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Preclinical evaluation of Hematide, a novel erythropoiesis stimulating agent, for the treatment of anemia

Qing Fan^a, Kerstin K. Leuther^a, Christopher P. Holmes^a,
Kei-lai Fong^b, Jim Zhang^a, Svetlana Velkovska^a, Min-jia Chen^a, Richard B. Mortensen^a,
Karen Leu^a, Jennifer M. Green^a, Peter J. Schatz^a, and Kathryn W. Woodburn^a

^aAffymax, Inc, Palo Alto, Calif., USA; ^bPennsylvania Biolab, Inc, Radnor, Penn., USA

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Objective. To evaluate the preclinical erythropoiesis stimulating properties of Hematide, a novel, PEGylated, synthetic peptide for the treatment of anemia associated with chronic kidney disease and cancer.

Methods. The *in vitro* activity of Hematide was assessed in competitive binding, proliferation, signal transduction, and apoptosis assays, and in erythroid colony-forming assays with CD34⁺ cells purified from human bone marrow. Erythropoiesis and pharmacokinetics were evaluated in rat, monkey, and a rat chronic renal insufficiency (CRI) model following single administration. Erythropoiesis and immunogenicity were also evaluated following repeat administration in rats.

Results. Hematide binds and activates the erythropoietin receptor and causes proliferation and differentiation of erythroid progenitor cells. Sustained circulatory persistence of Hematide is observed in rats and monkeys. In a rat CRI model, Hematide exhibited twofold lower clearance than in the normal rat, with hypothesis consistent with Hematide being cleared, at least partially, via the kidney. A dose-dependent rise in hemoglobin (Hgb) and duration of response was observed following single administration in rats and monkeys. Hematide was able to alleviate anemia in an experimental CRI rodent model. Repeat intravenous (IV) and subcutaneous (SC) administration in rats yielded similar erythrogenic responses, with no anti-Hematide antibodies being detected.

Conclusions. Hematide is a potent erythropoiesis stimulating agent with a prolonged half-life and slow clearance times. It is anticipated that similar prolonged clearance and activity will be observed in the clinic, potentially enabling dosing intervals of 3 to 4 weeks that may translate into improved patient convenience for the treatment of anemia. © 2006 International Society for Experimental Hematology. Published by Elsevier Inc.

Anemia is a hematological disorder prevalent in cancer patients receiving chemotherapy and patients suffering from chronic kidney disease and end-stage renal disease [1,2]. Erythropoietin (EPO) is a glycosylated protein hormone which maintains erythropoiesis homeostasis. Current anemia management uses recombinant human erythropoietin, rHuEPO or epoetin, which is administered up to three times weekly, or darbepoetin alfa (Aranesp), which is typically given weekly or biweekly.

Hematide, a peptidic erythropoiesis stimulating agent (ESA) coupled to polyethylene glycol (PEG), is being developed to overcome the shortcomings of currently approved erythropoietic agents, namely frequent administration of temperature-sensitive recombinant proteins. We have previously reported the discovery of novel erythropoietic synthetic peptides possessing no sequence homology to EPO [3], and subsequent extensive chemical analoging and architectural modifications including dimerization [4] has resulted in novel peptides now equipotent to EPO in functional cell-based assays.

In this paper, we demonstrate that Hematide is a potent stimulator of erythropoiesis with sustained activity. The PEG moiety confers increased peptide solubility and

Offprint requests to: Kathryn W. Woodburn, Ph.D., Affymax, Inc, 4001 Miranda Avenue, Palo Alto, CA 94304; E-mail: Kathryn_woodburn@affymax.com

stability and reduced renal clearance, thereby increasing plasma persistence [5,6]. Hematide is a full agonist of the EPO receptor in several *in vitro* cellular assays. Since Hematide lacks the immunological epitopes of EPO, it is much less likely to induce the cross-reactive immune response against recombinant or endogenous EPO that has been found to cause serious side effects and loss of potency of rHuEPO therapeutics [7]. Also, while antibodies to Hematide may result in loss of efficacy, patients would be expected to respond to currently approved ESAs. In view of the robust and prolonged *in vivo* activity of Hematide, use of this drug in anemia management may allow for less frequent dosing, thereby increasing patient convenience.

Materials and methods

Erythropoiesis stimulating agents

Hematide is a dimeric peptide, weighing approximately 5000 amu, and was synthesized via traditional solid-phase synthesis followed by a single site-specific conjugation to PEG. The sequence of the peptide portion of Hematide is unrelated to rHuEPO or to the sequences of any other known human proteins. rHuEPO was obtained from R&D Systems (Minneapolis, MN, USA) or from Ortho Biotech (Procrit; Raritan, NJ, USA). Aranesp was obtained from Amgen (Thousand Oaks, CA, USA) while ^{125}I -EPO was obtained from Amersham Biosciences (Piscataway, NJ, USA).

Binding assays

The competition binding assay was carried out by incubating 100 pg of HuEPOr-Fc (R&D Systems) with serial dilutions of competitor and 20,000 cpm ^{125}I -EPO, followed by incubation, addition of Protein G beads, washing, and counting, using conditions based on a method for the detection of anti-EPO antibodies [8]. The specificity of the binding properties of Hematide was evaluated by MDS Pharma Services (Taipei, Taiwan) in a screen of 66 diverse receptors (LeadProfilingScreen with addition of IL-2, IL-6, Leptin, TGF- β , and TNF receptors).

Proliferation assays

The EPO-responsive UT-7/EPO cell line was obtained from Dr. N. Komatsu [9] and maintained in IMDM supplemented with 10% fetal bovine serum (FBS), 1 U/mL recombinant human EPO, and 2 mM glutamine. Cells were resuspended in Starvation Medium (IMDM, 10% FBS, and 2 mM glutamine) and incubated for 24 hours at 37°C in a 5% CO₂ incubator. The next day, the cells were plated at 5000 cells/well in 96-well tissue culture plates, and serially diluted agonists were added. Proliferation was assessed after 4 days using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [10]. The data were analyzed using GraphPad Prism software to calculate EC₅₀ values from a 4-parameter logistic equation.

Ba/F3 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and transfected with the genes encoding a HuEPOr extracellular domain chimera with the intracellular domain of the human G-CSF receptor, the human G-CSF receptor, or the human TPO receptor. These cells were maintained in DMEM/F12 containing 10% FBS, 2 mM glutamine, and 2% WEHI conditioned medium. The night before the experiment,

cells were washed; resuspended in medium containing DMEM/F12, 10% FBS, 2 mM glutamine, and 0.01% WEHI conditioned medium; and incubated at 37°C in a 5% CO₂ incubator. On the day of the assay, cells were resuspended in assay medium that consisted of DMEM/F12, 10% FBS, and 2 mM glutamine and plated at 50,000 cells/well in 96-well tissue culture plates. Serially diluted agonists were added, the cells were incubated for 48 hours, and proliferation was assessed after 48 hours using MTT.

In vitro proliferation assays were also conducted by MDS Pharma Services (Taipei, Taiwan), using 0.1 to 1000 nM Hematide, in the following human tumor lines; MCF-7 breast adenocarcinoma, HT-29 colon adenocarcinoma, A549 lung carcinoma, SK-MEL-5 melanoma, MES-SA uterus sarcoma, PC-3 prostate adenocarcinoma, and OVCAR-3 ovarian adenocarcinoma in addition to human TF-1 erythroblasts.

Apoptosis assays

UT-7/EPO cells were washed and resuspended at 2.5×10^5 cells/mL in Starvation Medium. ESAs were added for 48 to 96 hours followed by cell staining using the Annexin V-FITC Apoptosis Detection Kit and propidium iodide (BD Biosciences, San Diego, CA, USA) using the manufacturer's protocol. Alternatively, cells were stained with FITC-conjugated anti-PARP cleavage site-specific antibodies using the manufacturer's protocol (Biosource International, Camarillo, CA, USA). Stained cells were analyzed by flow cytometry.

Signal transduction

UT-7/EPO cells were washed and resuspended in Starvation Medium before incubation overnight. Cells were treated with ESAs for 10 minutes in a 37°C sandbath. The cells were lysed in an equal volume of 2 \times lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2% Triton X-100, 2 mM Na₃VO₄, 40 mM NaF, 0.1 mM Na₂MoO₄, 1 mM Na₂H₂P₂O₇, 50 ng/mL calyculin A, 25 $\mu\text{g}/\text{mL}$ aprotinin, 25 $\mu\text{g}/\text{mL}$ leupeptin, and 500 μM phenylmethylsulfonyl fluoride for 1 to 2 hours at 4°C and then clarified by centrifugation at 14,000g for 5 minutes. Clarified cell lysates were immunoprecipitated by rotation overnight with antibodies to JAK2, STAT5A, STAT5B, or AKT (Upstate, Lake Placid, NY, USA; R&D Systems, Minneapolis, MN, USA) and 75 μL 1:1 slurry of Gamma Bind plus Sepharose (Pharmacia Biotech, Piscataway, NJ, USA). After extensive washing, the immunoprecipitates were eluted from the beads and analyzed by SDS-PAGE and Western blotting with the use of antibodies to phosphotyrosine or phospho-AKT (Ser473) (Upstate, Lake Placid, NY, USA). Peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham, Piscataway, NJ, USA) were used for development.

Erythroid (BFU-E) colony assay

Human CD34⁺ cells (Cambrex, Walkersville, MD, USA) were resuspended at 15,000 cells/mL in IMDM with 10% FBS. ESAs were added to a methylcellulose-based colony assay media (MethoCult GF H4534, StemCell Technologies, Vancouver, BC, Canada) supplemented with rhSCF, rhGM-CSF, and rhIL-3. Cells were added to the methylcellulose and plated with subsequent incubation at 37°C, in a 5% CO₂ incubator for 12 to 14 days. Colonies containing over 50 cells were counted by phase-contrast microscopy.

Animals

All animals received care in compliance with Guide for the Care and Use of Laboratory Animals (NIH Publication, 1996). Male Sprague-Dawley rats, 9 to 11 weeks, were obtained from Charles River Laboratories (Hollister, CA, USA). Rats, surgically prepared with jugular and femoral vein cannulae implants (Hilltop Laboratories, Inc, Scottsdale, PA, USA), were utilized for the normocytic rat pharmacokinetic studies. The 5/6 nephrectomized rats, known here also as the chronic renal insufficiency rats (Charles River Laboratories, Raleigh, NC, USA and Hollister, CA, USA) were placed on study 25 days following the nephrectomy procedure. Male *Cynomolgus* monkeys were obtained from Covance (Denver, PA, USA) and Charles River Laboratories (Worcester, MA, USA). All administrations were at 1 mL/kg in acetate buffered saline, pH 5.5.

Rat pharmacology

Normocytic rats, 4 per group, received a single intravenous (IV) dose of Hematide at 0, 0.01, 0.05, 0.1, 1, 10, and 50 mg/kg via the tail vein. Erythropoiesis was also assessed following repeated IV or subcutaneous (interscapular) injections in rats, 4 per group, using doses of 0, 0.135, and 1.35 mg/kg weekly for 6 weeks or every other week for 3 administrations. Blood (0.5 mL) was collected for hematology by retro-orbital puncture of mefotane-anesthetized rats into EDTA-containing tubes on days 3, 5, 9, 14, 19, 28, 38, and 43 for the single-dose experiment and on days 15, 29, 43, 50, and 64 for the once-weekly cohort and on days 15, 29, 43, and 57 for the every-other-week cohort. Anti-Hematide antibody assessment was performed on serum (0.25 mL) at day 43 for all dose groups, at day 78 for the IV groups, and at day 85 for the subcutaneous (SC) dose groups.

Partially nephrectomized (CRI) rats were dosed IV at 0, 0.1, 1, and 10 mg Hematide/kg via the tail vein with hematologic parameters measured pretest and on days 3, 5, 9, 15, 19, 23, 29, 33, 38, and 44.

Rat pharmacokinetics

Hematide was administered at doses of 0.138, 0.69, 1.38, 6.9, or 13.8 mg/kg IV via the tail vein with 5 animals per group. Blood samples (0.4 mL) were collected from each animal at predose and 0.083, 0.5, 1, 4, 10, 24, 72, and 96 hours postdose into heparin-containing tubes. For studies in CRI rats, 11 rats were divided into two blood sampling cohorts (A and B). Hematide was administered IV via the tail vein at 9.87 mg/kg with blood samples (0.2 mL) collected from each animal in cohort A at predose and 0.5, 4, 24, and 96 hours postdose, and for Cohort B, at 0.25, 1, 10, 72, and 120 hours.

Monkey pharmacokinetics and pharmacology

Hematide was administered IV to *Cynomolgus* monkeys at 0.02, 0.1, 0.475, and 1.35 mg/kg with 3 animals per group. For the 0.02, 0.1, and 0.475 mg/kg dosing cohorts blood samples (0.5 mL) were collected from each animal at predose and 0.25, 2, 6, 12, 24, 48, 72, 120, 144, and 168 hours postdose for pharmacokinetics. Blood samples (0.5 mL) for hematology were collected prior to dosing, and on days 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 35, and 43. For the 1.35 mg/kg cohort, pharmacokinetic blood samples were collected at 0, 0.083, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, and 168 hours. Blood was also collected

for hematology evaluation at predose and on days 0.5, 1, 2, 3, 4, 5, 6, 7, 9, 11, 14, 17, 21, 24, 28, 31, 35, 38, 42, and 45.

Laboratory analysis

Complete blood counts including reticulocytes were measured on an AdviaR120 hematology analyzer (Bayer Diagnostics, Tarrytown, NY, USA). Renal function was evaluated by measuring serum levels of blood urea nitrogen (BUN) and creatinine by standard urease and alkaline picrate methods on a Hitachi 747 clinical chemistry analyzer (Roche, Indianapolis, IN, USA).

Hematide sample evaluation

Hematide plasma concentration was determined using a competition enzyme-linked immunosorbent assay (ELISA) with a quantification limit of 31.5 ng/mL. This method involves preincubation of sample dilutions, and a Hematide standard curve, with a high-affinity anti-Hematide rabbit polyclonal antibody preparation, followed by incubation in microtiter wells coated with Hematide, washing, and development with anti-rabbit IgG secondary antibody conjugate. The assay detects both PEG-conjugated peptide and free peptide. Noncompartmental pharmacokinetic parameters were calculated from plasma concentration-time profiles of Hematide using WinNonlin software (version 4.1, Pharsight, Mountain View, CA, USA). Anti-Hematide antibody determination was performed using an ELISA with wells directly coated with Hematide followed by application of sera with subsequent detection with goat anti-rat IgG alkaline phosphatase conjugate (Biosource International). The sensitivity of the assay for Hematide-specific IgG was estimated to be 120 to 320 ng/mL using several different positive control mouse monoclonal antibodies to Hematide that were obtained from mice immunized in incomplete Freund's adjuvant with the peptide component of Hematide conjugated to human serum albumin or keyhole limpet hemocyanin.

Statistical analyses

Data are expressed as mean with 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.

Results

In vitro pharmacology

In a HuEPOr/¹²⁵I-EPO competition binding assay, rHuEPO and Aranesp, non-PEGylated peptide precursor of Hematide (dimer), and Hematide yielded IC₅₀ values of 7, 12, 7, and 37 pM, respectively. Thus, the dimer binds to a cross-competitive site on HuEPOr with affinity equivalent to that of the natural ligand. Conjugation to PEG causes approximately a fivefold loss in binding potency. Hematide binding is highly specific for the EPO receptor since little or no pharmacologically relevant binding of Hematide was detected against a panel of 66 diverse receptors. Additionally, Hematide did not significantly stimulate the growth of seven human tumor cell lines but did have a cytoproliferative effect on human EPO-responsive TF-1 erythroblast cells with an EC₅₀ of 0.9 nM (see Materials and methods).

Hematide stimulated EPO-responsive UT-7/EPO cells [9] through the JAK/STAT pathway in a manner equivalent to EPO as evidenced by phosphorylation of JAK2, STAT5A, and STAT5B (Fig. 1A). EPO, Aranesp, dimer, and Hematide stimulated cell proliferation with EC₅₀s of 36, 17, 260, and 460 pM, respectively (Fig. 1B). Hematide does not stimulate cell proliferation of Ba/F3 cells transfected with the human receptors for the related hematopoietic cytokines TPO and G-CSF, whereas it does activate

Ba/F3 cells transfected with a gene encoding a HuEPOr extracellular domain chimera with the intracellular domain of the human G-CSF receptor. The EC₅₀s of EPO, Aranesp, dimer, and Hematide in the latter cells were 14, 3.4, 11, and 180 pM, respectively.

When deprived of HuEPOr agonist activity, UT-7/EPO cells undergo apoptosis [11–13]. Like EPO, Hematide blocked apoptosis (Fig. 1C) and prevented caspase activation (Fig. 1D). Finally, Hematide stimulated the anti-apoptotic

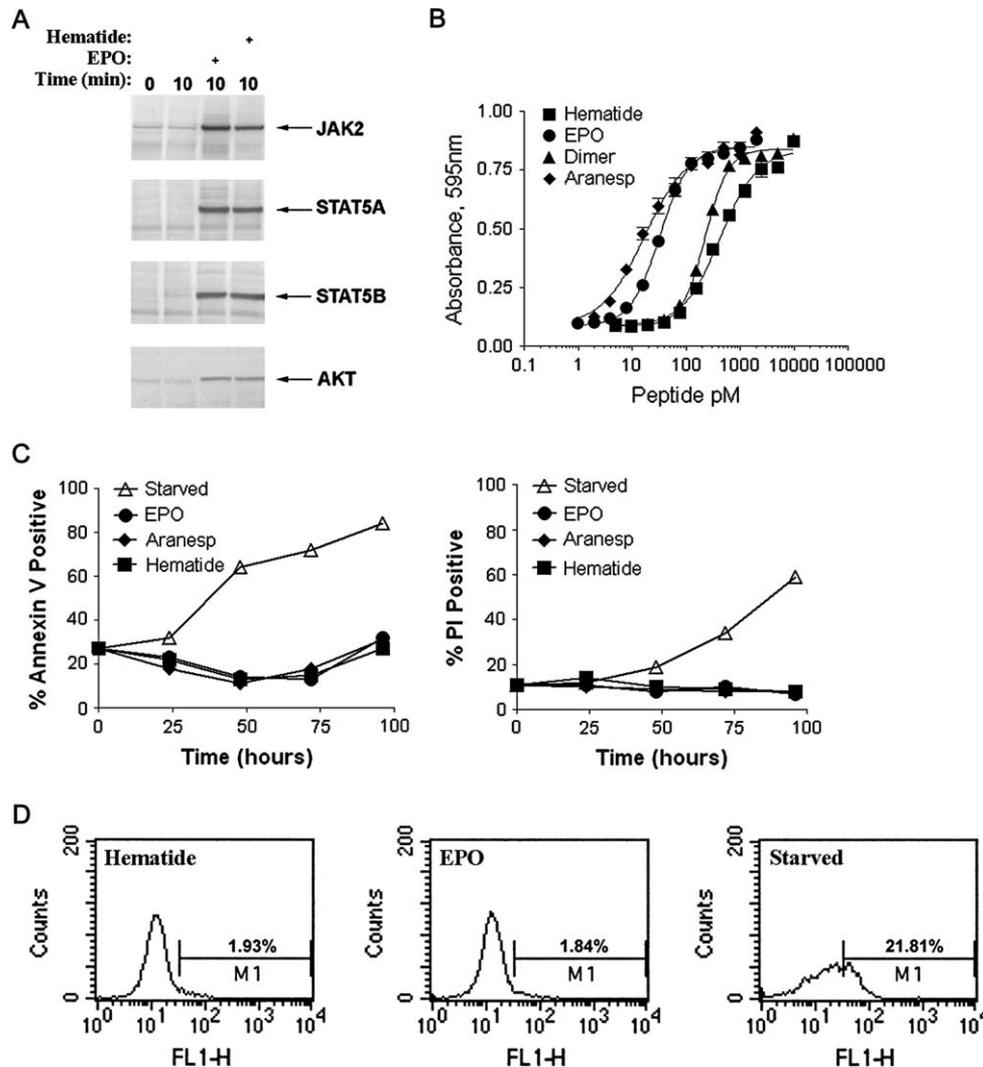


Figure 1. Hematide stimulates the proliferation and differentiation, and blocks apoptosis, of HuEPOr-expressing cells. (A) JAK2, STAT5A, STAT5B, and AKT were immunoprecipitated from lysates prepared from UT-7/EPO cells stimulated for 10 minutes with vehicle, 10 nM Hematide, or 10 U/mL Procrit. Protein extracts were quantified by bicinchoninic acid (BCA) to ensure that equal amounts of protein were present in each immunoprecipitation. These were analyzed by SDS-PAGE and Western blotting with the use of anti-phosphotyrosine or anti-phospho-AKT pSer473 antibodies. (B) Serially diluted recombinant human EPO, Aranesp, non-PEGylated peptide, and Hematide were added to 5000 UT-7/EPO cells/well in 96-well tissue culture plates and incubated at 37°C for 4 days. Cell proliferation was assessed using MTT. (C) UT-7/EPO cells (2.5×10^5 cells/mL) were cultured for 96 hours in the presence of 50 nM Hematide, 11 U/mL EPO, 11 U/mL Aranesp, or with vehicle (starved cells). An aliquot of cells was removed at various time points for staining with FITC-conjugated annexin V (left) and PI (right). Flow cytometry was used to assess the percentage of cells undergoing apoptosis. (D) Alternatively, UT-7/EPO cells (2.5×10^5 cells/mL) were cultured for 48 hours in the presence of 10 nM Hematide, 10 U/mL Procrit, or vehicle (starved cells). The cells were then stained with FITC-conjugated anti-PARP cleavage site-specific antibodies followed by flow cytometry analysis. As a control to ensure antibody specificity, staining was performed in the presence of excess PARP cleavage site peptide. The positive staining observed in the starved cells was blocked (data not shown).

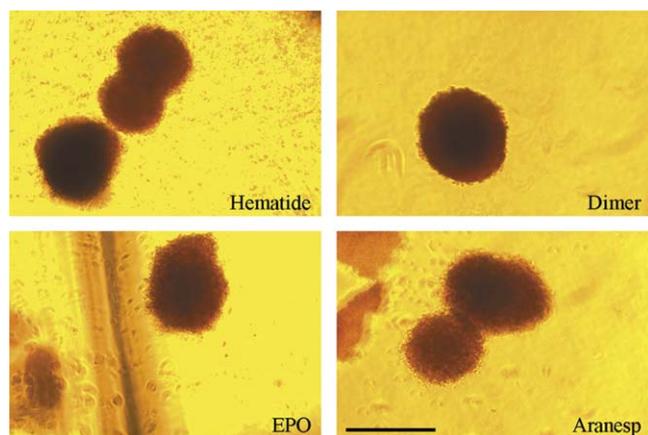


Figure 2. Hematide stimulates the differentiation of CD34⁺ bone marrow cells into BFU-E colonies. Human CD34⁺ cells were incubated for 14 days in methylcellulose-based colony assay media containing 100 nM Hematide, 100 nM non-PEGylated peptide precursor of Hematide (Dimer), 0.8 nM recombinant human EPO, or 6.8 nM Aranesp. Colonies containing over 50 cells were counted by phase-contrast microscopy. No BFU-E colonies grew in the absence of a HuEPO α agonist. Images were taken using 100 \times magnification, scale bar equals 300 μ m.

PI 3-kinase pathway leading to serine phosphorylation of the downstream effector AKT/protein kinase B (Fig. 1A).

Hematide caused proliferation and differentiation of primary human CD34⁺ bone marrow cells into erythroid colonies (BFU-E) of similar size and morphology to those produced by EPO and Aranesp (Fig. 2). The EC_{90S} of EPO, Aranesp, dimer, and Hematide were approximately 180, 400, 4000, and 3000 pM, respectively.

Pharmacokinetics

The pharmacokinetic parameters of Hematide following single IV administration are summarized in Table 1. In rats, the terminal half-life was generally similar at all dose levels, ranging from 21.5 to 30.7 hours with dose-related and dose-proportional increases of C_{max} and AUC (area under the curve) values. The nephrectomy rat model

is reflective of the human renal insufficiency condition [14,15]. Animals possessed significant renal impairment characterized by a 2.9-fold increase in BUN levels (66.8 mg/dL vs 22.9 mg/dL for controls), a twofold increase in creatinine (1.3 mg/dL vs 0.58 mg/dL for controls), and a 2.34 g/dL decrease in Hgb (12.64 vs 14.98 g/dL). In CRI rats, Hematide exhibited a biphasic plasma decline (Fig. 3) with a half-life of 47.8 hours and clearance of 0.763 mL/h•kg. When compared to normocythemic rats, the half-life of Hematide was 1.6 times longer in the CRI rats; similarly, clearance was twofold lower (Fig. 3, Table 1), suggesting that Hematide is being cleared, at least partially, via the kidney.

In monkeys, elimination of Hematide from plasma was in a bi-exponential fashion, with a short initial phase followed by a dominant, prolonged terminal phase. There were dose-proportional increases of C_{max}. However, the increases in AUC values were greater than dose proportional and are indicative of saturation of clearance mechanisms at higher doses. Mean residence times (MRTs) were also dose dependent. Consistent with the MRTs and clearance values, the half-life observed at the high dose (59.7 h) was 4 times longer than that at the low dose (14.6 h). The volume of distribution at steady state (V_{ss}) for the three lower-dosed groups were essentially the same, ranging from 27.4 to 31.3 mL/kg, while the higher-dosed group was 75.2 mL/kg.

Erythropoiesis pharmacology

Hematide administered as a single IV injection led to a dose-dependent increase in reticulocytes with subsequent Hgb generation in normocythemic (Fig. 4) and CRI rats (Fig. 5). Increases in Hgb, at day 14 above concurrent controls, for the normocythemic rats were 0.63, 2.0, and 3.73 g/dL for 0.1, 1.0, and 10 mg/kg respectively, while Hgb increases of 2.06, 6.01, and 5.89 g Hgb/dL were observed at day 15 for the CRI rats, indicating a greater pharmacological response to Hematide in anemic animals with compromised renal function.

Table 1. Summary of pharmacokinetic data for Hematide following single bolus intravenous administration

Species	Dose (mg/kg)	C _{max} (ng/mL)	AUC(inf) (μ g·h/mL)	t _{1/2} (h)	CL (mL/h·kg)	V _{ss} (mL/kg)	MRT (h)
Rat	0.138	2685 (405.5)	60.0 (8.4)	23.1 (4.3)	2.34 (0.38)	65.6 (6.5)	28.6 (5.2)
	0.69	13143 (2177)	373.0 (71.9)	21.5 (3.6)	1.91 (0.37)	56.1 (7.9)	30.5 (7.8)
	1.38	36990 (9668)	924.8 (171.0)	22.2 (1.7)	1.54 (0.3)	42.7 (9.7)	27.7 (1.8)
	6.9	194571 (60629)	5552.5 (1273.7)	27.5 (3.7)	1.30 (0.3)	44.1 (8.5)	34.3 (4.0)
	13.8	324727 (73929)	9921.8 (2259.0)	30.7 (2.6)	1.44 (0.3)	56.8 (12.9)	39.3 (3.1)
Chronic renal insufficiency rat*	9.87	233000	128883	47.8	0.763	50.3	65.9
Monkey	0.02	516.2 (21.2)	11.1 (1.8)	14.6 (4.3)	1.74 (0.29)	31.3 (4.02)	18.5 (4.90)
	0.1	3290 (700.5)	98.8 (25.5)	16.4 (1.7)	1.00 (0.23)	28.8 (4.86)	29.0 (2.0)
	0.475	15395 (1747)	851.8 (267.1)	29.9 (9.4)	0.60 (0.21)	27.4 (2.45)	48.3 (12.2)
	1.35	25998 (3720)	1486.2 (180.9)	59.7 (2.9)	0.9 (0.1)	75.2 (5.5)	82.3 (4.4)

Data expressed as mean values with standard deviation (SD) shown in parentheses; *composite sampling design so SDs not applicable. Five normocythemic rats, 4–5 CRI rats, and 3 monkeys were utilized for each time point. Abbreviations: AUC, area under the curve; CL, plasma clearance; C_{max}, maximum observed concentration; MRT, mean residence time; t_{1/2}, half-life; V_{ss}, volume of distribution at steady state.

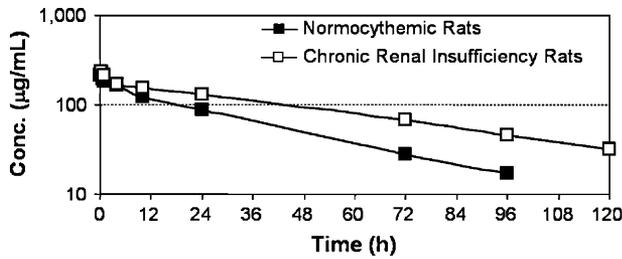


Figure 3. Plasma Hematide clearance is lower in chronic renal insufficiency rats compared to nonnephrectomized (normocythemic) rats. Data represents plasma concentration time profiles for Hematide following IV administration in normocythemic (13.8 mg/kg) and chronic renal insufficiency (9.87 mg/kg) rats. Each point represents the mean of five animals.

Erythropoiesis in response to a single IV administration of Hematide in monkeys is depicted in Figure 6. The increases in Hgb at day 15, from predose levels, were 0.57, 1.3, 2.44, and 3.7 g/dL for 0.02, 0.10, 0.475, and 1.35 mg Hematide/kg, respectively. The largest Hgb change in the 1.35 mg/kg cohort was at day 35, with an increase of 4.8 g/dL.

Hematide at 0.135 and 1.35 mg/kg caused a time-dependent increase in erythropoiesis in the rat following weekly (QW) and every-two-weeks dosing (Q2W). Both IV and SC routes of administration were examined (Fig. 7) with no apparent erythropoietic difference detected. No antibodies to Hematide were detected.

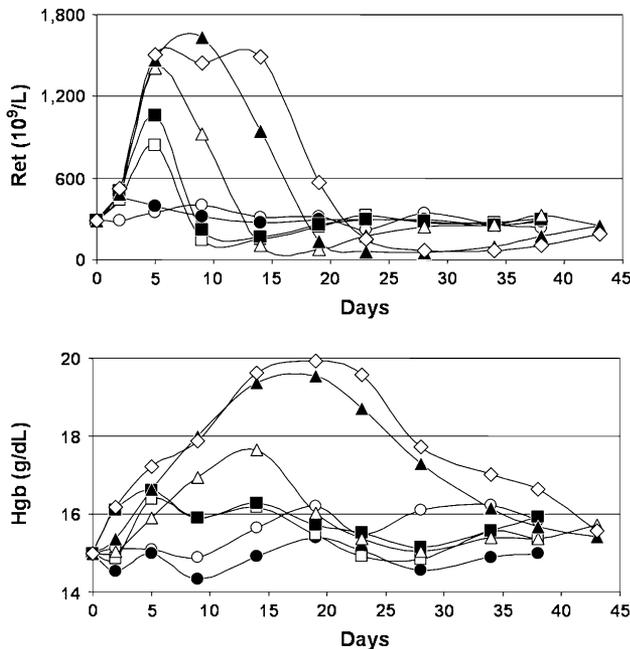


Figure 4. Dose- and time-dependent mean increase in reticulocyte (Ret) stimulation and hemoglobin (Hgb) production by Hematide in normocythemic male Sprague-Dawley rats. Rats received a single IV administration of vehicle (○), 0.01 (●), 0.05 (□), 0.1 (■), 1 (△), 10 (▲), and 50 (◇) mg Hematide/kg, respectively. Each point represents the mean of four animals.

Discussion

Hematide is a PEGylated peptide-based erythropoiesis stimulating agent with potent in vitro and in vivo erythropoietic activities. Hematide binds to and activates the human EPO receptor at sub-nanomolar concentrations and induces BFU-E colony formation by human CD34⁺ bone marrow cells at low nanomolar concentrations. These results demonstrate the similarity in potency of Hematide and native and recombinant erythropoietins in binding and functional activation of HuEPOr, and in the ability to cause proliferation and differentiation of erythroid progenitor cells. Hematide is highly specific for the EPO receptor since little or no pharmacologically relevant binding of Hematide was detected against a panel of 66 diverse receptors and, moreover, proliferation was not observed in seven human tumor cell lines.

PEGylation is a proven strategy to improve the pharmacokinetic, pharmacodynamic, and toxicity profiles of proteins [6,16–18]. Hematide pharmacokinetic analyses in the rat (normocythemic and chronic renal insufficiency model) and monkey revealed the volume of distribution to be slightly smaller than blood volume, indicating limited distribution outside the vascular compartment. Hematide possesses half-lives of approximately 22 to 31 hours in the rat, which are substantially longer than those published for rHuEPO (2.5 h) and darbepoetin alfa (6.9 h) [19], and 14.6 to 59.7 hours in the monkey.

Hematide induced robust erythropoiesis in the rat and monkey. Prolonged therapeutic efficacy of Hematide, due in part to PEGylation of the peptide moiety, is evidenced by the extended persistence of erythropoietic factors, such as reticulocytes and hemoglobin. Per given dose of Hematide, chronic renal insufficiency rats were more erythropoietically responsive, as measured by Hgb production.

Hematide plasma clearance in the CRI rats was twofold lower than clearance in rats with normal renal function, resulting in higher exposure and consistent with Hematide being cleared via the kidney. These findings suggest that target reticulocyte and hemoglobin levels in patients with compromised renal functions may be achieved with lower doses of Hematide than predicted from studies in normal animals and in healthy volunteers.

Single doses of Hematide also resulted in potent erythropoietic activity in the monkey, producing increases in reticulocytes that translated into Hgb increases ranging from 0.57 to 3.7 g/dL over the dose range tested (0.02–1.35 mg/kg). The longer duration of polycythemia in monkeys compared to rats is likely due to the longer erythrocyte lifespan in this species [20,21].

Hematide also produced marked and sustained hematologic activity in rats following repeat administration. Hematide-induced effects on reticulocytes and Hgb were statistically comparable when delivered by either the IV or the SC route. No Hematide-specific antibodies were detected in any of the rats repeatedly injected with Hematide

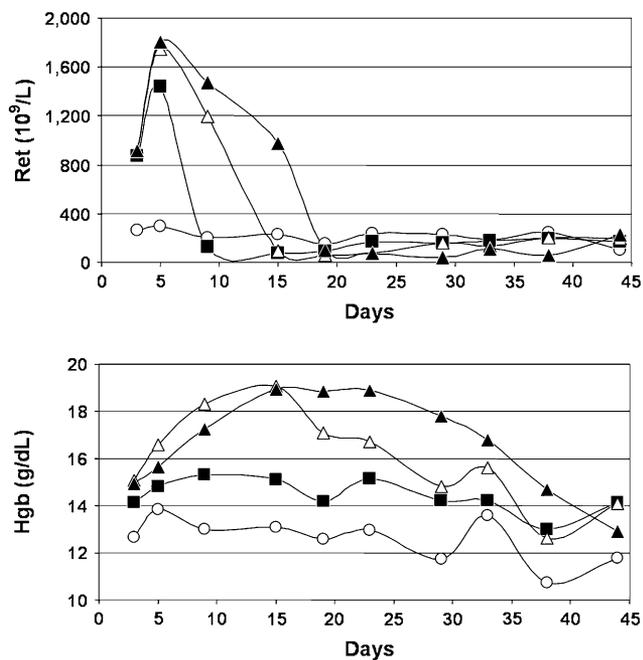


Figure 5. Dose- and time-dependent mean increases in reticulocytes (Ret) and hemoglobin (Hgb) generation by Hematide in chronic renal insufficiency rats. Rats received a single IV administration of vehicle (○), 0.1 (■), 1.0 (△) or 10 (▲) mg Hematide/kg. Three to eight rats were sampled per dose group for each time point.

either via the IV or SC route, suggesting that Hematide is not immunogenic in rats. Therefore, the pharmacology and pharmacokinetics associated with repeated dosing with Hematide in these studies was not affected by the presence of antibodies as can occur in animals receiving rHuEPO or darbepoetin alfa.

In conclusion, Hematide is a synthetic erythropoiesis stimulating agent that binds to and activates the human EPO receptor and stimulates erythropoiesis in human red cell precursors in vitro in a manner similar to native and recombinant EPOs. Hematide stimulates reticulocyte synthesis in a dose-dependent manner and results in the prolonged formation of red blood cells. Hematide was able to alleviate anemia in an experimental chronic renal insufficiency rodent model. IV and SC administration yielded similar erythropoietic responses. The substantially longer half-life of Hematide compared to rHuEPO or darbepoetin alfa suggests that use of Hematide in anemia management may allow for less frequent dosing compared to recombinant EPO products. These data supported the design of studies to evaluate the effect of Hematide in normal healthy volunteers (completed) and subsequent on-going Phase 2 studies in patients with anemia related to cancer and renal insufficiency [22].

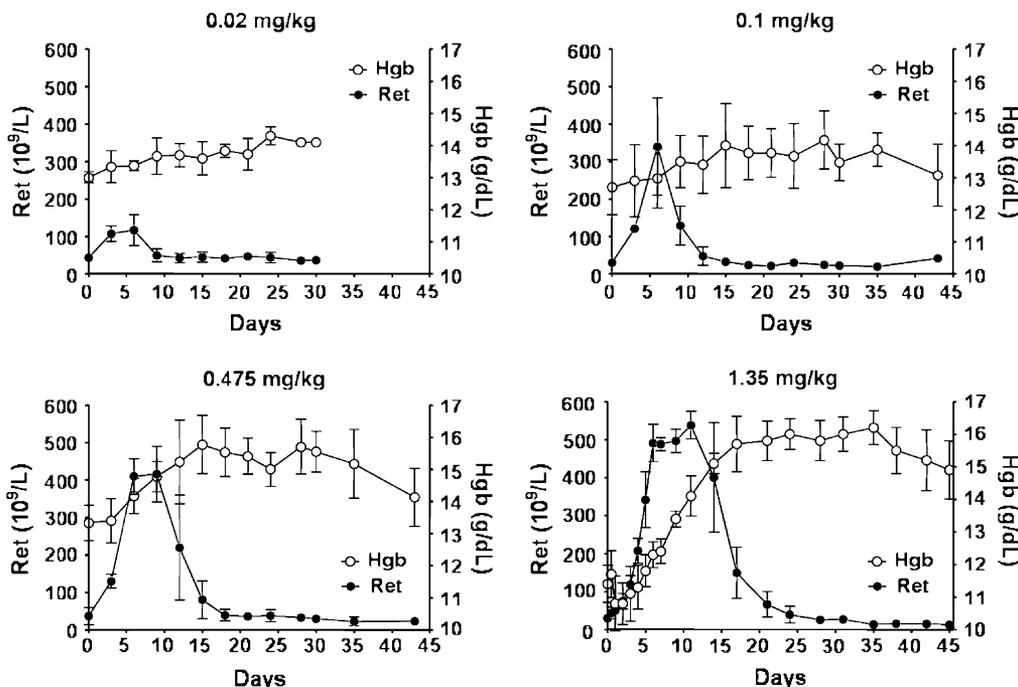


Figure 6. Hematide-induced erythropoiesis stimulation in the monkey following a single dose. Mean reticulocyte (Ret) counts and hemoglobin (Hgb) levels are shown over time following single IV bolus injections of 0.02, 0.1, 0.475, and 1.35 mg/kg of Hematide in male *Cynomolgus* monkeys (three monkeys per time point). The early Hgb decrease, from baseline through to day 4, in the 1.35 mg/kg cohort was likely due to the high frequency of blood sampling for pharmacokinetic and hematology assessments in this cohort (see Materials and methods for sampling times).

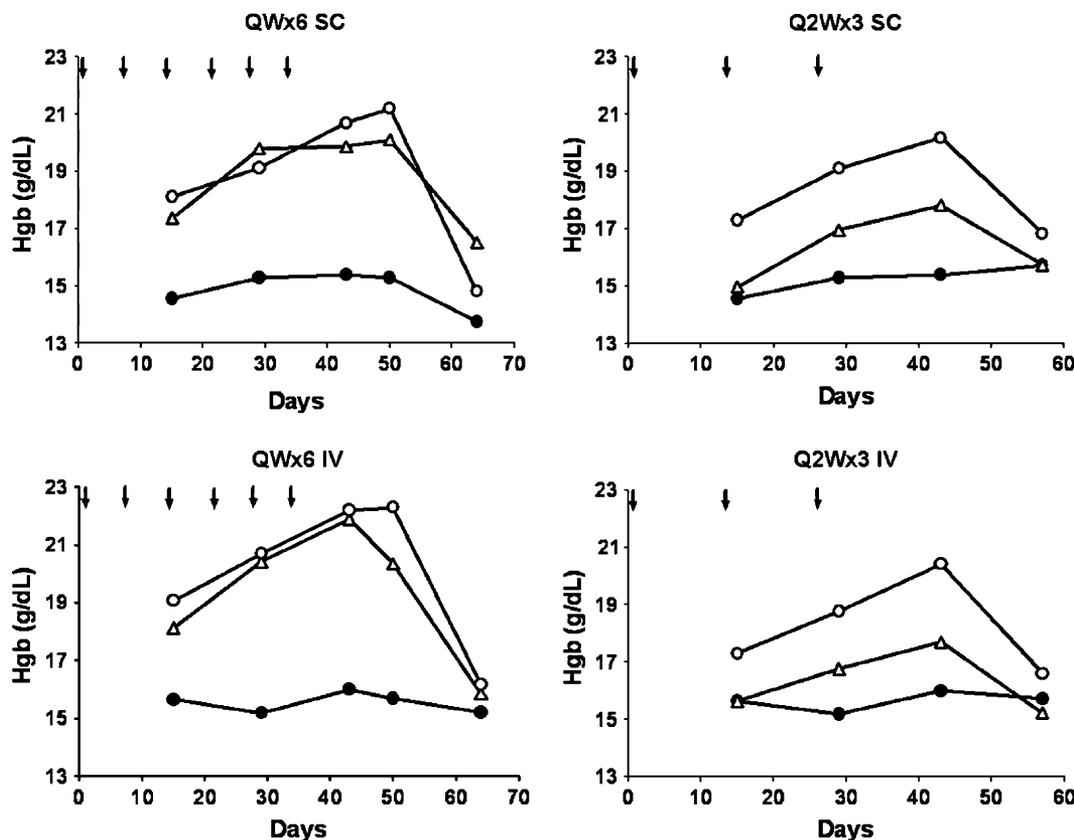


Figure 7. Robust erythropoiesis activity following repeat Hematide dosing in the rat. Hematide was intravenously and subcutaneously administered to normocythemic Sprague-Dawley rats (vehicle, ●; 0.135 mg/kg, △; 1.35 mg/kg, ○) every week for 6 weeks (QW×6) or every two weeks for a total of 3 injections (Q2W×3). Each point represents the mean of four animals. The arrowheads denote the days of administration.

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