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Toxicology and Safety Determination for a Novel Therapeutic Dual Carbon Monoxide and Oxygen Delivery Agent

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Abstract

Conditions in patients with hemoglobinopathies are far more complex and variable than in healthy humans due to the cascade of injury from hypoxia. Resulting complications can lead to tissue death, organ dysfunction and even death. Multi-faceted treatment is needed to address the array of complications. SANGUINATE™ (PEGylated, bovine carboxyhemoglobin) has multiple mechanisms of action that may prove effective. It acts both as a carbon monoxide-releasing molecule and as an oxygen transfer agent and is designed to safely perfuse the microvasculature to oxygenate tissue. Designing toxicology studies must not only deal with ensuring the safety of its mechanisms but deal with the potential safety issues of using bovine hemoglobin for both acute and chronic administration. Similar products have previously been shown to have effects on inflammation, vasoactivity, cardiac toxicity and nephrotoxicity as well as pro-coagulant activity. Therefore, studies were designed to thoroughly address the potential of these effects in conjunction with the traditional toxicology and safety pharmacology studies.

These toxicology and safety studies were designed to address the FDA's particular concerns of this novel drug candidate. There were several unique features to this preclinical program including a renal functional study, immunohistochemical and special staining of tissues, measurement of hemoglobin in the urine, measurement of troponin in the serum, inclusion of high dose/high volume groups, and analysis of interference for clinical pathology parameters. To address toxicity and safety concerns of its use as a single or repeating dose therapeutic, SANGUINATE was tested in pivotal studies using three species in repeating doses. There were no adverse effects identified for any doses and therefore, a no observed adverse effect level (NOAEL) was not determined even at dosage levels of 1200 mg/kg (monkey), 1600 mg/kg (pig) and 2400 mg/kg (rat). The completion of these studies permitted SANGUINATE to move into clinical trials.

Keywords PEGylation; Toxicology; Safety; SANGUINATE; Hemoglobin; Preclinical

Introduction

SANGUINATE (PEGylated bovine carboxyhemoglobin) is a dual action carbon monoxide (CO) releasing/oxygen (O_2) transfer agent whose functional components (CO, bovine hemoglobin and polyethylene glycol) contribute to its unique mechanisms of action. Due to the unique properties of its components, SANGUINATE has the potential to reduce or prevent the effects of hypoxia and protect the vasculature thereby preventing or reducing the extent of tissue damage.

While SANGUINATE is not traditional hemoglobin (Hb)-based oxygen carrier (HBOC), the Guidance for Industry: Criteria for Safety and Efficacy Evaluation of Oxygen Therapeutics as Red Blood Cell Substitute [1] provides a basis for the preclinical program design. Cell-free Hb has well-known effects on the vasculature. It readily extravagates and scavenges vascular endothelial nitric oxide (NO). NO is an important endogenous intercellular messenger that modulates blood flow, thrombosis and neural activity. Scavenging of NO by free Hb can result in systemic vasoconstriction, decreased blood flow, increased release of proinflammatory mediators and potent vasoconstrictors, and a loss of platelet inactivation. Free Hb also rapidly oxidizes to methemoglobin, which through its reactive iron moiety, causes oxidative damage to the blood vasculature and organs. Cell free Hb is known to cause iron deposition in tissue. The toxicity associated with iron is due to its role in catalyzing the generation of radicals which in turn damage cellular macromolecules and cause cell death and tissue injury.

In the 1990s, HBOCs were under development and then discontinued as a number of safety-related problems arose resulting in effects such as inflammation, vasoactivity, cardiac toxicity and nephrotoxicity as well as pro-coagulant activity [2-7]. Although not a conventional HBOC, SANGUINATE is designed to address these issues through the use of PEGylation [8,9] and carboxylation [10]. The modification of Hb with polyethylene glycol increases the effective molecular size, thereby preventing extravasation and the scavenging of NO. The release of CO may have therapeutic activity through the inhibition of vasoconstriction as well as by its anti-inflammatory activity.

Although this is a standard toxicology program in many ways, there are several unique features that were incorporated based on the novelty of the product. For this type of product, it was necessary to carefully design the studies to address traditional toxicology and safety concerns, pharmacokinetic (PK) and pharmacological effects, as well as to specifically address the theoretical concerns of inflammation,

vasoactivity, cardiac toxicity and nephrotoxicity, and pro-coagulant activity. Therefore, the toxicology and safety study design incorporated assessments to address issues that are unique to this class of products (Table 1). Unique features to this program included a renal glomerular filtration rate and renal blood flow study, immunohistochemical staining for tumor necrosis factor alpha (TNF-α, inflammatory marker), malondialdehyde (MDA, oxidative marker), and Prussian Blue iron staining for kidneys, myocardium, vasculature, and brain (cerebrum and cerebellum). Additional clinical pathology assessments included measurement of the cardiac diagnostic marker, troponin, in the serum. Elimination of SANGUINATE was assessed by measuring the levels of hemoglobin in the urine. High dose/high volume groups were included to further increase the dosing levels of SANGUINATE and to provide an increased safety margin. The cardiovascular safety study in monkey included additional clinical pathology and histopathology toxicity assessments. Interference studies were used to identify and correct for the interference of hemoglobin with the proper assessment of clinical pathology parameters that are based on colorimetric methods, and is a complex analysis that will be elaborated upon in a separate manuscript. This paper will focus on the toxicology and safety aspects of this program and the unique designs that enabled FDA approval of SANGUINATE for clinical trials.

Challenge	Testing Approach
Inflammation and oxidative stress	Immunohistochemistry for TNF-α and MDA in kidneys, myocardium, vasculature, and brain
Cardiac Toxicity	Electrocardiograms, clinical chemistry including Troponin, histopathology, and full cardiovascular and pulmonary assessment in telemetered monkeys
Nephrotoxicity	Renal glomerular filtration rate and renal blood flow study; clinical chemistry; urinalysis; histopathology; measured presence of hemoglobin in urine
Safety Margin	High dose/high volume groups included to maximize dosing levels
Clinical Pathology Interference	Interference assessment and correction for affected parameters

Table 1: Challenges and Unique Features in the SANGUINATE Toxicity and Safety Program.

Methodology

The SANGUINATE preclinical program consisted minimally of PK, genotoxicity, and toxicity and safety studies (several other studies were conducted during drug development). PK studies were performed in both rat and pig species. Animals were dosed with 40 mg/ml SANGUINATE by IV with a single dose at 2, 4, and 8 ml/kg. Blood was collected at timepoints out to 144 hours post dosing, and samples were analyzed for SANGUINATE concentration.

Genotoxicity was performed per OECD guidelines. SANGUINATE was evaluated for its ability to induce chromosomal aberrations (mitotic index evaluation and definitive chromosomal aberration assay) in cultured human peripheral blood lymphocytes *in vitro* in the presence and absence of metabolic activation. The *Salmonella typhimurium* and *Escherichia coli* Reverse Mutation Assay (Ames Assay) was conducted to evaluate the potential of SANGUINATE to induce reverse mutations in histidine (his^- to his^+) and tryptophan ($tryp^-$ to $tryp^+$) genes in *S. typhimurium* and *E. coli*. The Rodent Bone Marrow Micronucleus Assay was evaluated for induction of a statistically significant increase in the frequency of micronuclei in the bone marrow erythrocytes of mice.

The *in vivo* toxicity and safety program for SANGUINATE included toxicity and safety studies across 3 different species: Sprague Dawley Rats, Gottingen Minipigs, and Cynomolgus Monkeys (Table 2). Parameters evaluated for the assessment of toxicity included clinical observations, body weights, ophthalmic observations, food consumption, hematology, clinical chemistry, coagulation, urinalysis (Table 3), and organ weights and histopathology (Table 4). Additional assessments included in some of the studies were special histopathology staining, toxicokinetics, functional observational battery, immunogenicity, and cardiovascular measurements (Table 4).

Study	Animals	SANGUINATE Dose Levels / Regimen	Parameters	Observations
Determination of Glomerular Filtration Rate and Renal Blood Flow Following Single Intravenous Administration of SANGUINATE in Rats	Rat (n=222)	160, 280, 400 mg/kg Single dose IV	Glomerular Filtration Rate (GFR) and Renal Blood Flow (RBF)	There were no abnormal clinical observations within the study time period to suggest any acute toxic effect of treatment with the test article. Within 24 hours of SANGUINATE single-dose intravenous administration, no changes were observed in glomerular filtration rate (GFR) or renal blood flow (RBF) measured clearance.
Five−Day Repeat Dose Toxicity Study of SANGUINATE (PEGylated Bovine Hemoglobin) in Rats with a 14−Day Recovery Period	Rat (n=294)	100, 200, 400 mg/kg 5-day repeat dose; 5 min iv infusion	Food consumption, body and organ weight measurements, clinical observations, functional observational battery, hematology, blood chemistry, coagulation, urinalysis, immunogenicity,	No test article related effects on standard parameters. Significant differences for clinical chemistry parameters including increased creatinine, decreased albumin, and decreased alkaline phosphatase activity. Histopathological results revealed no macroscopic and/or microscopic treatment related findings, and no target organs and /or treatment related findings. Iron staining

			and histopathology including special staining.	was exclusively in the brain (minimal to mild) and in the kidneys (minimal to moderate), but was not considered biologically relevant. SANGUINATE was well tolerated up to and including 400 mg/kg after single and after 5 days intravenous administration. No NOAEL.
Maximum Feasible Dose Study of SANGUINATE in Rats	Rat (n=20)	2400 mg/kg 5-day repeat dose (MFD); slow iv push	Body weights and clinical observations, blood and urine collected for clinical pathology, gross necropsy and select target organs for histopathology.	Treatment related findings were noted in clinical observations, and in the evaluation of clinical pathology and histopathology. No adverse effects on body weights. Urine discolored and slight increase in the WBC and RBC. Clinical signs included excretion of red/brown fluid from the urogenital area and red fluid/staining around eyes, and piloerection. There were changes in the percentage of lymphocytes, and an increase in the percentage of neutrophils in both sexes and monocytes in males. A decrease in hemoglobin was also seen. The values of total bilirubin and creatinine were increased, and albumin and total protein were significantly decreased. Microscopic evaluation showed treatment related findings that were limited to the kidneys and heart in both sexes. The evaluations showed overt signs of systemic toxicity.
Six Months Repeat Dose Toxicity Study of SANGUINATE (PEGylated Bovine Hemoglobin) in Rats With a 30−Day Recovery Period	Rat (n=506)	100, 200, 400, 2400 mg/k 6-mo monthly, repeat dose; 5 min iv infusion	Food consumption, body and organ weight measurements, clinical and ophthalmic observations, clinical signs of neurotoxicity, hematology, serum chemistry, coagulation, urinalysis, immunogenicity, and histopathology including special staining.	Prolonged bleeding was noted in groups receiving SANGUINATE, and the negative controls DPH and Hextend, following intravenous dosing and/or retro-orbital blood sample collection. This procedure-related bleeding was not seen in the NaCl control groups. Significant, dose-dependent, albumin, total protein, total bilirubin, AST, ALP, amylase, calcium, creatinine and BUN effects were seen, not on all days. Recovery groups presented no significant abnormalities, indicating recovery from any treatment-related effects. Microscopic evaluation of liver and kidneys did not confirm any test article related effects on these or other organs compared to the controls.
Nine Months Repeat Dose Toxicity Study of SANGUINATE (PEGylated Bovine Hemoglobin) in Minipigs with a 30−Day Recovery Period	Pig (n=86)	100, 200, 400, 1600 mg/kg 9-mo monthly; 10-15 min iv infusion	Food consumption, body and organ weight measurements, clinical and ophthalmic observations, electrocardiographic exams, hematology, serum chemistry, coagulation, urinalysis, immunogenicity, and histopathology including special staining.	Majority of assessments showed no specific effects including body weights, ophthalmic exams, the amount of oxy- and deoxy-hemoglobin in whole blood, electrocardiographic exams, and immunogenicity. Some differences in organ weights (heart, liver, adrenal, and brain) were observed, but were not considered biologically relevant. Iron staining was observed in the brain and kidney and in one animal in the carotid aortas and jugular vein, but was not considered biologically relevant. TNF-α and MDA staining had no clear dose trend. The only clinical sign considered related was diarrhea, observed in 25% of the animals assigned to the 1600 mg/kg SANGUINATE group. Some differences in hematology and clinical chemistry parameters were observed. A significant increase in prothrombin time and activated partial thromboplastin time was observed but not clinically relevant. A NOAEL could not be determined.
Evaluation of Cardiovascular (Hemodynamic) and Pulmonary Function Following Intravenous Administration of SANGUINATE™ (PEGylated Bovine Hemoglobin) in Conscious Telemetered Male Cynomolgus Monkeys	Monkey (n=4)	100, 200, 400, 1200 mg/kg 5-day repeat dose; 5-10 min iv injection	Clinical observations, clinical pathology, histopathology, toxicokinetics, ECG, hemodynamics, and pulmonary parameters	Observations included loose or soft feces, delayed/ prolonged bleeding times, facial and inguinal erythema, pink skin color, petichiae on the leg, decreased activity and white foamy/frothy feces, small red areas on the abdomen and extremities. Clinical chemistry showed no Troponin, increased creatinine, increased BUN, decreased amylase , decreased ALK, and slightly decreased ALT. There were no microscopic findings that indicated direct test article toxicity. 100 and 200 mg/kg were not associated with changes in heart rate, blood pressure, ECG or pulmonary parameters. 400 mg/kg was associated with minimal increases in arterial pressure after the first dose and slight increases in heart rate and arterial pressure following the fifth dose. 1200 mg/kg/day was associated with decreased heart rate and increased arterial pressure. Following the fifth day of dosing at 1200 mg/kg/day, increases in heart rate, arterial pressure and QTc were noted as well as decreases in respiratory rate.

Table 2: Summary of SANGUINATE Toxicity and Safety Program.

Hematology	Clinical Chemistry	Coagulation
Differential White Blood Cell Count	Alanine Aminotransferase (ALT)	Activated Partial Thromboplastin Time (APTT
Hematocrit (HCT)	Albumin (ALB)	Prothrombin Time (PT)
Hemoglobin (HGB)	Albumin/Globulin Ratio (A/G)	
Mean Corpuscular Hemoglobin (MCH)	Alkaline Phosphatase (ALK)	Urinalysis
Mean Corpuscular Hemoglobin Concentration (MCHC)	Amylase	Appearance – color, clarity, volume
Mean Corpuscular Volume (MCV	Aspartate Aminotransferase (AST)	Bilirubin
Oxy-Carboxy and Met-hemoglobin	Blood Urea Nitrogen (BUN)	Glucose
Platelet Count (PLA)	Calcium (Ca)	Ketones
Red Blood Cell (Erythrocyte) Count (RBC)	Chloride (Cl)	Leucocytes
White Blood Cell (Leukocyte) Count (WBC)	Cholesterol (CHOL)	Microscopic examination of sediment
	Creatinine (CREAT)	Nitrites
	Gamma Glutamyltransferase (GGT)	Occult Blood
	Globulin	pH
	Glucose	Specific Gravity
	Inorganic Phosphorus (PHOS)	Total Protein
	Lipase	Urinary hemoglobin
	Potassium (K)	Urobilinogen
	Sodium (Na)	Volume
	Total Bilirubin	
	Total Protein (TP)	
	Triglycerides (TRIG)	

Table 3: General Clinical Pathology Parameters: Hematology, Clinical Chemistry, Coagulation, and Urinalysis.

Adrenal gland	Small intestine, duodenum	Small intestine, ileum
Aorta	Lung (with mainstem bronchi)	Small intestine, jejunum
Bone marrow	Lymph node, mesenteric	Spinal cord (cervical, mid-thoracic, lumbar)
Bone with articular surface, femur	Lymph nodes, submandibular (L/R)	Spleen
Brain (cerebrum, cerebellum, medulla/pons)	Mammary gland	Stomach
Epididymis (males)	Nasal turbinate	Testes (males, paired)
Esophagus	Nerve, sciatic	Thymus
Eyes (w/optic nerve)	Ovaries (females, paired)	Thyroid (with parathyroid)
Heart	Pancreas	Tongue
Injection Site	Pituitary gland	Trachea
Kidney	Prostate gland (males)	Ureter (paired)
Large intestine, cecum	Salivary gland, mandibular (L/R)	Urinary bladder

Large intestine, colon	Seminal Vesicles	Uterus (females)
Large intestine, rectum	Skeletal muscle	Cervix
Liver	Skin	Vagina (females)

Table 4: General Tissues Used in Histopathology Study.

A study was performed to analyze the effects of SANGUINATE (160, 280, and 400 mg/kg) on renal glomerular filtration rate (GFR) and renal blood flow (RBF) following single intravenous administration to Sprague Dawley rats, based on the plasma clearance of surrogate markers. Inulin, [Carboxyl-^{14}C], was utilized in the measurement of GFR and Aminohippuric Acid, P-[Glycyl-1-^{14}C], was used in the measurement of RBF. The control article was USP 0.9% Sodium Chloride for Injection (NaCl). Animals were administered inulin or aminohippuric acid as tracers. There were a total of 7 groups. In each group, a subset of animals was administered inulin, and another subset received p-aminohippuric acid. The animals were intravenously administered the specified dose of test or control article on study Day 1. Before tracer administration, animals were weighed and hydrated by oral administration of NaCl at a dose level of 25 mL/kg. The urinary bladder was expressed by induced micturition through manual pressure applied above the pubic area. Radiolabeled inulin or aminohippuric acid tracer was given intravenously at a dose level of 500 mg/kg within 24 ± 2 hours following treatment with the test or control article. Animals were placed into metabolic cages with food and water withheld for the remaining duration of the study. Samples of arterial blood and urine (as available) were collected from each animal at 30 ± 5 and 60 ± 5 minutes. The time and volume of urine collection were recorded for the determination of urine output. Animals were humanely sacrificed following the final sample collection. Levels of radioactivity were determined through sample analysis in duplicate on a scintillation counter. Blood samples were solubilized to produce a solution compatible with the liquid scintillation cocktail in the determination of radioactivity. Urine samples were measured directly on the scintillation counter. Renal glomerular filtration rate (GFR) and renal blood flow (RBF) were calculated based on the clearance of inulin or p-aminohippuric acid, respectively.

An evaluation was performed of the potential toxicity and the toxicokinetic profile of the test article, SANGUINATE, after single dose and after five days repeated IV administration to Sprague Dawley rats at the doses of 0, 100, 200 and 400 mg/kg, and the reversibility of any toxic effect after a 14–day recovery period. One group was treated with 200 mg/kg of Purified Bovine Hemoglobin (positive control group). Two additional groups (negative controls) were treated with 200 mg/kg of deoxy-PEGylated bovine hemoglobin (DPH) or 5.0 mL/kg Hextend (Hospira). There were 15 male and 15 female animals per group. On Days 2, 6 and 20, ten animals in each group (5 males and 5 females) were sacrificed. To study the toxicokinetic profile of SANGUINATE, an additional 6 males and 6 females per group were used. Parameters evaluated for the assessment of toxicity included food consumption, body and organ weight measurements, clinical observations, clinical signs of neurotoxicity, hematology, blood chemistry, coagulation, urinalysis, immunogenicity, and histopathology. Histopathological analysis included special staining to indicate TNF-α expression, oxidative damage (MDA staining) or iron accumulation (Prussian Blue staining).

The potential toxicity using repeat dosing with a maximum feasible high dose 2400 mg/kg of SANGUINATE at a dose volume of 60 mL/kg/day was assessed for 5 consecutive days. Twenty (20) rats were used in the study and were placed into 5 dose groups each with 2 males and 2 females. Each animal received 30 mL/kg via an intravenous injection twice a day of the following test or control articles: NaCl, SANGUINATE, PEGylated bovine hemoglobin (PBH), DPH, or Hextend. Body weights were taken daily and animals were observed for clinical signs of toxicity prior to the initial dose and following the last dose of the day. Blood and urine were collected for clinical pathology evaluation at the end of the study. A gross necropsy was performed on all animals and select target organs were collected for microscopic examination. Parameters evaluated included food consumption, body and organ weight measurements, clinical and ophthalmic observations, clinical signs of neurotoxicity, hematology, serum chemistry, coagulation, urinalysis, immunogenicity, and histopathology.

A six month study was performed in Sprague Dawley Rats to evaluate the potential toxicity and the toxicokinetic profile of Sanguinate after once a month IV administration, and the reversibility of any toxic effect after a 30-day recovery period. Animals received doses of 0, 100, 200 and 400 mg/kg. One group was treated with 200 mg/kg of PBH (positive control), and two groups (negative controls) were treated with 200 mg/kg of DPH or 5.0 mL/kg Hextend. Two additional groups received a high volume dose of 60 mL/kg of the vehicle control or 2400 mg/kg of Sanguinate. Some animals per sex receiving the high volume control or 2400 mg/kg of Sanguinate were sacrificed on Day 61 and some animals per sex receiving 2400 mg/kg were sacrificed on Day 74. To study the toxicokinetic profile of Sanguinate, 6 or 9 animals/group/sex were used with groups surviving 60 days or 180 days, respectively. Parameters evaluated for the assessment of toxicity included food consumption, body and organ weight measurements, clinical and ophthalmic observations, functional observational battery (FOB) exams, hematology, serum chemistry, coagulation, urinalysis, immunogenicity, and histopathology. Interference for clinical pathology parameters was characterized and corrections were applied.

A nine month study in Gottingen Minipigs was performed to evaluate the potential toxicity and the toxicokinetic profile of the test article, Sanguinate, after once a month IV administration, and the reversibility of any toxic effect after a 30–day recovery period. Animals received doses of 0, 100, 200, and 400 mg/kg. One group was treated with 200 mg/kg of PBH (positive control) and two groups (negative controls) were treated with 200 mg/kg of DPH or 5.0 mL/kg Hextend. Two additional groups received a high volume dose of 40 mL/kg of the vehicle control or 1600 mg/kg of Sanguinate. On Day 276, some animals per sex in each group receiving 0, 100, 200, or 400 mg/kg of Sanguinate, PBH, DPH, or Hextend were sacrificed. On Day 300, some animals per sex receiving 0 or 400 mg/kg of Sanguinate were sacrificed. Some animals per sex receiving the high volume control or 1600 mg/kg of Sanguinate were sacrificed on Day 91 and some animals

per sex receiving 1600 mg/kg were sacrificed on Day 105. To study the toxicokinetic profile of Sanguinate, blood samples from 4 animals per sex per group were obtained on Days 1, 90 and 270 (0, 100, 200, and 400 mg/kg Sanguinate, PBH, DPH and Hextend groups) and from 3 animals per sex assigned to 1600 mg/kg Sanguinate on Days 1 and 90. Parameters evaluated for the assessment of toxicity included food consumption, body and organ weight measurements, clinical and ophthalmic observations, electrocardiographic exams, hematology, serum chemistry, coagulation, urinalysis, immunogenicity, and histopathology. Interference for clinical pathology parameters was characterized and corrections were applied.

A study was performed to determine the potential acute and repeat dose, intermittent dose effects of SANGUINATE on cardiac, circulatory and pulmonary functions and electrocardiograms (ECG) of conscious telemetered male Cynomogus monkeys (Table 5).

Cardiovascular Parameters
Systolic arterial pressure
Diastolic arterial pressure
Mean arterial pressure
Heart rate
P duration
PR interval
QRS interval
R amplitude
QT interval
Pulmonary Parameters
Respiration rate
Minimum
Maximum
Inspiration time
Expiration time
Depth

Table 5: Cardiovascular and Pulmonary Parameters.

There was one treatment group of 4 Cynomolgus monkeys each receiving the PBH (positive control), DPH (negative control), Hextend (negative control) the vehicle and four doses of SANGUINATE (100 mg/kg/day, 200 mg/kg/day, 400 mg/kg/day and 600 mg/kg/2x per day) with a washout period in between dosing regimens. Treatments were administered once per day for 5 days except for the vehicle control #2 and the 1200 mg/kg/day total dose which were administered twice daily approximately 6 hours apart. The 1200 mg/kg/day total high dose was also administered on Day 114, for toxicokinetic purposes, and again on Day 127. One-minute means of hemodynamic, pulmonary and ECG parameters were measured on the first and last dosing day of each 5-day treatment arm as well as on Day 127. One minute tracings of the ECGs were printed at 15 minutes prior to dosing and at 30 minutes, 1, 2, 4, 8, 12 and 22 hours post-dose on each recording day for review by a veterinary cardiologist. Blood for evaluation of serum chemistry was collected from all animals prior to treatment initiation and on each dosing day prior to dosing and on Day 128 prior to terminal sacrifice. Blood for toxicokinetic evaluation was collected on Day 114 at selected timepoints. Selected tissues were harvested at necropsy on Day 128 from all animals and were evaluated microscopically. Interference for clinical pathology parameters was characterized and corrections were applied.

Results

Pharmacokinetic Studies

In both rat and pig species, a dose-dependent pattern in terms of C_{max} and AUC was observed. When adjusted for dose, there appeared to be a solid linear response in both C_{max} and AUC. In the rat, the estimated half-life was 12 hr in all groups. In the pig, the estimated half-life was 12 hours in the low and mid-dose groups and 22 hours in the high dose group.

Genotoxicity Studies

In the Chromosomal Aberration assay the results showed that the SANGUINATE at concentrations of 5, 1.66, and 0.55 mg/mL did not induce statistically significant chromosomal aberrations (does not include chromatid gaps or polyploidy cells) as compared to the negative control group both in the presence and absence of metabolic activation. The confirmatory assay, involving extended exposure of cells to the test substance for 23 hours in the absence of metabolic activation, also showed lack of induction of chromosomal aberrations by the test substance. SANGUINATE was considered non-clastogenic in human peripheral blood lymphocytes. The results of the definitive Ames assay showed that the SANGUINATE did not increase the frequency of revertants at any of the test concentrations in any of the strains tested both in the presence and absence of metabolic activation. The results of the confirmatory assay were consistent with the negative results from the definitive assay. Under the test conditions, the test substance was non-mutagenic in the test species. In the Micronucleus assay there was no statistically significant increase in the frequency of micronucleated PCEs in the test substance dose group as compared to the negative control group. SANGUINATE was considered non-clastogenic, under the experimental conditions.

Renal Glomerular Filtration Rate

There were no abnormal clinical observations within the study time period to suggest any acute toxic effect of treatment with the test article. Calculated GFR and RBF values were evaluated by ANOVA followed by post hoc group comparison. There were no statistically significant differences in the RBF among groups in males and females at 30 and 60 minutes. For GFR analysis, data from males and females were combined. In general, no statistically significant differences were observed at 30 minutes when groups were compared. Analysis at 60 minutes was not possible due to the small number of samples. Therefore, the SANGUINATE was not observed to produce changes in the inulin and p-aminohippuric acid clearance at the analyzed time points, within 24 hours of single dose intravenous administration in rats.

Repeat dose toxicity and safety studies

The five-day repeat dose study in rats had no test article related effects on body and organ weight, clinical observations, functional

observational battery assessments, food consumption, hematology, coagulation, urinalysis, histopathology, or immunogenicity. Statistically significant differences were observed for several clinical chemistry parameters including increased creatinine (Days 3, 4, 5, 6 and 7), decreased albumin (Days 4 and 5), and decreased alkaline phosphatase activity (Days 6 and 7). No dose trend was recognized on Days 14 and 20. Histopathological results revealed that there were no macroscopic and/or microscopic treatment related findings, and no target organs and /or treatment related findings were determined. Iron staining was observed exclusively in the brain and in the kidneys. The staining intensity detected was minimal to mild in the brain and minimal to moderate in the kidneys. Given the absence of meaningful findings during the functional observational battery assessments and the absence of any test article related microscopic findings, the iron staining observed in the kidney and brain was not considered biologically relevant. The toxicokinetics demonstrated a dose dependent increase in C_{min} values in both males and females, particularly in the Day 5 animals, suggesting a minor risk of accumulation following 5 consecutive days of intravenous dosing of SANGUINATE to rats. Based upon evaluation criteria used for the study, SANGUINATE was considered to be well tolerated up to and including 400 mg/kg after single and after 5 days intravenous administration to Sprague Dawley rats. The NOAEL of SANGUINATE in this study could not be determined as there were no adverse effects at the highest dose level.

In the maximum feasible dose study, treatment related findings were noted in clinical observations, and in the evaluation of clinical pathology and histopathology. There were no adverse effects on body weights. Urine was discolored and there was a slight increase in the WBC and RBC in animals receiving SANGUINATE, PBH, and DPH relative to those receiving NaCl. Clinical signs attributed to treatment within their respective groups included excretion of red/brown fluid from the urogenital area and red fluid/staining around eyes of animals receiving SANGUINATE, PBH and DPH; piloerection in those receiving SANGUINATE and DPH; and abnormal breathing, lethargy, and dehydration were observed in animals receiving DPH. There were changes in hematology parameters considered biologically meaningful and attributed to treatment. When compared to historical values by sex and to the NaCl treated control group, animals treated with SANGUINATE and DPH had changes in the percentage of lymphocytes, an increase in the percentage of neutrophils in both sexes and monocytes in males. A decrease in hemoglobin was also seen after treatment with SANGUINATE, DPH and Hextend. Changes in clinical chemistry parameters and coagulation factors that were significantly different from the NaCl group and did not fall within historical ranges were considered biologically meaningful and attributed to treatment with SANGUINATE, DPH or Hextend. The values of total bilirubin and creatinine were increased and were above ranges for both sexes. Albumin and total protein were significantly decreased in groups treated with SANGUINATE, DPH, and Hextend and cholesterol levels were decreased in Hextend treated females. Microscopic evaluation showed treatment related findings that were limited to the kidneys and heart in both sexes in groups receiving SANGUINATE and DPH. The evaluations defined in the procedure showed that there were overt signs of systemic toxicity following treatment of a 2.4 g/kg dose of SANGUINATE, PBH, and DPH and 60 mL/kg of Hextend.

The majority of assessments evaluated in the six month study in rat showed no specific effects related to Sanguinate. These included body weights, functional observational battery assessments, ophthalmic exams, food consumption, the amount of oxy- and deoxy-hemoglobin in whole blood, Troponin I, and immunogenicity. Significant adverse clinical observations included prolonged bleeding, which was dose dependent and considered related to treatment with Sanguinate and DPH. Clinical observations in the moribund animals correlated to the prolonged bleeding and may have contributed to the morbidity seen and clinical chemistry findings. Morbidity was considered test article related. Toxicokinetic analysis (Figures 1 and 2) showed a dose dependent pattern in terms of C_{max} and AUC with both reaching peaks in the 2400 mg/kg dose group on Day 1 and in the 400 mg/kg dose group on Days 90 and 180. When adjusted for dose, there appeared to be a linear response in both C_{max} and AUC. The detection of measurable plasma concentrations of PEG-Hemoglobin at the 144 hour time points in the 2400 mg/kg dose groups suggested the possibility of a minor risk of accumulation following a 5 minute intravenous infusion of very high dose Sanguinate to rats. Females of the high volume/high dose test article groups had significant change in clotting parameters while males had a strong trend toward increased clotting times. Urine was brown and contained significant levels of hemoglobin in the high dose/ high volume test article treated groups. Urine volume showed a significant decrease in male animals, however only was significant on Days 121 and 181 in the high dose female group. In the high volume/high dose groups, urine volume decrease was significantly different from the control for both sexes and was attributed to treatment with Sanguinate. This effect was fully recoverable in all groups tested. Statistically significant differences in clinical chemistry included many parameters. Given the moderate and transient effect of some observations, most of these differences were not considered biologically significant. Significant, dose dependent albumin, total protein, total bilirubin, AST, ALP, amylase, calcium, creatinine and BUN effects were seen in both sexes. Recovery groups presented no significant clinical chemistry parameter abnormalities. Liver and kidneys had significant increases in organ weights in relationship to dose. TNF-α findings were not considered specific to treatment, and MDA staining was similarly inconclusive. Iron staining was observed in the brain and kidneys of study animals. Staining in the brain was not considered biologically relevant, but staining of the kidney was much higher for the high volume/high dose Group. Microscopic evaluation of liver and kidneys did not confirm any test article related effects on these or other organs. The immunogenicity data indicated that Sanguinate did not induce an immunogenic response, which was consistent with other PEGylated proteins that have been long approved by the FDA [11]. To summarize, test article related effects were seen with bleeding, early deaths, monocytes, urine volume, clinical chemistry, organ weights, and iron staining. The identification of a NOAEL dose level and target organs for toxicity was inconclusive.

The majority of assessments evaluated in the nine month study in pig showed no specific effects related to SANGUINATE. These included body weights, ophthalmic exams, and the amount of oxy- and deoxy-hemoglobin in whole blood, electrocardiographic exams, and immunogenicity. Some differences in organ weights (heart, liver, adrenal, and brain) were observed, but those differences were not considered biologically relevant. Iron staining was observed in the brain and kidney and in one animal in the carotid aortas and jugular vein, but it was not considered biologically relevant. Immunostaining for TNF-α and MDA in all examined tissues had no clear dose trend and scattered positivity was interpreted as non-test article related. The only clinical sign considered related to treatment with SANGUINATE was diarrhea, observed in 25% of the animals assigned to the 1600

mg/kg SANGUINATE group. The toxicokinetics data (Figures 1 and 2) showed a dose dependent pattern in terms of C_{max} and AUC with both reaching peaks in the 1600 mg/kg dose group on Day 1 and 90 and in the 400 mg/kg dose group on Day 270. When adjusted for dose, there appeared to be a solid linear response in both C_{max} and AUC. Measurable plasma concentrations of PEG-Hb at the 144 hour time points in the 1600 mg/kg dose group were detected on Days 1 and 90. Although slightly elevated plasma concentrations were observed in SANGUINATE (200 mg/kg) treated animals compared to DPH treated animals on Days 90 and 270, there was no remarkable difference observed between the $t_{½}$, Tmax, dose adjusted C_{max}, and $AUC_{0-\infty}$ between SANGUINATE and DPH treated animals. Selected hematology and clinical chemistry parameters were corrected to account for SANGUINATE interference. Some differences in hematology and clinical chemistry parameters were observed. In addition, a significant increase in prothrombin time and activated partial thromboplastin time was observed in all the test article treated groups compared to their respective controls. None of these differences were considered clinically relevant, given the absence of abnormal clinical observations and histopathological correlates. The immunogenicity data indicated that Sanguinate did not induce an immunogenic response, which was consistent with other PEGylated proteins that have been long approved by the FDA [11]. According with the parameters for the study, there were no adverse effects identified for any dose in this study; therefore, a no observed adverse effects level (NOAEL) could not be determined.

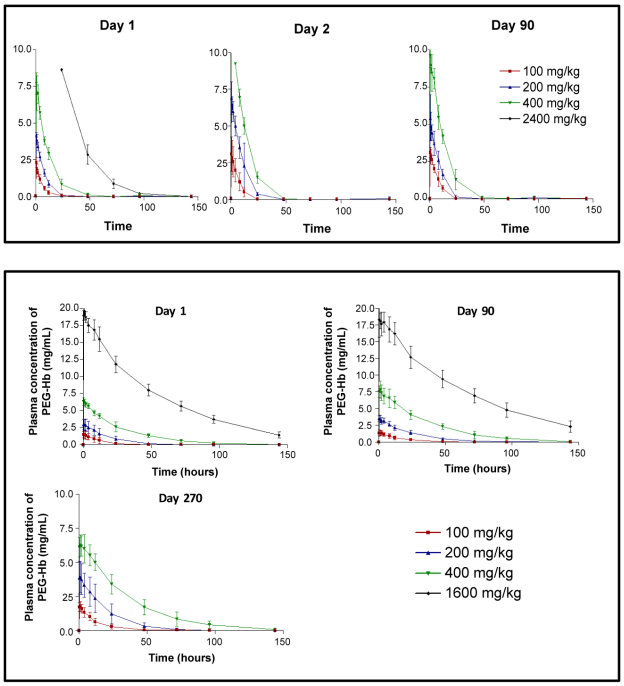

Figure 1: Mean Plasma Concentration of SANGUINATE in Rats (6 month study) and Pigs (9 month study).

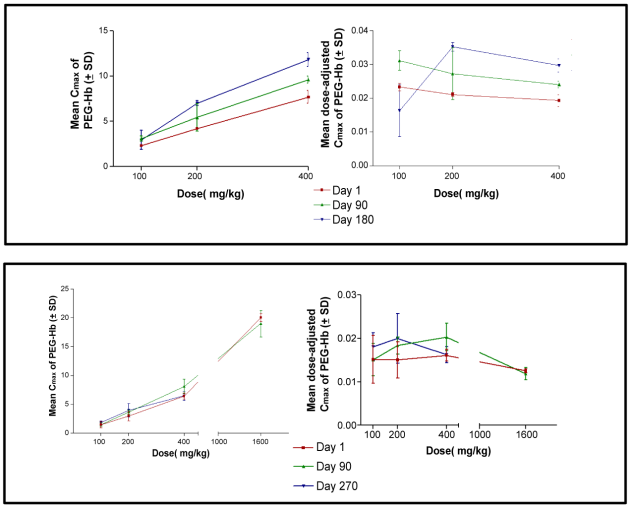

Figure 2: Mean C_{max} of SANGUINATE in Rats (6 month study) and Pigs (9 month study).

The acute and repeat dose, intermittent dose effects of SANGUINATE on cardiac, circulatory and pulmonary functions and electrocardiograms (ECG) of conscious telemetered male Cynomogus monkeys were examined. Loose or soft feces were noted in two of four animals on Day 44 24-hours following the first administration of the high dose SANGUINATE at 400 mg/kg. Following the twice a day administration of the total high dose of SANGUINATE (1200 mg/kg/day), observations included delayed/prolonged bleeding times, facial and inguinal erythema, pink skin color, petichiae on the leg, decreased activity and white foamy/frothy feces. Following the additional total high dose of 1200 mg/kg/day on Day 114, clinical observations were limited to small red areas on the abdomen and extremities. Further, no clinical observations were noted on Day 127 with the exception of loose feces for one animal. Clinical chemistry showed no detectable levels of Troponin, increased creatinine, increased BUN, decreased amylase, decreased ALK, and slightly decreased ALT. There were no microscopic findings that indicated direct test article toxicity. Some of the findings may be considered an indication of toxicity in dosed animals from non-instrumentation studies, but there were no untreated or sham control study specific animals to compare microscopic findings against. Administration of 100 and 200 mg/kg SANGUINATE as either single doses or 5 daily doses were not associated with any definitive changes in heart rate, blood pressure, ECG or pulmonary parameters. Administration of 400 mg/kg SANGUINATE was associated with minimal increases in arterial pressure following the first dose and slight increases in heart rate and arterial pressure following the fifth dose. No definitive changes in ECG or pulmonary parameters were noted at the 400 mg/kg dose level. The first day of dosing of the total high dose of SANGUINATE (1200 mg/kg/day) was associated with decreased heart rate and increased arterial pressure but no biologically relevant changes in ECG or pulmonary pressures. Following the fifth day of dosing at 1200 mg/kg/day, increases in heart rate, arterial pressure and QTc were noted as well as decreases in respiratory rate. The additional doses of 1200 mg/kg/day on Day 127 were associated with increased heart rate and pressure but no definitive changes in ECG or pulmonary parameters (Figure 3).

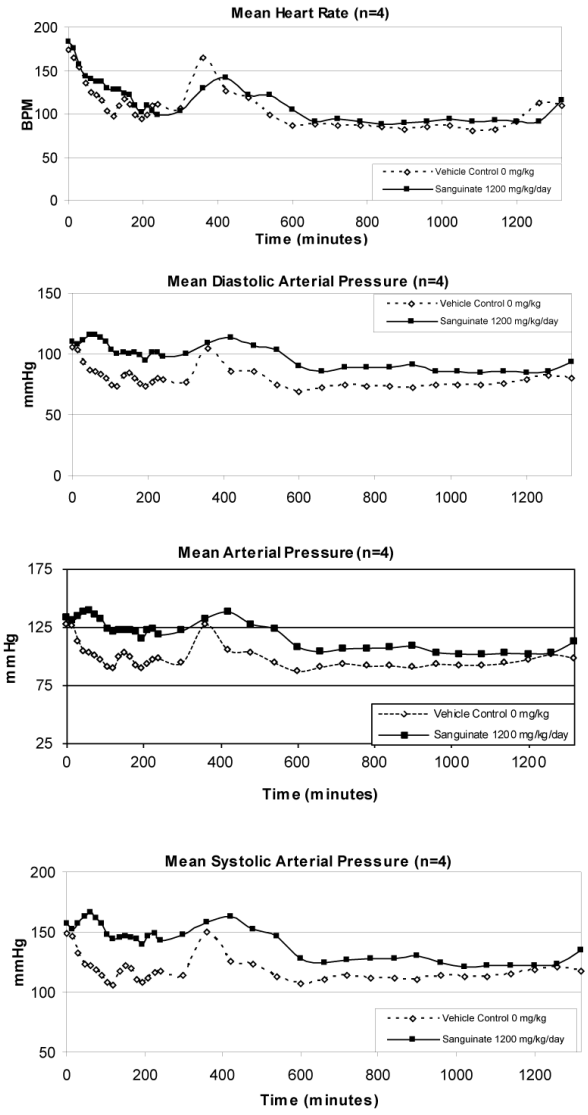

Figure 3: Effects of Intravenous Administration of 1200 mg/kg/day SANGUINATE™ Upon Cardiovascular Parameters (Day 127).

Discussion

Unlike HBOCs, SANGUINATE is both a CO and oxygen delivery agent. Historically, oxygen carriers were under development primarily as blood substitutes for trauma indications. However due to SANGUINATE's unique mechanisms of action, it is under development as an acute and chronic therapeutic for specific indications. As such, the preclinical development program not only had to incorporate the theoretical toxicity concerns of PEG-Hb but address both toxicity concerns of repeat and chronic dosing. Critical to the success of this testing program was not only addressing the concerns that arose from the experiences of HBOC development but establishing a collaborative effort with feedback from the FDA to design a program that would move the program into clinical trials. Unique features to this program included a renal glomerular filtration rate and blood flow study; immunohistochemical staining for TNF-α (inflammatory marker), malondialdehyde (oxidative marker), and Prussian Blue iron staining in kidneys, myocardium, vasculature, and brain (cerebrum and cerebellum); troponin in the serum; hemoglobin in the urine; high dose/high volume groups; inclusion of toxicity parameters within the cardiovascular safety study in monkey; and interference characterization and correction.

To address the issue as to whether the Hb moiety of SANGUINATE induced inflammation or oxidative stress, two well-characterized markers, TNF-α (an inflammatory marker) and MDA (an oxidative marker) were assessed in the 6 and 9 month rat and pig studies. TNF-α and MDA staining had no dose trend and was not considered to be significant in either the rat or pig, indicating that SANGUINATE is not causing inflammation or oxidative effects in these tissues. To determine whether SANGUINATE contributed to iron deposition, tissue sections from kidneys, myocardium, vasculature, and brain were stained using Prussian blue techniques. Iron staining was present in the brain and kidneys of both rat and pig, particularly in the high dose/high volume group. However, these findings were not considered biologically relevant as further microscopic evaluation of liver and kidneys did not confirm any test article related effects.

The colloidal nature of PEGylated proteins allows SANGUINATE to act as a plasma expander, and dosing levels of SANGUINATE are limited by the volume administered. Therefore, high dose/high volume groups were added to both the 6 and 9 month rat and pig toxicity studies in an attempt to increase the exposure and produce some signs of toxicity. The potential for cardiotoxicity was specifically addressed through the use of clinical chemistry including Troponin, histopathology, ECGs, and a cardiovascular and pulmonary safety study in monkeys. Clinical chemistry or histopathological findings were reversible or were not considered biologically relevant. Although there were some changes in heart rate and blood pressure, there were no definitive changes in ECG or pulmonary parameters. The potential for nephrotoxicity was specifically addressed through a renal glomerular filtration rate and blood flow study, clinical chemistry, urinalysis, measurement of hemoglobin in the urine, the high dose/high volume group, and histopathology. There were no changes in renal glomerular filtration rate or blood flow. Clinical chemistry or histopathological findings were reversible or were not considered biologically relevant. Although urine contained hemoglobin and there were some changes in urine volume, these changes were reversible. Overall, cardiotoxic and nephrotoxic signs were not significant. It is also relevant to note that SANGUINATE was non-immunogenic in both rat and pig.

Despite the history of adverse effects seen with HBOCs, SANGUINATE was well tolerated and safe at doses anticipated for human clinical trials. At dosages up to 400 mg/kg, no significant renal, cardiovascular or pulmonary toxicity was observed nor were there any abnormal body and organ weight measurements, clinical and ophthalmic observations, hematology, serum chemistry, coagulation, urinalysis and histopathology results. At doses of 1200 and 1600 mg/kg, changes in heart rate, arterial pressure and respiration were noted. Although no NOAEL could be determined for SANGUINATE within a study, it is likely to fall between 1200 and 2400 mg/kg. While there were signs of toxicity following dosing at the highest levels, the highest intended dose level for humans is 320 mg/kg infused over 2 hours which is 7 times lower than highest doses provided to animals. Recently three compassionate use eINDs were approved by US-FDA

for the treatment of patients with hemoglobin levels below 4 g/dL. These patients received multiple infusions of SANGUINATE and no adverse effects attributable to SANGUINATE were observed. Following infusion of SANGUIANTE, improved cerebral oximetry and status (i.e. ability to responds to commands, reports of "feeling better", discharge from ICU) were observed.

Conclusion

Chronic toxicology studies used the highest doses and the largest volumes that were permitted by the governing Animal Ethics Committee. As can be seen from the listed reports, Prolong has performed a comprehensive array of short-term and longer-term nonclinical toxicology studies, as well as pharmacology/toxicokinetics studies. Due to its lack of toxicity, a NOAEL could not be determined from these studies, even at doses up to 2400 mg/kg. Toxicity-related findings from the studies, including doses and method of administration, are provided in Table 2. A summary report of histopathology findings from the three long-term preclinical studies (in rat, mini-pig, and non-human primate) explained that, "…no direct toxic test article related macroscopic or microscopic findings were found in the protocol-specified blinded tissues [brain, kidneys, lungs] from these three studies. As no specific test article related dose effect was noted, no NOAEL could be determined for these studies".

As such, the program outlined above was successful. Based on the safety profile, the US FDA as well as other regulatory agencies have approved clinical testing of SANGUINATE in human patients for multiple indications. A phase I study has been completed and is consistent with the preclinical studies. SANGUINATE was found to be safe and well-tolerated in healthy human volunteers at doses of 80 mg/kg, 120 mg/kg, and 160 mg/kg [12]. Multiple doses of SANGUINATE have been used in Sickle Cell patients as well [13,14]. By using rational study design and including additional features to address specific concerns a comprehensive preclinical toxicity and safety program enabled the approval of SANGUINATE for Phase II clinical use worldwide for multiple indications.

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