to EPOr, internalization or dissociation of the molecule from the receptor can occur. Once internalized, the ligand may be degraded or recycled intact to the cell surface and released. Peginesatide has been shown to have high affinity for the EPOr (Fan et al., 2006). Internalization, however, has not yet been shown.

Agoram et al. (2009) suggest that non-EPOr-mediated pathways, including clearance in the interstitium or lymphatic system, may contribute to elimination and that non-EPOr-mediated pathways may play a greater role than RME for hyperglycosylated and PEGylated ESAs. A 30-kDa PEGylated epoetin beta (Mircera) does not appear to be internalized when incubated with EPO receptor-bearing UT-7 cells, unlike epoietin beta [EMEA, 2010 (http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000739/human_med_000905.jsp&mid=WCOb01ac058001d124)]. The findings

<table>
<thead>
<tr>
<th>Compound</th>
<th>Urine</th>
<th>Feces</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-168 h</td>
<td>168-336 h</td>
</tr>
<tr>
<td>Total radioactivity</td>
<td>47.7 (100)</td>
<td>12.0 (100)</td>
</tr>
<tr>
<td>Peginesatide</td>
<td>35.2 (70.8)</td>
<td>7.4 (61.7)</td>
</tr>
<tr>
<td>Mono-PEG</td>
<td>12.1 (28.3)</td>
<td>3.8 (31.7)</td>
</tr>
<tr>
<td>Other</td>
<td>0.4 (0.9)</td>
<td>0.8 (6.6)</td>
</tr>
</tbody>
</table>

BLQ, below the lower limit of quantitation; PEG, polyethylene glycol.

Fig. 9. Plasma concentration-versus-time profile for total $^{14}$C-radioactivity, $[^{14}$C]peginesatide, and $[^{14}$C]mono-PEG in monkey plasma following a single i.v. administration of 5 mg/kg $[^{14}$C]peginesatide.
Pharmacokinetic parameters of peginesatide and related compounds following a single i.v. dose of 5 mg/kg [14C]peginesatide to cynomolgus monkeys

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cmax</th>
<th>AUC0–336 h</th>
<th>CL</th>
<th>t1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg equiv./ml</td>
<td>µg equiv./h/ml</td>
<td>ml/kg/h</td>
<td>h</td>
</tr>
<tr>
<td>Total radioactivity</td>
<td>140</td>
<td>11,005 (100)</td>
<td>0.43</td>
<td>65.1</td>
</tr>
<tr>
<td>Peginesatide</td>
<td>118</td>
<td>9580 (87.1)</td>
<td>0.436</td>
<td>64.3</td>
</tr>
<tr>
<td>Mono-PEG</td>
<td>16.4</td>
<td>596 (5.4)</td>
<td>1.05</td>
<td>34.7</td>
</tr>
<tr>
<td>Other</td>
<td>ND</td>
<td>829 (7.5)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

AUC, area under the plasma concentration-time curve; CL, clearance; ND, not detected; PEG, polyethylene glycol.

are consistent with the literature suggesting that PEGylation may lower RME (Webster et al., 2007) due to lower receptor affinity. A 64% decrease in clearance associated with a 50-fold increase in peginesatide was observed at higher doses in the normal monkey and may reflect saturation of receptor- and nonreceptor-mediated mechanisms. The decrease in peginesatide clearance in the rat renal-failure model may reflect saturation of receptor- and nonreceptor-mediated mechanisms to a greater or lesser extent, in addition to decreased GFR.

Monkey distribution data should be interpreted and extrapolated to humans within the context of dose differences. While a 0.1 mg/kg dose in the monkey is 2.5-fold higher than the human starting dose of 0.04 mg/kg administered every 4 weeks, the 5 mg/kg dose is 125-fold higher than the human starting dose. The nonclinical doses were selected to obtain a recommended targeted radioactive dose of 16–300 µCi/kg (Solon et al., 2010) and based on the achievable specific activity of [14C]peginesatide. Administration of lower clinically relevant doses would not have delivered adequate radioactivity for the determination of tissue distribution using both QWBA and MARG analyses.

Radioactivity concentrations were decreasing in most tissues in the monkey at 1 week postdose. The increased radioactivity concentration in spleen, lymph node, bone marrow, adrenal gland, and urine suggests a partitioning of peginesatide into these tissues or increased excretion (urine). The relatively high peginesatide-associated radioactivity levels in the lymphatic tissues may reflect the uptake or removal of the macromolecule, transport to the draining lymph nodes, and the potential processing and/or degradation of the molecule (Porter et al., 2001).

The ability to collect potentially meaningful quantitative tissue distribution data with peginesatide is in contrast to approved protein-based ESAs. Radiolabeling of proteins, such as HuEPO and darbepoetin alfa, with iodine-125 can lead to results confounded by label instability with cleavage and uptake of free label into various tissues by transporters (Solon, 2012). For peginesatide, 14C can be used to radiolabel the lysine linker between the peptide dimer and the two 20-kDa PEG chains.

Peginesatide biodistribution data may potentially provide insight into the biodistribution of ESAs and perhaps even EPOr sites. The data suggest that peginesatide is localized to known EPOr sites (bone marrow), sites that may have functional EPOr (e.g., splenic red pulp), and putative EPOr sites such as the renal medulla and papilla (Westenfelder et al., 1999). There is considerable controversy, however, with respect to not only EPOr distribution, but whether the EPOr is functional if expressed on cells other than hematopoietic progenitor cells.

The bone marrow is the primary hematopoietic site in monkeys. Unlike rodents, in which notable EMH occurs throughout the animal’s lifespan, EMH does not normally occur in nonhuman and human primates. (Greaves and Faccini, 1992; O’Malley et al., 2005). Furthermore, unlike in rodents (Woodburn et al., 2009), EMH has not been shown to occur in monkeys administered peginesatide up to 20 mg/kg i.v. every 3 weeks for 9 months (Woodburn et al., 2008a,b). Under certain pathologic conditions and in response to certain biologic modifiers, EMH has been shown to occur in the spleen, lymph node, thymus, adrenal gland, pituitary, and kidney of monkeys (Welte et al., 1987; Okasaki et al., 2002; Starost et al., 2004) and in the spleen of humans (O’Malley et al., 2005). The EMH in rodents and, under certain conditions in primates, may involve the erythroid lineage, which suggests that EPOr occurs at these sites and may be or may become functional. The peginesatide data suggest that although drug may bind to splenic EPOr, the receptor was not functional, under the conditions of the study, since no erythropoietic effect was noted in the monkeys.

Following i.v. administration of peginesatide, the pronounced, sustained erythropoietic response increased with increasing dose and exposure across animal species and in normal healthy volunteers (Fan et al., 2006; Stead et al., 2006). A pharmacologically active i.v. peginesatide dose of 0.1 mg/kg resulted in an average maximum change in baseline hemoglobin of 1.36 g/dl in normal healthy volunteers (Stead et al., 2006) and of 1.4 g/dl in monkeys. The apparently more pronounced effect in rodents likely reflects the robust peginesatide-associated EMH response in the spleen and liver.

A greater erythropoietic response in 5/6 nephrectomized rats compared with normal animals is likely due to at least two factors, including altered PK (i.e., a 2-fold slower clearance and higher exposures) and diminished activity in nephrectomized rats of the negative feedback controls on erythropoiesis that would come into play following administration of peginesatide in normal animals (Fan et al., 2006). The nephrectomized rat findings are consistent with data in clinical trials of peginesatide showing an enhanced pharmacological response, decreased CL, and an increase in half-life in CKD patients compared with normal healthy volunteers.

The ADME and pharmacology of peginesatide have been extensively evaluated in monkeys. The similarity in PK and pharmacology parameters for monkeys and humans suggests that the monkey data can be extrapolated to humans and that the monkey is an appropriate species for evaluating peginesatide effects. The robust, sustained erythropoietic response in monkeys is consistent with the clinical data demonstrating that once monthly dosing of peginesatide maintains hemoglobin in dialysis patients.

Authorship Contributions

Participated in research design: Woodburn, Fong, Wilson, Sloneker, Moriya, Tagawa.

Conducted experiments: Sloneker, Strzemienski, Solon, Moriya, Tagawa.

Performed data analysis: Woodburn, Fong, Sloneker, Strzemienski, Solon, Moriya, Tagawa.

Wrote or contributed to the writing of the manuscript: Woodburn, Fong, Wilson, Sloneker, Moriya, Tagawa.

References


