CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER:

125513Orig1s000

PHARMACOLOGY REVIEW(S)

Comments on BLA 125-513 asphotase alpha

From: A Jacobs, AD

Date: 9/11/15

1. There are no pharm-tox-related approval issues
2. I have conveyed several comments to the reviewer and they will be addressed as appropriate.

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/s/

ABIGAIL C JACOBS
09/11/2015

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH

PHARMACOLOGY/TOXICOLOGY BLA REVIEW AND EVALUATION

Application number:	125-513
Supporting document/s:	0003
Applicant's letter date:	June 30, 2014
CDER stamp date:	June 30, 2014
Product:	STRENSIQ™ (Asfotase alfa)
Indication:	Treatment of hypophosphatasia (HPP).
Applicant:	Alexion Pharmaceuticals, Inc.
Review Division:	Division of Gastroenterology and Inborn Errors products (DGIEP)
Reviewer:	Dinesh Gautam, Ph.D.
Supervisor/Team Leader:	Sushanta Chakder, Ph.D.
Division Director:	Donna Griebel, M.D.
Project Manager:	Kevin B. Bugin, MS, RAC

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Executive Summary

1.1 Recommendations

1.1.1 Approvability

There are no pharm-tox approval issues.

1.1.2 Additional Non Clinical Recommendations

None

1.1.3 Labeling

The draft labeling of STRENSIQ™ conforms to the format specified under 21CFR 201.57(c)(14) Requirements for PLR (Physician's Labeling Rule) Prescription Drug Labeling.

8.1 Pregnancy

Sponsor's version:

Risk Summary

(b) (4)

Data

(b) (4)

Animal Data

(b) (4)

Evaluation: The text should be modified as proposed below.

Risk Summary

There are no available human data on STRENSIQ use in pregnant women to inform a drug associated risk. In animal reproduction studies, asfotase alfa administered intravenously to pregnant rats and rabbits during the period of organogenesis showed no evidence of fetotoxicity, embryolethality or teratogenicity at doses causing plasma exposures up to 21 and 24 times, respectively, the exposure at the recommended human dose *[see Data].*

In the U.S. general population, the estimated background risk of major birth defects and miscarriage in clinically recognized pregnancies is 2 to 4% and 15 to 20%, respectively.

Data

Animal Data

Asfotase alfa administered during the period of organogenesis to rats (from gestation Day 6 to Day 19 post-partum) and rabbits (on gestation days 7 to 19) at intravenous doses up to 50 mg/kg/day, (approximately 21 and 24 times the human AUC of 65486 ng.h/mL at 2 mg/kg dose administered three times weekly for a 50 kg individual, respectively) did not cause any adverse effects on embryofetal development. A pre- and postnatal development study in pregnant rats showed no evidence of adverse effects on pre- and postnatal development at intravenous doses (from Day 6 of gestation to Day 19 postpartum) of asfotase alfa up to 50 mg/kg/day (approximately 21 times the human AUC of 65486 ng.h/mL at 2 mg/kg dose administered three times weekly for a 50 kg individual).

13. NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Sponsor's version:

(b) (4)

[redacted]

Evaluation: The text should be modified as proposed below.

Long-term studies in animals to evaluate carcinogenic potential or studies to evaluate mutagenic potential have not been performed with asfotase alfa. Asfotase alfa at intravenous doses up to 50 mg/kg/day administered daily in pregnant rats (approximately 21 times the human AUC of 65486 ng.h/mL at 2 mg/kg dose administered three times weekly for a 50 kg individual) was found to have no adverse effect on fertility and reproductive performance of male and female rats.

(b) (4)

[redacted]

1.2 Brief Discussion of Nonclinical Findings

In support of the BLA, the applicant submitted reports of primary pharmacology, safety pharmacology, pharmacokinetics, single and repeated dose toxicity studies in juvenile rats and juvenile monkeys, and reproductive and development toxicology studies in rats and rabbits.

ENB-0040 (asfotase alfa) is a fusion protein that consists of the catalytic domain of human tissue nonspecific alkaline phosphatase, human IgG1 Fc domain and a decamer aspartate peptide used as a bone targeting domain. *In vitro* pharmacology studies showed that asfotase alfa binds with a high affinity (up to 97%) to hydroxyapatite, the most common mineral component of bone. Asfotase alfa can rescue the inhibition of mineralization induced by inorganic pyrophosphate (PPi). The efficacy of asfotase alfa was evaluated in *Akp2*$^{-/-}$ (an HPP knockout mouse model) mice (immediately after birth to 15-day-old) following SC administration at dose levels of up to 15.2 mg/kg for up to 52 days. Following SC administration to *Akp2*$^{-/-}$ mice, asfotase alfa caused a reduction of plasma PPi (inorganic pyrophosphate) levels, and caused a significant increase in bone mineralization of the feet, rib cages and pelvic limbs, and improved body weight gain and survival rate. Following IV administration of single dose of asfotase alfa, to normal rats, it caused a bradypneic effect with a decrease in minute volume. In normal rats, treatment with asfotase alfa was associated with reduced motor activity, reduced reactivity to stimuli, abnormal gait and reduced mobility, altered landing foot splay and lower grip strength. Asfotase alfa had no effects on ECG parameters in juvenile Cynomolgus monkeys when administered by SC injection at dose levels up to 10 mg/kg.

The pharmacokinetics (PK) of asfotase alfa was characterized in mice, rats and monkeys. The plasma clearance (CL) of asfotase alfa ranged from 0.00504-0.0540 L/h/kg and apparent terminal half-lives ranged from 14-40 hours across the species. Distribution studies with radiolabeled asfotase alpha showed that it is distributed into peripheral tissues, including bones. The PK profiles following SC dosing suggest a slow absorption of the enzyme and the SC bioavailability ranged from 25 to 56% in the species tested.

The toxicity profile of asfotase alfa was assessed in a single-dose toxicity study in monkeys, and repeated dose toxicity studies of up to 6-months duration in rats and monkeys after intravenous and subcutaneous administration. Common clinical signs observed in rats were partly closed eyes, decreased muscle tone, lying on the side, hunched posture, cold to touch, uncoordinated movements, decreased activity, abnormal gait and/or blue, red and/or firm swollen hindpaws and/or forepaws and swollen muzzle. These observations were transient and did not occur on non-dosing days or during the recovery period. A dose related increase in alkaline phosphatase level was observed in all test article treated animals (rats and monkeys) throughout the treatment period. Since the test article is recombinant soluble form of tissue nonspecific alkaline phosphatase, this increase was due to the presence of the test article in the bloodstream of the animals. The NOAEL dose in juvenile rats in the 6-month IV toxicity

study was 13 mg/kg/day, and the NOAEL dose in the 6-month SC toxicology study in juvenile monkeys was 10 mg/kg/day.

Asfotase alfa was not fetotoxic, embryo lethal or teratogenic in rats and rabbits at up to 50 mg/kg/day IV doses. Asfotase alfa had no effect on fertility in rats at IV doses up to 50 mg/kg/day. It had no adverse effects on pre- and postnatal development in rats at up to 50 mg/kg/day IV doses, the highest dose tested.

2 Drug Information

2.1 Drug:

STRENSIQ™ (Asfotase alfa) SC injection

2.1.1 CAS Registry Number (Optional)

1174277-80-5

2.1.2 Generic Name

Asfotase alfa

2.1.3 Code Name

ENB-0040, sALP-FcD10 and ALXN 1215

2.1.4 Chemical Name

Human Recombinant Tissue Non-Specific Alkaline Phosphatase Fusion Protein (TNSALP)

2.1.5 Molecular Formula/Molecular Weight

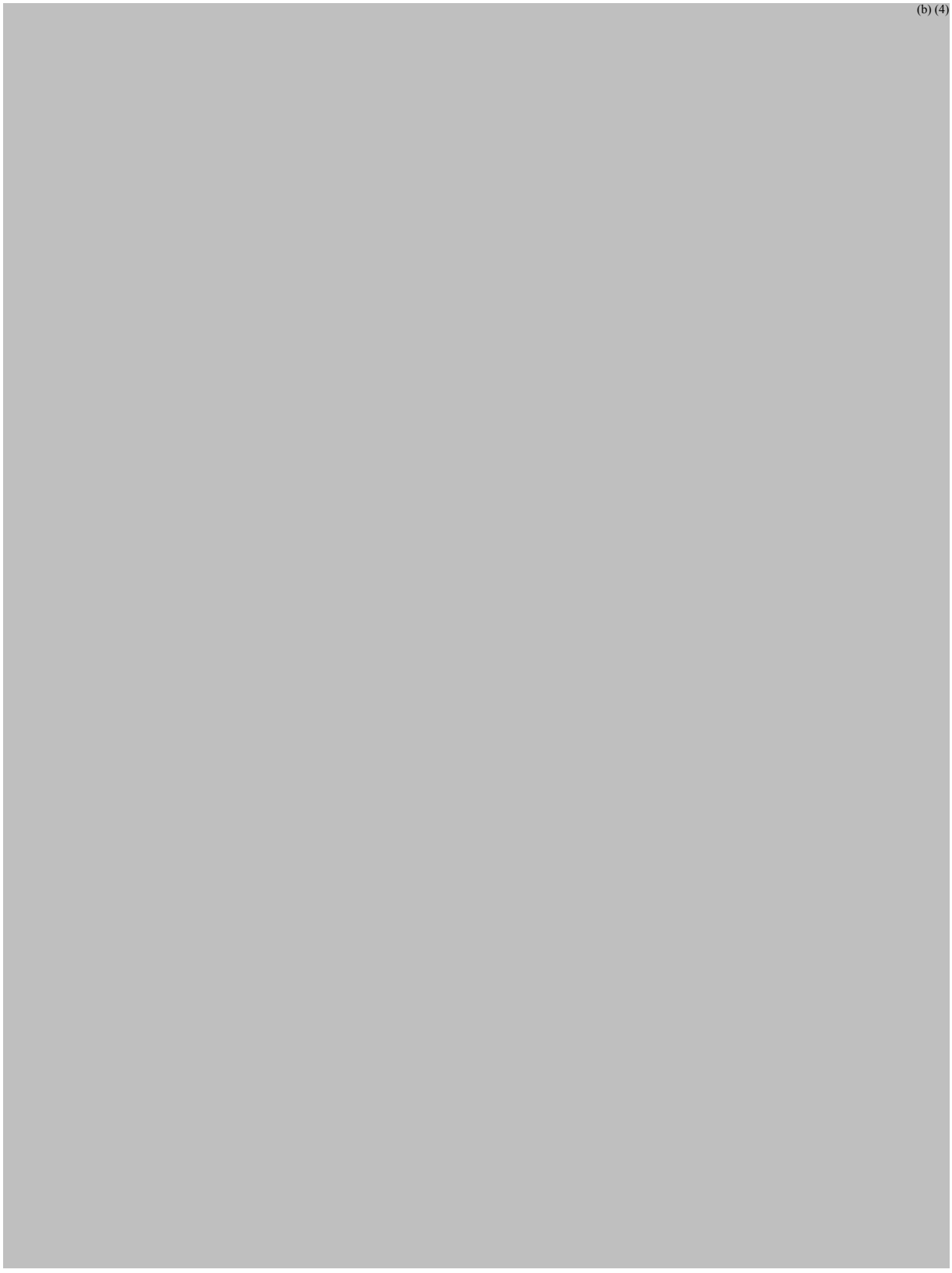
Molecular formula: (b) (4)

2.1.6 Structure

Asfotase alfa is a soluble IgG1 Fc fusion glycoprotein comprised of two identical polypeptide chains, each with a length of 726 amino acids. Each polypeptide chain is comprised of a soluble catalytic domain of human tissue non-specific alkaline phosphatase (sALP), a human immunoglobulin IgG1 Fc domain, a deca-aspartate peptide (D10) used as a bone-targeting domain, and two amino acid long linkers between these domains. Each polypeptide chain contains (b) (4)

(b) (4) The two polypeptides are covalently linked together by two disulfide bonds. The schematic Figure and amino acid sequences are shown below.

(b) (4)



2.1.7 Pharmacologic class

Enzyme replacement therapy.

2.2 Relevant IND/s, NDA/s, and DMF/s

IND 100,619

2.3 Clinical Formulation

2.3.1 Drug Formulation

STRENSIQ™ is a sterile, nonpyrogenic SC injectable formulation containing asfotase alfa at a concentration of 40 mg/mL or 100 mg/mL in (b) (4) sodium phosphate, (b) (4) sodium chloride in a 2 mL Type 1 glass vial. At the 40 mg/mL concentration, it is supplied as a single-use vial at (b) (4) 0.45, 0.70, and 1.0 mL volumes containing (b) (4) 18, 28, and 40 mg of asfotase alfa, respectively. At the 100 mg/mL concentration, it is supplied in a single-use vial of 0.80 mL volume containing 80 mg of asfotase alfa. The vials are stoppered with (b) (4) rubber stoppers and sealed with aluminum seals with (b) (4) caps. The composition of the drug product is shown in the applicant's Table below.

Table 1: Asfotase Alfa Drug Product 40 mg/mL Composition

Component (Formulation Concentration)	Quality Standard	Function	Available Quantity per Vial for Each Extractable Volume		
			(b) (4) 0.45 mL	0.70 mL	1.0 mL
asfotase alfa (40 mg/mL)	In-house	Active ingredient	18 mg	28 mg	40 mg
(b) (4) Sodium chloride (b) (4)	USP, Ph. Eur., JP	(b) (4)			(b) (4)
(b) (4) Dibasic sodium phosphate, (b) (4) (b) (4)	USP, Ph. Eur.				
(b) (4) Monobasic sodium phosphate, (b) (4) (b) (4)	USP, Ph. Eur.				
(b) (4)	USP, Ph. Eur., JP				

Table 2: Asfotase Alfa Drug Product 100 mg/mL Composition

Component (Formulation Concentration)	Quality Standard	Function	Available Quantity per Vial for Each Extractable Volume 0.80 mL
asfotase alfa (100 mg/mL)	In-house	Active ingredient	(b) (4)
(b) (4) Sodium chloride (b) (4)	USP, Ph. Eur., JP	(b) (4)	
(b) (4) Dibasic sodium phosphate, (b) (4) (b) (4)	USP, Ph. Eur.		
(b) (4) Monobasic sodium phosphate, (b) (4) (b) (4)	USP, Ph. Eur.		
(b) (4)	USP, Ph. Eur., JP		

2.3.2 Comments on Novel Excipients

No novel excipients are present in the drug product.

2.3.3 Comments on Impurities/Degradants of Concern

Asfotase alfa was tested for the potential process or product related impurities. Process related impurities encompass those derived from or introduced during the manufacturing process. These include impurities (b) (4) . Product related impurities are (b) (4) Eleven (b) (4) related impurities were identified. (b) (4) related impurity profile of asfotase alfa is listed in the applicant's Table below.

Table 20: Summary of the Eleven Process Related Impurities Determined in the Drug Substance

(b) (4)

Product related impurities include, (b) (4) Summary of the product related impurities is presented in the applicant's Table below.

Table 4: Summary of Asfotase Alfa Product Related Impurities

(b) (4)

Based on the ICH guidance, these impurities are within the acceptable limits and are qualified.

Evaluation of Leachable in STRENSIQ™ (Asfotase alfa):

The extractable studies of (b) (4) were conducted in the test solutions included; (b) (4) . The (b) (4) The resulting solutions were analyzed by (b) (4) Extractable studies identified (b) (4) as potential leachable compounds from (b) (4) Potential leachables identified during extractable studies are presented in the applicant's Table below:

(b) (4)

The drug substance lots (b) (4) were evaluated for leachables at (b) (4) month time points as indicated in the applicant's Table below.

Table 3: Drug Substance Leachable Data to Date

(b) (4)

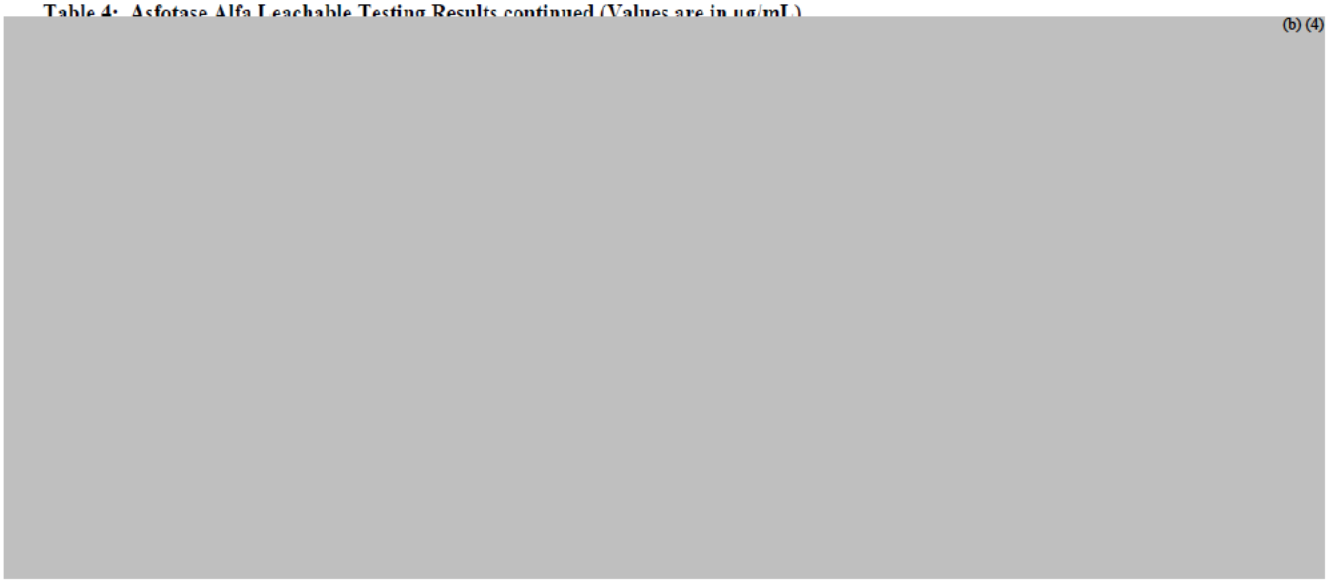
Results of the leachables analysis are summarized in the applicant's Table below.

Table 4: Asfotase Alfa Leachable Testing Results (Values are in µg/mL)

(b) (4)

ND = Not Detected

Table 4: Asfotase Alfa Leachable Testing Results continued (Values are in µg/mL)



In the long term leachable study, only two leachables, [redacted] were detected in all samples stored for up to [redacted] months; [redacted] was also detected in the two drug lots at the [redacted]-month time point. Other potential leachables, listed in the Table above were either not detected or below the limits of detection. The [redacted] levels ranged between [redacted] µg/mL. The [redacted] levels were in between [redacted] µg/mL and [redacted] µg/mL for all 5 lots. However, the highest level [redacted] µg/mL) of [redacted] was detected at the [redacted]-month time point (T= [redacted]) for lot 335332. At the [redacted]-month time point, simulating worst case leachable profile, the levels of [redacted] was [redacted] µg/mL. The level of [redacted] detected was [redacted] and [redacted] µg/mL at the [redacted] month time point from drug lots of 259248 and 260464, respectively. [redacted] was detected at levels between [redacted] and [redacted] µg/mL throughout the study period.

The maximum recommended daily dose of asfotase alfa is 2 mg/kg, and the total amount administered to a 50 kg individual is 100 mg or 1mL daily. According to ICH Q3C guidance, [redacted] the daily exposures of [redacted] mg/day or less would be acceptable without justification. The highest amount of [redacted] detected was [redacted] µg/mL, which is [redacted]-fold lower than the acceptable daily exposure of [redacted] The NOAEL dose of [redacted] is identified to be [redacted] mg/day by the EPA (http://www.epa.gov/iris/subst/0290.htm). The PDE of [redacted] would be [redacted] µg/day for a 50 kg individual [redacted]. The highest amount of [redacted] exposure to a 50 kg individual from 1 mL asfotase alfa would be [redacted] µg/day, which is [redacted]-fold lower than the calculated PDE. According to ICH Q3D guidance, [redacted] is classified as a Class [redacted] with the parenteral PDE of [redacted] µg/day. A 50 kg person is expected to receive a maximum amount of [redacted] µg/day of [redacted] from 1 mL of asfotase alfa. The expected daily exposure of [redacted] is > [redacted]-fold lower than its PDE. Thus, the levels of all potential leachables from the container closure system are within the recommended safety limit and appear to be acceptable.

2.4 Proposed Clinical Population and Dosing Regimen

STRENSIQ™ (Asfotase alfa) SC injection is indicated for (b) (4) in patients with infantile- and juvenile-onset hypophosphatasia (HPP). The proposed dosing regimen is 2 mg/kg of body weight administered subcutaneously three times per week, or 1 mg/kg of body weight administered six times per week.

2.5 Regulatory Background

Several pre-sBLA meetings were held between November 26, 2013 to January 14, 2014 regarding CMC, clinical pharmacology and clinical development. A pre-IND meeting was held on July 26, 2007, in which the agency requested the Sponsor to conduct toxicity studies in younger animals and also recommended safety pharmacology and reproductive toxicology studies with asfotase alfa.

3 Studies Submitted

The applicant submitted following pharmacology, pharmacokinetics and toxicological studies to support this BLA application.

2.6.3.1. Pharmacology

Overview			Test Article: Asfotase Alfa	
Type of Study	**Test System**	**Method of Administration**	**Testing Facility**	**Study Number**
Primary Pharmacodynamics				
Binding to hydroxyapatite	asfotase alfa	in vitro	Enobia Pharma Inc.	JG-60-Exp2
Rescue of PPi-induced mineralization inhibition in MC3T3-E1 cells	asfotase alfa	in vitro	Enobia Pharma Inc.	FB-06-Exp17
Prophylactic treatment in *Akp2*$^{-/-}$ mice (15 days) – effect on bone mineralization defects of the hindpaw and growth	*Akp2*$^{-/-}$ mice	SC	Enobia Pharma Inc.	ALP-PT-04
Phenotyping study in *Akp2*$^{-/-}$ mice – craniosynostosis	*Akp2*$^{-/-}$ mice	NA[a]	Enobia Pharma Inc.	HPP-PH-06
Prophylactic treatment in *Akp2*$^{-/-}$ mice (16 days) – effect on bone mineralization deficit of the enthesis	*Akp2*$^{-/-}$ mice	SC	Enobia Pharma Inc.	ALP-PT-22
Prophylactic treatment in *Akp2*$^{-/-}$ mice (15 days) – effect on plasma PPi	*Akp2*$^{-/-}$ mice	SC	Enobia Pharma Inc.	ALP-PT-24
Prophylactic treatment in *Akp2*$^{-/-}$ mice (9 days) – effect on plasma PLP	*Akp2*$^{-/-}$ mice	SC	Enobia Pharma Inc.	ALP-PT-25.1
Prophylactic treatment in *Akp2*$^{-/-}$ mice (52 days; daily dosing) – effect on bone mineralization defects of the hindpaw and survival	*Akp2*$^{-/-}$ mice	SC	Enobia Pharma Inc.	ALP-PT-03
Prophylactic treatment in *Akp2*$^{-/-}$ mice (52 days; 3 and 7 days dosing interval) – effect on bone mineralization defects of the hindpaw and survival	*Akp2*$^{-/-}$ mice	SC	Enobia Pharma Inc.	ALP-PT-05
Prophylactic treatment in *Akp2*$^{-/-}$ mice (43 days; 1, 3 and 7 days dosing interval) – effect on bone mineralization defects of the hindpaw and survival	*Akp2*$^{-/-}$ mice	SC	Enobia Pharma Inc.	ALP-PT-06
Prophylactic (43 days; daily dosing) and therapeutic (initiation of treatment on day 15) treatment in *Akp2*$^{-/-}$ mice – effect on bone mineralization defects of the hindpaw, growth and survival	*Akp2*$^{-/-}$ mice	SC	Enobia Pharma Inc.	ALP-PT-08
Therapeutic treatment in *Akp2*$^{-/-}$ mice (initiation of treatment on day 12) – effect on bone mineralization defects of the hindpaw and survival	*Akp2*$^{-/-}$ mice	SC	Enobia Pharma Inc.	ALP-PT-09
Prophylactic treatment in *Akp2*$^{-/-}$ mice (43 days; daily dosing) – effect on bone mineralization defects of the hindpaw, growth and survival	*Akp2*$^{-/-}$ mice	SC	Enobia Pharma Inc.	ALP-PT-11
Dose response modeling in *Akp2*$^{-/-}$ mice – dose response in relation to bone mineralization defects of the hindpaw and survival	*Akp2*$^{-/-}$ mice	SC	Enobia Pharma Inc.	ALP-PT-12
Prophylactic treatment in *Akp2*$^{-/-}$ mice (43 days; daily dosing) – effect on bone mineralization deficit of the enthesis	*Akp2*$^{-/-}$ mice	SC	Enobia Pharma Inc.	ALP-PT-23.2

Overview			Test Article: Asfotase Alfa	
Type of Study	Test System	Method of Administration	Testing Facility	Study Number
Primary Pharmacodynamics				
Prophylactic treatment in *Akp2*$^{-/-}$ mice (35 days; daily dosing) – effect of dosing cessation on bone mineralization defects of the hindpaw, growth and survival	*Akp2*$^{-/-}$ mice	SC	Enobia Pharma Inc.	ALP-PT-25.2
Secondary Pharmacodynamics				
No studies of this type were conducted.				
Safety Pharmacology				
Serum calcium and phosphorus	*Akp2*$^{-/-}$ mice	SC	Enobia Pharma Inc.	ALP-PT-10
Acute injection reaction	Sprague-Dawley rats	IV bolus; IV infusion; SC	(b) (4)	PK2008021951
Central nervous system	Sprague-Dawley rats	IV		1007-2491[b]
Respiratory function	Sprague-Dawley rats	IV		1007-2501[b]
Cardiovascular system[c]	Cynomolgus juvenile monkeys	SC		670388[b]
Pharmacodynamic Drug Interactions				
No studies of this type were conducted.				

[a] NA, not applicable
[b] report contains a GLP Compliance Statement
[c] conducted as part of the 6-month toxicology study

2.6.5.1. Pharmacokinetics Overview

			Test Article: Asfotase Alfa			
Type of Study	Test System	Method of Administration	Testing Facility	Study Number	Location	
					Vol.	Page
Pharmacokinetics: Absorption Single Dose						
Single Dose (Lot 070629)	C57BL/6 Mouse	IV, SC	Enobia Pharma Inc. (Montreal, Canada)	ALP-PC-09		
Pharmacokinetics: Absorption Single Dose						
Single Dose (PUR012G02)	*Akp2*$^{-/-}$ and WT Juvenile C57BL/6, IJ29 Mouse	SC	Enobia Pharma Inc. (Montreal, Canada)	ALP-PC-25		
Pharmacokinetics: Absorption Single Dose			(b) (4)			
Single Dose (Lot PUREA2_(1+2))	Sprague Dawley Rat	IV, SC		PK2008010951		
Pharmacokinetics: Absorption Single Dose						
Single Dose (Lot PUREA2_(1+2))	Cynomolgus Monkey	IV, SC		3008-0133		
Pharmacokinetics: Absorption Single Dose						
Single Dose (Lot 070629)	Juvenile Cynomolgus Monkey	IV, IV infusion		2007-0693		
Pharmacokinetics: Absorption Single Dose						
Single Dose (Lot 169466)*	New Zealand White Rabbit	IV, SC		902235		
Pharmacokinetics: Absorption Repeated Dose						
Repeated Dose Toxicity/TK (4-week)* (IV lot PUR012F01, SC Lot PUREA2 200L#3)	Juvenile Sprague Dawley Rat	IV, SC		70552		
Pharmacokinetics: Absorption Repeated Dose						
Repeated Dose Toxicity/TK (4-week)* CON025F02 (PUREA2_(1+2))	Juvenile Sprague Dawley Rat	IV		670314		
Pharmacokinetics: Absorption Repeated Dose						
Repeated Dose Toxicity/TK (26-week)* (Lot PUR012G01, FIL094G02, PUR012G02)	Juvenile Sprague Dawley Rat	IV		670315		
Pharmacokinetics: Absorption Repeated Dose						
Repeated Dose Toxicity/TK (4-week)* (Lot PURE A2(1+2))	Juvenile Cynomolgus Monkey	IV		1007-1503		

2.6.5.1. Pharmacokinetics Overview (Continued)

			Test Article: Asfotase Alfa			
Type of Study	**Test System**	**Method of Administration**	**Testing Facility**	**Study Number**	**Location**	
					Vol.	**Page**
Pharmacokinetics: Absorption Repeated Dose						
Repeated Dose Toxicity/TK (6-month)* (Lot PUR012F01)	Juvenile Cynomolgus Monkey	SC	(b) (4)	670388		
Pharmacokinetics: Organ Distribution						
Single Dose (Lot 060410)	Adult 129J Mouse	IV	Enobia Pharma Inc. (Montreal, Canada)	ALP-PD-01		
Pharmacokinetics: Organ Distribution						
Repeated Multiple Doses (unlabeled lot 07312, Iodinated lot 070405)	Newborn CD-1 Mouse	SC	Enobia Pharma Inc. (Montreal, Canada)	ALP-PD-02		
Pharmacokinetics: Study in Pregnant/Nursing Animals						
Single Dose (PUR012G01)	Pregnant CD-1 Mouse	SC	Enobia Pharma Inc. (Montreal, Canada)	ALP-PT-15		
Pharmacokinetics: Study in Pregnant/Nursing Animals						
Reproductive/Development TK(14-day)* (Lot 169466)	Pregnant Sprague Dawley Rat	IV	(b) (4)	902480		
Pharmacokinetics: Study in Pregnant/Nursing Animals						
Reproductive/Development TK(14-day)* (Lot 259248)	Pregnant Sprague Dawley Rat	IV		902238		
Pharmacokinetics: Study in Pregnant/Nursing Animals						
Reproductive/Development TK (13-day)* (Lot 169466)	Pregnant New Zealand White Rabbit	IV		902236		
Pharmacokinetics: Study in Pregnant/Nursing Animals						
Reproductive/Development TK (13-day)* (Lot 259248)	Pregnant New Zealand White Rabbit	IV		902237		

*GLP study.
Note: All IV administrations in this section are IV bolus unless specifically stated as IV infusion.

2.6.7.1. Toxicology Overview

Overview						**Test Article: Asfotase Alfa**			
Type of Study	**Species and Strain**	**Method of Administration**	**Duration of Dosing**	**Doses (mg/kg)**	**GLP Compliance**	**Testing Facility**	**Study Number**	**Location Vol.**	**Page**
Single-Dose Toxicity									
Maximum Tolerated Single-Dose Toxicity (within animal dose escalation design)	Monkey/ Cynomolgus (juvenile)	IV bolus and IV infusion single dose (weekly dose escalation)	29 days for TK satellite; 46 days for main group	5, 15(bolus), and 45 (3min), 90 (6 min), and 180 (12 min infusion)	No	(b) (4)	2007-0693		
Repeat-Dose Toxicity									
Subchronic Single-Dose Toxicity	Rat/Sprague-Dawley (juvenile)	IV (weekly)	4 weeks	10, 30, 90, 180	No		70385		
Subchronic	Rat/Sprague-Dawley (juvenile)	IV (weekly)	4 weeks	0, 2.6, 26, 77	Yes		670314		
Chronic	Rat/Sprague-Dawley (juvenile)	IV (daily)	6 months	0, 1, 3, 13	Yes		670315		
Subchronic	Monkey/ Cynomolgus (juvenile)	IV (weekly)	4 weeks	0, 5, 15, 45	Yes		1007-1503		
Chronic	Monkey/ Cynomolgus (juvenile)	SC (daily)	6 months	0, 0.43, 2.14, 10	Yes		670388		
Genotoxicity	No studies of this type were performed								
Carcinogenicity	No studies of this type were performed								
Reproductive and Developmental Toxicity									
Pilot Fertility Males	Rat/Sprague-Dawley	IV (daily)	15 days	0, 25, 50	Yes		902230		

Overview						Test Article: Asfotase Alfa			
Type of Study	Species and Strain	Method of Administration	Duration of Dosing	Doses (mg/kg)	GLP Compliance	Testing Facility	Study Number	Location Vol.	Page
Reproductive and Developmental Toxicity (Continued)									
Fertility	Rat/Sprague-Dawley	IV (daily)	4-5 weeks females, at least 9 weeks males	0, 10, 25, 50	Yes	(b) (4)	902231		
TK in pregnant rats	Rat/Sprague-Dawley	IV (daily)	GD[a] 6-19	13, 25, 50	Yes		902480		
Pilot Developmental	Rat/Sprague-Dawley	IV (daily)	GD 6-19	0, 13, 25, 50	Yes		902233		
Developmental	Rat/Sprague-Dawley	IV (daily)	GD 6-19	0, 13, 25, 50	Yes		902234		
TK in nonpregnant rabbits	Rabbit/New Zealand White	IV and SC (daily)	Single dose	5 and 50 for both routes	Yes		902235		
Pilot Developmental	Rabbit/New Zealand White	IV (daily)	PC[b] 7-19	0, 6, 13, 25, 50	Yes		902236		
Developmental	Rabbit/New Zealand White	IV (daily)	PC 7-19	0, 10, 25, 50	Yes		902237		
Perinatal/Postnatal	Rat/Sprague-Dawley	IV (daily)	GD 6-LD[c] 21	0, 10, 25, 50	Yes		902238		
Local Tolerance									
IV and SC Bridging Study	Rat/Sprague-Dawley (juvenile)	IV (weekly) and, SC (daily)	4 weeks	IV: 0, 3, 30, 90 (weekly) SC: 0, 0.84, 8.4, 25.2 (daily)	Yes		70552		
Other Toxicity Studies			No studies of this type were performed						

[a] GD = Gestation day; may also be designated as postcoitum (pc) in the study reports.
[b] PC = Postcoitum.
[c] LD = Lactation day.

3.1 Studies Reviewed

All submitted studies were reviewed.

3.2 Studies Not Reviewed

N/A

3.3 Previous Reviews Referenced

None.

4 Pharmacology

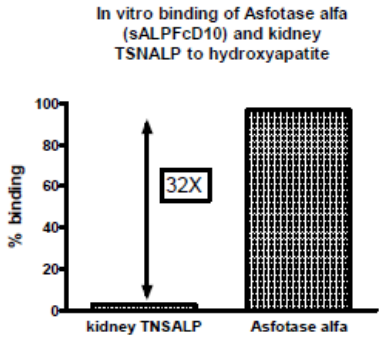
4.1 Primary Pharmacology

Binding of Asfotase Alfa and kidney TNSALP to purified hydroxyapatite (study # JG-60-Exp2).

The mineral component of bone represents up to 75% of bone tissue by weight and is composed of a group of phosphate minerals known collectively as apatite. The most prevalent component of apatite is hydroxyapatite (HA). Asfotase alfa (FcD10 fusion

protein) contains a deca-aspartate peptide moiety designed to bind specifically to hydroxyapatite. The binding affinity of asfotase alfa to hydroxyapatite was compared to that of non-FcD10 fusion protein (Kidney TNSALP, Tissue Non-Specific Alkaline Phosphatase). Asfotase alfa showed higher binding capacity to hydroxyapatite than kidney TNSALP. The percentage of binding for asfotase alfa was up to 97% compared to control (TNSALP), which was 3%. The result is presented in the applicant's Figure below.

Figure 1: Binding of Asfotase alfa and kidney TNSALP to hydroxyapatite



	% binding		
	Y1	Y2	Y3
kidney TNSALP	3.0	3.0	3.0
Asfotase alfa	97.0	96.0	97.0

Rescue of inorganic pyrophosphate (PPi)-induced mineralization inhibition in MC3T3-E1 cell cultures by Asfotase alfa (Study # FB-06-Exp17).

The osteoblastic cell line (MC3T3-E1) has been established from C57BL/6 mouse calvaria (skull), and have the ability to synthesize extracellular matrix (collagen) and differentiate into osteoblasts and osteocytes *in vitro*. Mineralization of MC3T3-E1 cells have been identified as hydroxyapatite in the presence of ascorbic acid and β-glycerol phosphate (β-GP). Mineralization of MC3T3-E1 cell cultures is inhibited by the addition of inorganic pyrophosphate (PPi) to the culture, and this inhibition can be reversed by administration of exogenous tissue non-specific alkaline phosphatase (TNSALP).

This study evaluated the ability of asfotase alfa to overcome PPi inhibition of mineralization in MC3T3-E1 cell cultures. Extracellular matrix deposition and mineralization was induced on day 1 with the addition of ascorbic acid (100μM final concentration) and β-glycerol phosphate (10 mM final concentration) to cell culture media. After 5 days in culture, various treatments (shown in the applicant's Table below; ±5 μM PPi final concentration) in the presence or absence of asfotase alfa (132 U/L) were initiated and the cells were incubated for another week. On day 14, extracellular matrix deposition and mineralization were assessed using a Picrosirius Red Stain Kit (collagen-specific, Polysciences Inc. Cat # 24901) and a calcium assay (Sekisui Diagnostics Cat # 140-20), respectively.

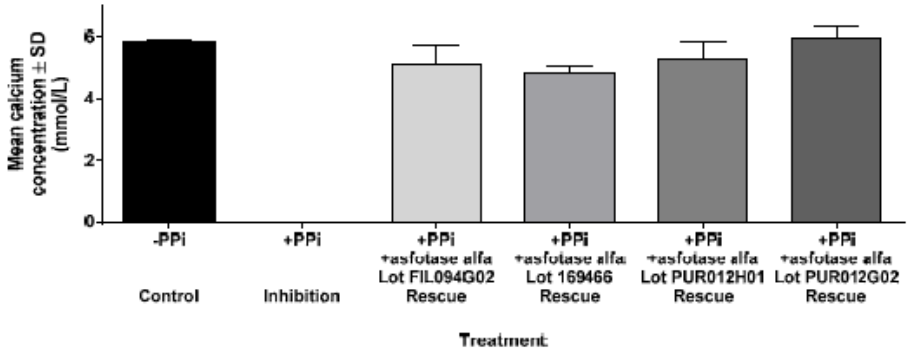
In the presence of PPi, the concentration of calcium was significantly decreased compared to untreated control cultures. When the cultures were incubated with both asfotase alfa and PPi, concentrations of calcium were similar to those found in the control cultures lacking PPi. The results demonstrated that asfotase alfa can rescue the inhibition of mineralization induced by PPi in MC3T3-E1 cells. Findings are presented in the applicant's Table and Figure below.

Table 2: Individual and mean calcium concentrations measured in MC3T3-E1 cells following various treatments with PPi and asfotase alfa

Sample ID	Treatments					
	− PPi	+ PPi	+ PPi	+ PPi	+ PPi	+ PPi
	− asfotase alfa	− asfotase alfa	+ asfotase alfa FIL094G02	+ asfotase alfa 169466	+ asfotase alfa PUR012H01	+ asfotase alfa PUR012G02
A	1.160	< LOQ	1.205	1.011	1.219	1.298
B	1.145	< LOQ	0.958	0.963	1.009	1.189
C	1.166	< LOQ	0.938	0.923	0.991	1.134
D	1.187	< LOQ	0.985	1.011	1.023	1.162
MEAN	**1.165**	**< LOQ**	**1.022**	**0.977**	**1.061**	**1.196**
SD	**0.02**	**NA**	**0.12**	**0.04**	**0.11**	**0.07**
% CV	**1.5**	**NA**	**12.1**	**4.4**	**10.0**	**6.0**

LOQ, lower limit of quantification; NA, not applicable

Figure 2: Quantification of mineralization in MC3T3-E1 extracellular matrix using a calcium assay after 14 days in cuolture and treatment with PPi and asfotase alfa



Phenotypic assessment of craniosynostosis in *Akp2*$^{-/-}$ mice (Study # HPP-PH-06):

Functional craniosynostosis and premature bony fusion of cranial sutures have been observed in patients with infantile and juvenile hypophosphatasia (HPP), respectively. The objective of the present study was to evaluate the presence and the severity of the craniosynostosis in *Akp2*$^{-/-}$ mice by linear craniofacial measurements and micro-CT evaluation.

The *Akp2*$^{-/-}$ mice is a murine knockout model of human HPP created by inactivating the TNSALP gene. The *Akp2*$^{-/-}$ mice and WT littermates received no treatment. The skulls from each animal were collected at necropsy (for surviving animals) or time of death to assess the presence and the severity of craniosynostosis. Two sets of specimens were analyzed: skulls of 15-day old *Akp2*$^{-/-}$ and WT mice and skulls of 20-24 day-old *Akp2*$^{-/-}$ and WT mice. Fifteen-day-old *Akp2*$^{-/-}$ mice were selected as standard duration for bone

phenotyping and 20-22-day-old *Akp2*$^{-/-}$ mice were selected as longest-lived *Akp2*$^{-/-}$ mice due to survival issues. A digital caliper was utilized to measure linear craniofacial measurements in *Akp2*$^{-/-}$ and WT mice in order to quantitate craniofacial abnormalities. These measurements included nasal, frontal and parietal bone length, skull length, and infraorbital and skull width, and skull height. At 15 days of age, the skulls of *Akp2*$^{-/-}$ mice were smaller (up to 11% in length and 7% in width) compared to WT littermates. Skull height was not significantly different between *Akp2*$^{-/-}$ and WT mice. When normalized to skull length, the nasal and frontal bone lengths were significantly shorter (24%, p <0.0001 and 7%, p <0.001, respectively) in severely affected *Akp2*$^{-/-}$ mice compared to WT littermates. No significant difference was observed in normalized parietal bone length. Normalized infraorbital width to skull width was significantly increased by 9% (p <0.0001).

At 20-22 days of age, the skulls of *Akp2*$^{-/-}$ mice were smaller (up to 21% in length and 8% in width) compared to 20-24-day old WT littermates. Skull height was not significantly different between *Akp2*$^{-/-}$ and WT mice. When normalized to skull length, the nasal and frontal bone lengths were significantly shorter (14% and 5%, respectively; p <0.01) in severely affected *Akp2*$^{-/-}$ mice compared to WT littermates. No significant difference was observed in normalized parietal bone length. Normalized infraorbital width to skull width was significantly increased by 8% (p <0.01).

Coronal suture fusion was assessed by two-dimensional microcomputed tomography (Micro-CT) in *Akp2*$^{-/-}$ mice. Assessments could not be performed in 15-day old *Akp2*$^{-/-}$ mice due to the tissue quality around the sutures which exhibits low and variable mineralization. Therefore, assessments of coronal suture was performed at 20 to 22 days of age when suture fusion was still incomplete. All *Akp2*$^{-/-}$ mice with a severe bone mineralization phenotype of bones of the hindpaw had a fused coronal suture at 20-22 days of age. *Akp2*$^{-/-}$ mice with less severe bone mineralization phenotype (moderate, slight and normal) displayed open coronal sutures.

These phenotypes support the relevance of the *Akp2*$^{-/-}$ mouse as a preclinical animal model of hypophosphatasia (HPP).

Evaluation of 15 Days of Treatment with a Daily Bolus Subcutaneous Injection of a Bone-Targeted Soluble Form of tissue non-specific alkaline phosphatase (TNAP) in *Akp2*$^{-/-}$ mice (Study # ALP-PT-04):

The objective of this study was to assess the effect of ENB-0040 on the bone mineralization defects observed in the *Akp2*$^{-/-}$ mice.

The *Akp2*$^{-/-}$ mice (n=17-20) received ENB-0040 (asfotase alfa, 8.2 mg/kg) or vehicle SC no later than 2 days after birth and up to 15 days of age, as described in the applicant's Table below, followed by necropsy at 24 hours after the last injection (i.e. 16 days).

Group Number	Group description	Treatment	Duration of treatment (day)	Dosing Interval	Dose Level (mg/kg)	Conc. (mg/ml)	Dose volume (ml/kg)
1	Vehicle	SC injection	15	Daily	0	0	3.2
2	Tx-1	SC injection	15	Daily	8.2	2.53	3.2
3	WT(*)	---	---	---	---	---	---

(*): WT (wild-type): normal littermates of *Akp2*$^{-/-}$ mice

Bone abnormalities were assessed by X-ray after 24 hours of last injection on study day 16. Hindpaw bones (metatarsals and phalanges) were evaluated in a blinded fashion and classified to one of the four classifications: severe, moderate, slight or normal.

Significant bone mineralization defects were observed 16 days after birth in vehicle-treated *Akp2*$^{-/-}$ mice. All WT littermates showed normal bone mineralization. Asfotase alfa (8.2 mg/kg) showed significantly increased bone mineralization after 15 days of treatment. No mice with severe mineralization defects were present in the group treated with ENB-0040. Severe mineralization defects were observed in 28% of *Akp2*$^{-/-}$ mice treated with the vehicle. Data are presented in the applicant's Table below.

Effect of 8.2 mg/kg Asfotase Alfa on Bone Mineralization of the Hindpaw from *Akp2*^-/- Mice After 15 Days of Prophylactic Treatment

Sample Size (n)	Daily Doses (mg/kg), SC	Classification of Bones of the Hindpaw at Day 16 Number (%) of Animals			
		Abnormal			Normal
		Severe	Moderate	Slight	
18	0	5 (28)	7 (39)	0 (0)	6 (33)
19	8.2	0 (0)	7 (37)	4 (21)	8 (42)[a]

The average concentration of asfotase alfa (ENB-0040) was 0.498 ± 0.514 ug/mL in the ENB treated group. The average concentration of endogenous total ALP (alkaline phosphatase) estimated in the WT group was 0.372 ± 0.077 ug/mL. Data are presented in the applicant's Figure below.

8.3.1 Test article concentrations in plasma

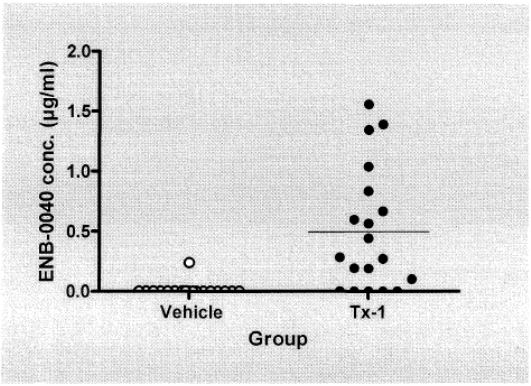


Figure 2 : ENB-0040 concentrations in plasma 24 hours after the last injection.

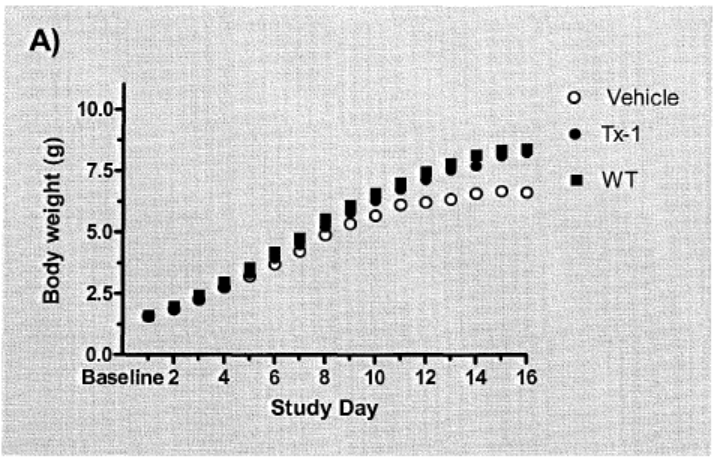
At day 16, the lengths of left tibiae and left femurs from the WT and asfotase alfa-treated animal groups were significantly longer than animals in the vehicle-treated group (WT, $p = 0.0006$ and $p = 0.0009$, respectively; asfotase alfa-treated *Akp2*$^{-/-}$ mice, $p = 0.0135$ and $p = 0.0267$, respectively). There were no significant differences between the tibiae or the femurs lengths, in the asfotase alfa-treated *Akp2*$^{-/-}$ and WT groups. Thus, bone growth was significantly greater in *Akp2*$^{-/-}$ mice treated with 8.2 mg/kg asfotase alfa compared to vehicle treated *Akp2*$^{-/-}$ mice after 15 days of prophylactic treatment. Data are presented in the applicant's Table below.

Table 6: Effect of 8.2 mg/kg Asfotase Alfa on Mean Bone Length of Left Tibia and Left Femur of *Akp2*^-/- Mice After 15 Days of Prophylactic Treatment

Study Number	Sample Size (n)	Mouse Strain	Daily Doses (mg/kg), SC	Bone Length (mm) Mean (SD)	
				Left tibia	Left femur
ALP-PT-04	18[a]	*Akp2*$^{-/-}$	0	11.71 (1.06)	8.58 (0.77)
ALP-PT-04	19	*Akp2*$^{-/-}$	8.2	12.59 (0.75)[b]	9.18 (0.42)[b]
ALP-PT-04	18[a]	WT	Not injected	13.06 (0.59)[c]	9.43 (0.39)[c]

[a] n = 17 for left femur.
[b] Difference with 0 mg/kg is statistically significant. $p = 0.0135$, left tibia. $p = 0.0267$, left femur.
[c] Difference with 0 mg/kg is statistically significant. $p = 0.0006$, left tibia. $p = 0.0009$, left femur.

Individual body weights of *Akp2*$^{-/-}$ and WT mice were measured daily and at necropsy on study day 16. At initiation of treatment (day 1), there was no significant difference in the body weights among vehicle-treated *Akp2*$^{-/-}$ mice, asfotase alfa-treated *Akp2*$^{-/-}$ mice and WT groups. On day 6, the difference in body weights between WT and vehicle-treated *Akp2*$^{-/-}$ groups (-12%) achieved statistical significance ($p = 0.0217$). Asfotase alfa at 8.2 mg/kg had a statistically significant, positive effect on the suppression of weight gain observed in vehicle-treated *Akp2*$^{-/-}$ mice. The difference in body weights between vehicle- and asfotase alfa-treated *Akp2*$^{-/-}$ mice achieved statistical significance on day 11 ($p = 0.0408$), and the difference remained significant at the end of the study on day 16 ($p = 0.0026$). There was no statistically significant difference in body weights between the asfotase alfa-treated *Akp2*$^{-/-}$ and WT groups at any time point. Subcutaneous administration of asfotase alfa (8.2 mg/kg) reversed suppression of the body weight gain in *Akp2*$^{-/-}$ mice following a 15-day prophylactic treatment period. The effect of asfotase alfa on body weight gain is shown in the Applicant's Figure below.



ALXN 1215: Evaluation of prophylactic and therapeutic treatment with asfotase alfa on bone volume at the Achilles tendon enthesis in *Akp2*$^{-/-}$ mice (Study # ALP-PT-22).

A marked to severe mineralization deficit in the calcified fibrocartilaginous zone of the calcaneus-Achilles tendon enthesis was observed in *Akp2*$^{-/-}$ mice, which is similar to the defects observed in patients with hypophosphatasia (HPP). The objective of this study was to evaluate the effect of asfotase alfa treatment on the enthesis mineralization deficit observed in *Akp2*$^{-/-}$ mice using both therapeutic and prophylactic treatment regimens.

Asfotase alfa or vehicle was subcutaneously administered to 15-day-old *Akp2*$^{-/-}$ mice at 8.2 mg/kg/day for 7 days. Animals were divided into three groups. Mice in Groups 1 and 3 were euthanized on day 15 post-partum and mice in group 2 received a daily SC injection of asfotase alfa at 8.2 mg/kg for 7 days before necropsy on day 22 post-partum. The study design is presented in the applicant's Table below.

Table 1: Study design - Therapeutic treatment (Phase 1)

Group No.	Group description	Strain	Sample size (n)[a]	Daily dose (mg/kg), SC	Dose conc. (mg/mL)	Dose volume (mL/kg	Duration of treatment (days) from day 15 post-partum
1	*Akp2*$^{-/-}$	*Akp2*$^{-/-}$	11	NA	NA	NA	NA
2	Asfotase alfa (RTx-8.2)	*Akp2*$^{-/-}$	22	8.2	1.75	4.7	7
3	WT	WT	3	NA	NA	NA	NA

NA, not applicable. Group 1 and Group 3 mice received no treatment and were euthanized on day 15 post-partum.
[a] Sample size at initiation of treatment (day 15 post-partum). Detailed information on sample size is provided in

In another group, one-day-old *Akp2*$^{-/-}$ mice received daily SC injection of vehicle or asfotase alfa at 8.2 mg/kg from day 1 (initiated at birth) and continued for 16 days. The study design is presented in the applicant's Table below.

Table 2: Study design - Prophylactic treatment (Phase 2)

Group No.	Group description	Strain	Sample size (n)[a]	Daily dose (mg/kg), SC	Dose conc. (mg/mL)	Dose volume (mL/kg	Duration of treatment (days)
1	Vehicle (V)	*Akp2*$^{-/-}$	30	0	0	4.7	16
2	Asfotase alfa (Tx-8.2)	*Akp2*$^{-/-}$	29	8.2	1.75	4.7	16
3	WT	WT	3	NA	NA	NA	NA

NA, not applicable. Group 3 mice received no treatment and served as reference controls for the study. Mice were euthanized on day 17 post-partum.

The enthesis mineralization of *Akp2*$^{-/-}$ mice was compared to WT mice. Treatment with 8.2 mg/kg/day asfotase alfa for 7 days initiated in 15-day old *Akp2*$^{-/-}$ mice had no effect on the mineralization deficit at the calcaneus-Achilles tendon enthesis or on the bone mineralization defects of the hindpaw. Data are presented in the applicant's Table below.

Table 8: Bone mineralization defects in the hindpaw from *Akp2*$^{-/-}$ mice after 7 days of therapeutic treatment initiated on day 15 post-partum

Group No.	Sample size (n)	Daily dose (mg/kg), SC	Time point (day post-partum)	Classification of bones of the hindpaw Number (%) of animals			
				Abnormal			Normal
				Severe	Moderate	Slight	
1	11	NA	14 ± 1	2 (18)	2 (18)	2 (18)	5 (46)
2	18	8.2	14 ± 1	7 (39)	1 (6)	6 (33)	4 (22)
			22	6 (33)	1 (6)	1 (6)	10 (55)

NA, not applicable. Animals received no treatment.

Phenotypic alterations were noted at the calcaneal entheses of both male and female *Akp2*$^{-/-}$ mice treated with the vehicle for 16 days. In contrast to the WT mice, all vehicle treated *Akp2*$^{-/-}$ mice showed decreased mineralization of the calcified fibrocartilage of the enthesis as well as in the adjacent secondary ossification centers. The severity of this mineralization defect was highly variable, with 5 out of 6 males and 2 out of 3 females presenting with a score of marked to severe. All A*kp2*$^{-/-}$ mice treated prophylactically for 16 days with 8.2 mg/kg/day asfotase alfa (9 males and 17 females assessed) showed some degree of improved mineralization at or adjacent to the enthesis, suggesting at least a partial prevention of mineralization defects by asfotase alfa. Two male and 4 female mice failed to respond to asfotase alfa, having no mineral at the enthesis. Semi-quantitative microscopic evaluation of the mineralization at the calcaneus-Achilles tendon enthesis from *Akp2*$^{-/-}$ and WT mice are presented in the applicant's Table below.

Table 9: Microscopic findings at the calcaneus-Achilles tendon enthesis in *Akp2*$^{-/-}$ mice after 16 days of prophylactic treatment

	Males			Females		
Genotype	*Akp2*$^{-/-}$	*Akp2*$^{-/-}$	WT	*Akp2*$^{-/-}$	*Akp2*$^{-/-}$	WT
asfotase alfa (mg/kg/day)	0	8.2	None	0	8.2	None
No. Animals Examined	6	10	2	3	17	1
Tissue (No. Examined)	6	9	2	3	17	1
Decreased mineral	(6)[a]	(9)	(0)	(3)	(17)	(0)
Minimal	1	2	–	–	1	–
Slight	–	3	–	1	8	–
Moderate	–	1	–	–	1	–
Marked	1	1	–	–	3	–
Severe	4	2	–	2	4	–

[a] Numbers in parentheses represent the number of animals with the finding.

Significant bone mineralization defects were observed 17 days after birth in vehicle treated *Akp2*$^{-/-}$ mice. All WT littermates were normal. Asfotase alfa (8.2 mg/kg/day) significantly increased bone mineralization after 16 days of prophylactic treatment. The distribution of normal and abnormal individuals between vehicle- and asfotase alfa-treated *Akp2*$^{-/-}$ mice was statistically significant (p = 0.0003).

In conclusion, therapeutic treatment with 8.2 mg/kg/day asfotase alfa for 7 days initiated in 15-day old *Akp2*$^{-/-}$ mice had no effect on the mineralization deficit at the calcaneus-Achilles tendon enthesis or on the bone mineralization defects of the hindpaw. However, prophylactic treatment with 8.2 mg/kg/day asfotase alfa for 16 days to *Akp2*$^{-/-}$ mice immediately after birth (1-day-old) promoted mineralization of bones of the

hindpaw and reversed suppression of bone growth, and partially prevented mineralization deficit at the calcaneus-Achilles tendon enthesis.

ALXN 1215: Evaluation of prophylactic treatment (43 days) with Asfotase alfa on bone volume at the Achilles tendon enthesis in *Akp2*$^{-/-}$ mice (Study # ALP-PT-23.2).

In the above study (ALP-PT-22), prophylactic treatment with asfotase alfa (8.2 mg/kg/day) for 16 days starting on day 1 post-partum promoted normal mineralization of bones of the hindpaw and partially prevented mineralization deficit at the calcaneus-Achilles tendon enthesis. The purpose of this study was to evaluate the effect of asfotase alfa for longer duration (43 day) on the enthesis mineralization deficit in *Akp2*$^{-/-}$ mice.

Asfotase alfa or vehicle was administered daily to Akp2$^{-/-}$ mice by SC injection from Day 1 after birth, and continued for 43 days. The study design is presented in the applicant's Table below.

Table 1: Study design - Prophylactic treatment

Group No.	Group description	Strain	Sample size (n)	Daily dose (mg/kg), SC	Dose conc. (mg/mL)	Dose volume (mL/kg	Duration of treatment (days)
1	Vehicle (V)	*Akp2*$^{-/-}$	7M/13F	0	0	4.0	43
3	Asfotase alfa (K1-4)[a]	*Akp2*$^{-/-}$	7M/13F	4	1.0	4.0	43
6	Asfotase alfa (K4-4)[b]	*Akp2*$^{-/-}$	9M/11F	4	1.0	4.0	43
7	WT	WT	5M/F[c]	NA	NA	NA	NA

NA, not applicable. Group 7 mice received no treatment and served as reference controls for the study. Mice were euthanized on day 44 post-partum; M, male; F, female.

[a] Lot 169466

[b] Lot 259248

[c] Ten out of the 20 WT mice enrolled in study ALP-PT-23 were selected for micro-CT evaluation of the enthesis.

On Day 44, animals were euthanized and mineralization of the calcaneus-Achilles tendon enthesis was determined by micro-CT evaluation and the mineralization defects in bones of the hindpaw was determined by radiography.

All vehicle-treated *Akp2*$^{-/-}$ mice lacked mineralization of the calcaneus-Achilles tendon enthesis and adjacent secondary ossification. The bone volume (BV) measured in vehicle-treated *Akp2*$^{-/-}$ mice was only 3% compared to WT mice. Treatment of *Akp2*$^{-/-}$ mice with 4 mg/kg asfotase alfa (group 3, Lot 169466, Group 3) or 4 mg/kg asfotase alfa (group 4, Lot 259248, Group 6) resulted in bone volume of 28% and 37%, respectively, compared to WT mice. This corresponds to an approximate 9- to 12-fold increase in mineralization compared to vehicle-treated *Akp2*$^{-/-}$ mice. The bone volumes (BV) for different groups are presented in the applicant's Table below.

Table 5: Effect of 4 mg/kg asfotase alfa on bone volume in the Achilles tendon enthesis and adjacent ossification centers in *Akp2*$^{-/-}$ mice after 43 days of prophylactic treatment

Group No.	Strain	Sample size (n)	Daily doses (mg/kg), SC	Bone volume (mm^3) Mean (SD)
1	*Akp2*$^{-/-}$	20	0	0.005445 (0.01718)[a]
3	*Akp2*$^{-/-}$	20	4	0.05059 (0.02622)[a b]
6	*Akp2*$^{-/-}$	20	4	0.06552 (0.02909)[a b]
7	WT	10	Not injected	0.1778 (0.02914) [b]

[a] Difference with WT animals is statistically significant. $p < 0.0001$.
[b] Difference with 0 mg/kg is statistically significant. $p < 0.0001$.

Bone mineralization was determined in hindpaws by X-Ray. A significant bone mineralization defects were observed in vehicle-treated *Akp2*$^{-/-}$ mice. All WT littermates showed normal bone mineralization. Asfotase alfa (4 mg/kg/day) significantly increased bone mineralization after 43 days of prophylactic treatment. A comparative analysis of the distribution of normal and abnormal between vehicle and asfotase-treated *Akp2*$^{-/-}$ mice was statistically significant (p <0.001) for both lots. Data are presented in the applicant's Table below.

Table 6: Bone mineralization defects in the hindpaw from *Akp2*$^{-/-}$ mice after 43 days of prophylactic treatment

Group No.	Sample size (n)	Daily dose (mg/kg), SC	Classification of bones of the hindpaw at necropsy[a] Number (%) of animals			
			Abnormal			Normal
			Severe	Moderate	Slight	
1	20	0	7 (35)	3 (15)	8 (40)	2 (10)
3	20	4	0 (0)	0 (0)	3 (15)	17 (85)[b]
6	20	4	1 (5)	0 (0)	0 (0)	19 (95) [b]

[a] Bone abnormalities were assessed at the end of treatment (for surviving animals) or time of death.
[b] Difference with 0 mg/kg is statistically significant. $p < 0.001$.

In conclusion, micro-CT measurements of bone volume at the calcaneus-Achilles tendon enthesis in vehicle- and asfotase alfa-treated *Akp2*$^{-/-}$ mice showed a partial improvement in mineralization, which was a 9- to 12-fold increase in enthesis mineralization compared to vehicle-treated *Akp2*$^{-/-}$ mice. However, prophylactic treatment with 4 mg/kg/day asfotase alfa for 43 days caused normal mineralization of bones of the hindpaw.

ALXN 1215: Prophylactic treatment with Asfotase Alfa in *Akp2*$^{-/-}$ mice: Duration of pharmacologic activity following the cessation of dosing (Study # ALP-PT-25.2).

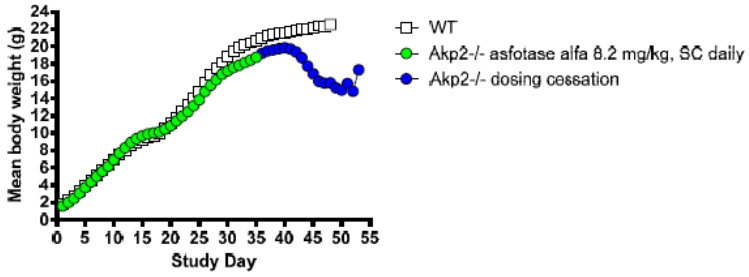
The aim of this study was to evaluate the effect of asfotase alfa on mineralization of hindpow, growth, and survival of *Akp2*$^{-/-}$ knockout mice following 35 days of prophylactic treatment.

Akp2$^{-/-}$ mice received daily SC injections of asfotase alfa at 8.2 mg/kg at birth and continued for 35 days. Treatment was ceased on Day 36, and animals were followed until death. Age-matched untreated WT mice served as reference control. A mineralization defect in bones of the hindpaw was determined by radiography. Individual body weight (growth) was measured daily and survival rate was determined

after daily observation. Bone abnormalities were assessed by X-Ray at time of death after cessation of dosing. All *Akp2*$^{-/-}$ mice were dead by day 53 (18 days after cessation of dosing). All animals treated with 8.2 mg/kg asfotase alfa from birth to day 35, mineralization of the bones of the hindpaw was scored as normal at the time of death.

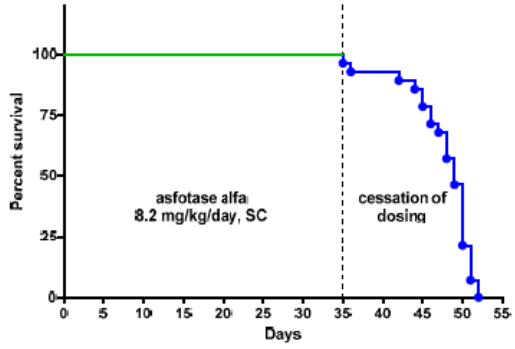
Individual body weights of the mice were measured daily. After 7 days without treatment, average body weight began to decrease and clinical symptoms (such as decreased activity, dehydration, hunched back posture, and seizures) worsened in all *Akp2*$^{-/-}$ mice. The changes in body weight are shown in the applicant's Figure below.

Figure 1: Body Weight Curve of *Akp2*$^{-/-}$ Mice Treated with Asfotase Alfa at Birth for 35 Days Followed by Dosing Cessation on Day 36



Within 18 days after cessation of dosing (on day 36), all *Akp2*$^{-/-}$ mice had died. Median survival time after dosing cessation was 14 days. Data are presented in the applicant's Figure below.

Figure 2: Survival curve of *Akp2*$^{-/-}$ mice treated with asfotase alfa at birth for 35 days followed by dosing cessation on day 36



In conclusion, dosing of *Akp2*$^{-/-}$ mice with 8.2 mg/kg asfotase alfa daily by the SC route from birth for 35 days followed by cessation of dosing had no impact on bone mineralization in hindpow at time of death. However, cessation of treatment on Day 36 reversed the beneficial effects of asfotase alfa on weight gain and survival rate.

ALXN 1215: Evaluation of prophylactic treatment with asfotase alfa on plasma inorganic pyrophosphate in *Akp2*$^{-/-}$ mice (Study # ALP-PT-24).

Inorganic pyrophosphate (PPi) accumulation in hypophosphatasia (HPP) contributes to impaired skeletal mineralization. *Akp2*$^{-/-}$ mice exhibit accumulation of PPi in plasma. The objective of this study was to evaluate the effect of prophylactic treatment with asfotase alfa on plasma PPi concentrations in *Akp2*$^{-/-}$ mice.

Akp2$^{-/-}$ mice received a daily SC injection of the vehicle or asfotase alfa at 8.2 mg/kg for 14 days. PPi concentrations in the plasma were measured at different time points (0, 12, 24, 48 and 96 hours) after the last injection. However, due to the limited number of vehicle-treated *Akp2*$^{-/-}$ mice to serve as controls for baseline PPi values, the effect of asfotase alfa treatment was evaluated only at the 12 hours post-dose time point.

Akp2$^{-/-}$ mice treated with the vehicle, showed a significant increase in plasma PPi concentrations compared to age-matched WT littermates (p = 0.0210). Treatment with asfotase alfa completely prevented the increase in plasma PPi compared to vehicle-treated animals. Data are presented in the applicant's Table below.

Effect of 8.2 mg/kg Asfotase Alfa on PPi Concentrations in Plasma Samples from 15-Day Old *Akp2*^-/- mice After 15 Days of Prophylactic Treatment

	Strain	Sample Size (n)	Daily Doses (mg/kg), SC	PPi Concentration (µM) Mean (SD)
	Akp2$^{-/-}$	15	0	4.34 (1.86)
	Akp2$^{-/-}$	15	8.2	2.31 (0.41)[a]
	WT	14	Not injected	2.93 (1.11)[b]

ALXN 1215: Evaluation of prophylactic treatment with asfotase alfa on plasma pyridoxal-5'phosphate in *Akp2*$^{-/-}$ mice (Study # ALP-PT-25.1).

The pyridoxal-5' phosphate (PLP) is a cofactor for over 140 enzymatic reactions. Tissue uptake of circulating plasma PLP requires prior removal of the 5'-phosphate group by alkaline phosphatase (ALP). The tissue uptake is impaired in patients with hypophosphatasia (HPP), and there is an accumulation of PLP in the blood. The objective of this study was to evaluate the effect of prophylactic treatment with asfotase alfa on plasma PLP concentrations in *Akp2*$^{-/-}$ mice.

The *Akp2*$^{-/-}$ mice received SC injections of the vehicle or asfotase alfa at 8.2 mg/kg for up to 9 days in the absence of pyridoxine supplementation in the diet. WT mice received no treatment and served as a reference group.

Vehicle treated *Akp2*$^{-/-}$ mice showed a significant increase in plasma PLP concentrations compared to age-matched WT littermates (p <0.0001). Treatment with asfotase alfa prevented the increase in plasma PLP in *Akp2*$^{-/-}$ mice compared to the vehicle-treated animals. Data are presented in the applicant's Table below.

Table 4: PLP concentrations in plasma samples from *Akp2*$^{-/-}$ and WT mice after 9 days of prophylactic treatment

Group No.	Strain	Sample size (n)	Daily doses (mg/kg), SC	PLP concentration (ng/mL) Mean (SD)
1	*Akp2*$^{-/-}$	43	0	484 (146)
2	*Akp2*$^{-/-}$	25	8.2	LOQ, ND[a]
3	WT	9	Not injected	82.2 (11.2)[b]

[a] LOQ, ND, 14/25 sample results were below limit of quantification (LOQ) (2.5 ng/mL), 11/25 sample results were ND (not detected).
[b] Difference with 0 mg/kg is statistically significant. $p < 0.0001$.

Evaluation of a 52 day bolus subcutaneous injection regimen of a bone-targeted soluble form of tissue non-specific alkaline phosphatase (ENB-0040) in *Akp2*$^{-/-}$ mice (Study # ALP-PT-03).

The aim of this study was to assess the effect of daily SC administration of ENB-0040 on the survival and the bone mineralization defects in *Akp*$^{-/-}$ mice.

In a 52-day study, ENB-0040 was administered SC at the dose of 8.2 mg/kg/day to *Akp*$^{-/-}$ mice (n=17). Another group of *Akp*$^{-/-}$ mice (n=16) received the vehicle (Sodium phosphate-sodium chloride), and a third group of WT mice (14 *Akp2*$^{+/+}$ and 1 *Akp2*$^{+/-}$ mice) were included as reference animals without any treatments. All animals were sacrificed on Day 53.

The median survival for the mice in the vehicle treated group was 18.5 days; no mice in the vehicle treated group survived beyond 24 days. Seventy five percent of the animals in the ENB-0040 treated group (Tx-1) survived. The survival of the ENB-0040 treated group was statistically significant compared to vehicle treated group. Data are presented in the applicant's Figure below.

8.2 Primary endpoint: Survival

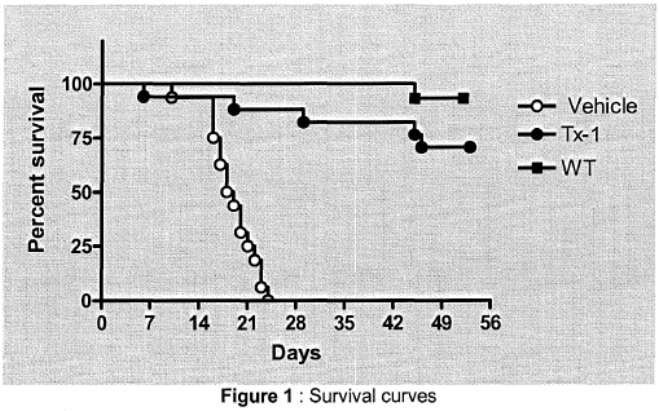


Figure 1 : Survival curves

There was no statistically significant difference in body weights between different groups at Day 1. The difference in body weights between WT (average: 4.2±0.6 g) and vehicle treated mice (average: 3.7±0.6 g) achieved statistical significance (p=0.0331) at day 6. The difference in body weights between WT mice (average: 14.4±2.4 g) and ENB-0040 treated mice (average: 12.2±2.4 g) reached statistical significance at Day 28 (p=0.0274) and was maintained until the end of the study. The differences in the

increase in body weights between ENB-0040 treated mice (average, 8.4±2.0 g) and vehicle treated mice (average, 6.6±0.5 g) reached to statistical significance at Day 21 (p=0.0267). At Day 53, the body weight of ENB-0040 treated mice was significantly (p=0.0019) lower by 25% (average, 16.7±2.0 g) compared to WT group (average, 20.9±2.5 g). Changes are shown in the applicant's Figures below.

8.3.2 Body weights

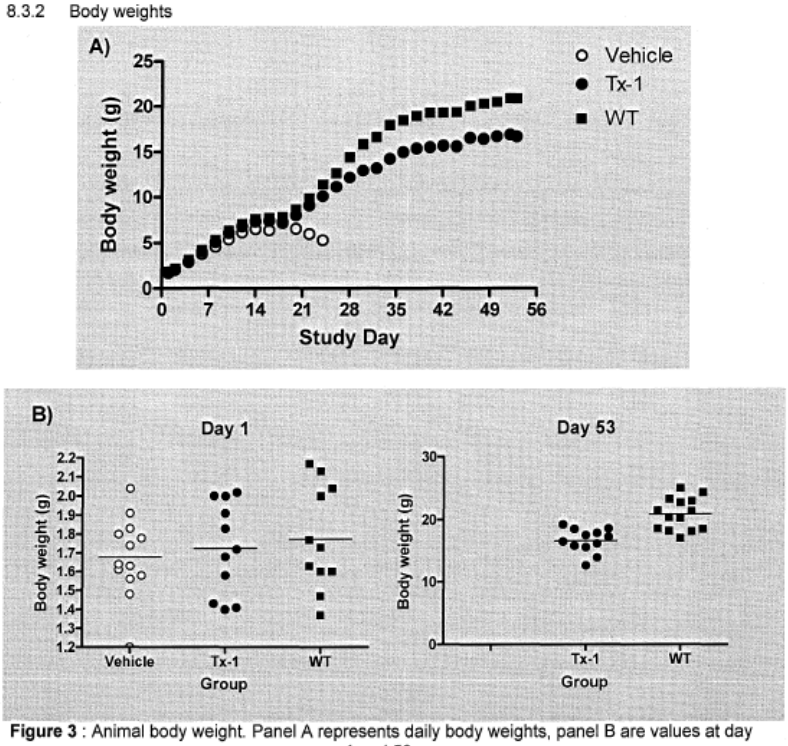


Figure 3 : Animal body weight. Panel A represents daily body weights, panel B are values at day 1 and 53.

At the end of the study, the length of the left tibia in ENB-0040 treated mice (Tx-1) was significantly (p= 0.0126) shorter by 6% (average: 15.7±1.1 mm) compared to WT (average: 16.7±0.9 mm). Similarly, at the end of the treatment, a statistically significant difference (p=0.0456) was also observed when the lengths of the femur were compared between ENB-0040 treated mice (average: 13.3 ± 1.6 mm) and WT (average: 14.0±0.4 mm) mice.

Assessment of bone mineralization was performed by radiograph of the left foot of each animal. No difference in bone mineralization was detected between the ENB-0040 treated (Tx-1) animals and WT animals.

In conclusion, enzymatic replacement therapy with ENB-0040 significantly improved survival and improved the mineralization defects in the mouse model of hypophosphatasia (*Akp*$^{-/-}$ mice). The differences in bone length between ENB-0040 treated and WT mice were statistically significant, they were relatively small compared to the 25% decrease in body weight and were not associated with any radiographic abnormalities.

Evaluation of the Efficacy of Two Dosing Intervals of ENB-0040 in *Akp2*$^{-/-}$ Mice (Study # ALP-PT-05).

The objective of this study was to evaluate the effect of two ENB-0040 dose intervals on survival and bone mineralization of *Akp2*$^{-/-}$ mice. The *Akp2*$^{-/-}$ mice received ENB-0040 (8.2 mg/kg/dose) in every 3 or 7 days. ENB-0040 was administered SC from Day 1 (study day) to 50 to 52 days. WT mice were selected as reference animals without any treatment.

Bone mineralization was determined by radiography. ENB-0040 administered at 8.2 mg/kg every 3 days (Tx-3) normalized the bone mineralization defects in 62% of mice. The percentage of normalization was significantly different (p=0.0029) from the normalization of 16% observed after weekly treatment (Tx-7) with ENB-0040. Radiography images in WT mice were all normal. Data are presented in the applicant's Table below.

Group	Abnormal	Normal
Tx-3 (N=21)	8 (38)	13 (62)
Tx-7 (N=19)	16 (84)	3 (16)

Table 1 : Distribution of radiographic images of the feet. Values between brackets are percentages.

The median survivals of mice were 26 and 24 days in the every 3 and 7 days dosing interval groups, respectively, which was not significantly different. However, both ENB-0040 regimens significantly improved the survival rate when compared with their vehicle-treated mice in study ALP-PT-03 (median: 18.5 days). Data are presented in the applicant's Table below.

				P values		
				Dose (mg/kg/day)		
Group	Median Survival (Day)	Dose & Regimen	Normalized Dose (mg/kg/day)	Vehicle (ALP-PT-03)	1.2	2.7
Vehicle (ALP-PT-03)	18.5	0 mg/kg daily	0			
Tx-7	24	8.2mg/kg weekly	1.2	0.0009 *		
Tx-3	26	8.2mg/kg every 3 days	2.7	< 0.0001 *	0.1908	

Table 2 : Median survival and statistical comparison according to the administered dose.

In conclusion, ENB-0040 prevented bone mineralization defects in mice when administered every 3 days at a dose of 8.2 mg/kg. ENB-0040, administered once weekly was not effective in preventing the bone mineralization defects. However, both ENB-0040 dosing regimens significantly improved survival rates.

Evaluation of the Efficacy of Three Dosing Intervals of ENB-0040 in *Akp2*$^{-/-}$ Mice (Study # ALP-PT-06).

The aim of this study was to assess the effects of three ENB-0040 dosing intervals on survival and bone mineralization of *Akp2*$^{-/-}$ mice.

Akp2$^{-/-}$ mice received SC doses of ENB-0040 daily (4.3 mg/kg) or every 3 (15.2 mg/kg) or 7 days (15.2 mg/kg).

Hindpaw mineralization abnormalities were assessed by X-ray following 24 hours after the last injection. On Day 23, bone mineralization was normal in 67% of mice treated daily with ENB-0040 at 4.3 mg/kg/day. Similarly, mice treated with ENB-0040 at 15.2 mg/kg once every 3 days (Tx-3) and every 7 days (Tx-7) showed normal bone mineralization of 79% and 50%, respectively. At the end of the study (Day 43), the bone mineralization was normal in 83%, 100% and 85% of mice in the Tx-1, Tx-3 and Tx-7 dose groups, respectively. Radiograph images of WT mice were all normal on Day 23 and at the end of study. Data are presented in the applicant's Table below. Numbers in parenthesis represent percentage.

A)

Mid-Study (D23)		
Group	**Abnormal**	**Normal**
Tx-1 (N=18)	6 (33)	12 (67)
Tx-3 (N=19)	4 (21)	15 (79)
Tx-7 (N=20)	10 (50)	10 (50)

B)

D23-45		
Group	**Abnormal**	**Normal**
Tx-1 (N=18)	3 (17)	15 (83)
Tx-3 (N=19)	0 (0)	19 (100)
Tx-7 (N=20)	3 (15)	17(85)

Table 1: Distribution of radiographs of the feet.

The survival rates of three groups (Tx-1, Tx-3, Tx-7) of mice treated with ENB-0040 were statistically significant ($p<0.0001$) when compared to the vehicle treated *Akp2*$^{-/-}$ mice in study ALP-PT-03. However, there was no difference in the survival rates between Tx-1, Tx-3 and Tx-7 groups. Survival for different groups is presented in the applicant's Figure below.

8.3.1 Survival

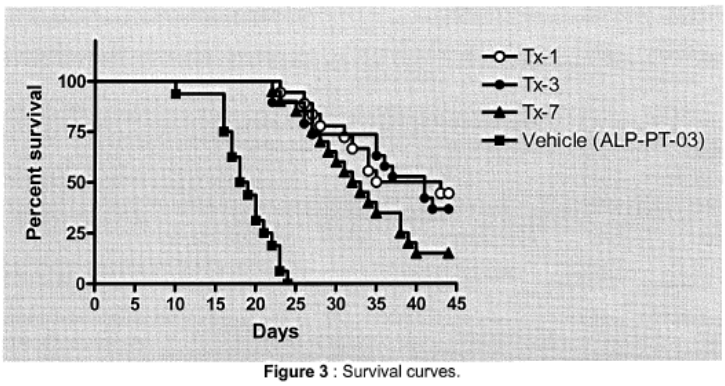


Figure 3 : Survival curves.

In conclusion, longer dosing intervals with ENB-0040 could prevent the mineralization defects and improve survival rates in the *Akp2*$^{-/-}$ mouse model of hypophosphatasia.

Evaluation of Daily Bolus Subcutaneous Injection of ENB-0040 on Prevention and Rescue of the HPP Bone Phenotype in *Akp2*$^{-/-}$ Mice (Study # ALP-PT-08).

The objective of this study was to evaluate the efficacy of ENB-0040 in restoring bone mineralization in *Akp2*$^{-/-}$ mice once hypomineralization of bone was established. Hypomineralization was established in *Akp2*$^{-/-}$ mice 15 days after birth without any treatment.

Animals were divided into 4 groups. The mice in group 1 (Tx-1) were subcutaneously administered ENB-0040 at 8.2 mg/kg/day from 1 day to 43 days of age. Animals in group 2 (RVehicle) received vehicle from day 15 to at least 43 days of age. *Akp2*$^{-/-}$ mice in group 3 (RTx-1) received SC injections of ENB-0040 at a dose of 8.2 mg/kg/day from day 15 to at least 43 days of age. WT animals in group 4 served as reference animals and did not receive any treatment. The experimental design is presented below (Sponsor's submission).

1.4 Experimental design

Group 1 (Tx-1): 21 *Akp2*$^{-/-}$ mice received daily ENB-0040 injections (8.2 mg/kg) from day 1
Group 2 (RVehicle): 16 *Akp2*$^{-/-}$ mice received daily vehicle injections from day 15
Group 3 (RTx-1): 17 *Akp2*$^{-/-}$ mice received daily ENB-0040 injections (8.2 mg/kg) from day 15
Groupe 4 (WT): 26 *Akp2*$^{+/+}$ and 4 *Akp2*$^{+/-}$ mice served as reference, they did not receive injections.

On examination of the radiographs of the feet, 41% of the animals in RTx-1 group showed normal feet mineralization compared to 12% in RVehicle group, which was statistically significant. Animals injected ENB-0040 daily from day 1 at a dose of 8.2 mg/kg (Tx-1) showed 100% normal feet mineralization. Data are presented in the applicant's Table below.

Group	Abnormal	Normal
Tx-1 (N=21)	0 (0)	21 (100)
RVehicle (N=16)	14 (88)	2 (12)
RTx-1 (N=17)	10 (59)	7 (41)

Table 1: Distribution of radiographs of the feet.

Compared to animals in the RVehicle group, animals in Tx-1 (<0.0001) and RTx-1 (p=0.0002) groups showed improved survival rates. Median survival was 39 and 20 days in RTx-1 and vehicle groups, respectively. The survival data are presented in the applicant's Figure below.

8.3.1 Survival

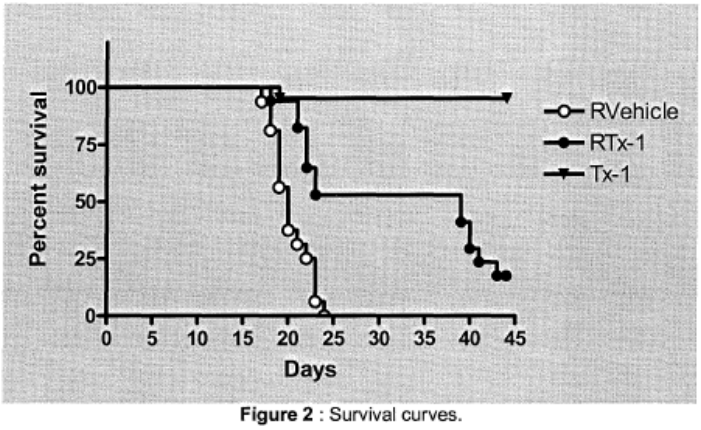
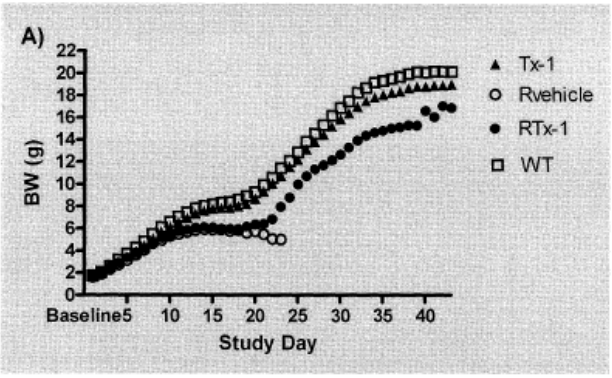


Figure 2 : Survival curves.

At the start of the study (day 1), no significant difference in body weights was observed between WT, RTx-1 and Tx-1groups. However, the difference in body weights between RVehicle and WT mice were statistically significant from Day 1 to rest of the study period. At the start of treatment (day 15), body weight of animals in RTx-1 (average: 6.1±1.4 g) and RVehicle (5.9±1.1 g) were similar; whereas, a higher body weight (average: 8.2±1.3 g) was noted in the WT group. While the average body weight of RVehicle group continued to decrease until death, RTx-1 mice started to gain weight 4-5 days following initiation of treatment and kept gaining weight until the end of the study. Body weights of RTx-1animals, with a few exceptions, were constantly significantly different from the body weights of WT mice during treatment. Average body weights of Tx-1 mice were always lower compared to average body weights of WT mice. Data are presented in the applicant's Figure below.

8.3.2 Body weight



In conclusion, treatment of *Akp2*$^{-/-}$ mice daily from day 15 (after establishing hypomineralization) with ENB-0040 improved feet mineralization and increased survival rate and body weight gain. This study shows that ENB-0040 can rescue the hypomineralization observed at day 15 in this animal model of hypophosphatasia.

Evaluation of the Efficacy of Three Dosing Intervals of ENB-0040 on Rescue of the HPP Bone Phenotype in *Akp2*$^{-/-}$ Mice (Study # ALP-PT-09).

Previous studies (ALP-PT-03 and ALP-PT-04) showed that daily SC administration of ENB-0040 significantly improved survival and prevented the mineralization defect observed in Akp2$^{-/-}$ mice. The treatment was initiated on Day 1 after birth. Similarly, in another study (ALP-PT-08) in Akp2$^{-/-}$ mice, the treatment with ENB-0040 was initiated on Day 15 after birth (after establishing hypomineralization). In this study, survival rate and bone mineralization was improved significantly.

The objective of this study was to evaluate the efficacy of three dosing intervals of ENB-0040 in rescuing bone mineralization defects in *Akp2*$^{-/-}$ mice. The treatment was initiated at day 12 after birth to maximize chances of survival.

Akp2$^{-/-}$ mice received the vehicle or the test article subcutaneously daily from day 12 after birth for 7 days. Then, group 1 (RVehicle) received the vehicle every 7 days until day 47; group 2 (RTx-7) received the test article every 7 days until day 47; group 3 (RTx-3) received the test article every 3 days until day 46; group 4 (RTx-1) received the test article daily until day 47, and group 5 (WT) served as reference animals and did not receive any treatment. The study design is described in the applicant's Table below:

Group Number	Group description	Treatment	Duration of treatment (day)	Dosing Interval	Dose Level (mg/kg)	Conc. (mg/ml)	Dose volume (ml/kg)
1	RVehicle	SC injection	36	Daily for 7 days fd by every 7 days	0	0	3.3
2	RTx-7	SC injection	36	Daily for 7 days fd by every 7 days	8.2 for 7 days fd by 57.4 every 7 days	2.50 for 7 days fd by 17.5 every 7 days	3.3
3	RTx-3	SC injection	35	Daily for 7 days fd by every 3 days	8.2 for 7 days fd by 24.6 every 3 days	2.50 for 7 days fd by 7.5 every 3 days	3.3
4	RTx-1	SC injection	36	Daily	8.2	2.50	3.3
5	WT*	---	---	---	---	---	---

WT (wild-type): normal littermates of *Akp2*$^{-/-}$ mice
Fd: followed
* WT associated with group no. 1, 2 and 3 were only used to evaluate the overall health condition of the litter; they were not sacrificed and radiographed at the end of the study. They were released in the colony.

Bone mineralization was determined by radiographs of the left foot of each animal upon completion of treatment. The result showed that 35% of animals in Group 1 (RVehicle) had normal mineralization, which was higher than previous findings (Studies # ALP-PT-04 and ALP-PT-08). Chi Square test showed that the bone mineralization compared among RVehicle, RTx-1, RTx-3 and RTx-7 groups were not significant (p=0.3248). The normalization of mineralization defects appears to improve with more frequent dosing. Radiograph images in WT mice were normal at the end of study. The distribution between normal and abnormal bone mineralization is shown in the applicant's Table below.

Group	Abnormal (%)	Normal (%)	RVehicle	RTx-7	RTx-3
RVehicle (N=17)	11 (65)	6 (35)			
RTx-7 (N=18)	11 (61)	7 (39)	p= 0.8259		
RTx-3 (N=20)	10 (50)	10 (50)	p= 0.3682	p= 0.4916	
RTx-1 (N=19)	7 (37)	12 (63)	p= 0.0951	p=0.1399	p=0.4075

The survival of animals in all the treatment arms, (RTx-7, p=0.0054; RTx-3, p<0.0001; RTx-1 p<0.0001) were significantly different from the RVehicle group. The survival of RTx-3 (p=0.0549) and RTx-1 (p=0.0010) animals were significantly different compared to the survival of RTx-7 (p=0.0010) animals. There was no significant difference in survival between RTx-3 and RTx-1 (p=0.2514) animals. The survival curve and median

survival for the treated groups are presented in the applicant's Figure and Table below, respectively.

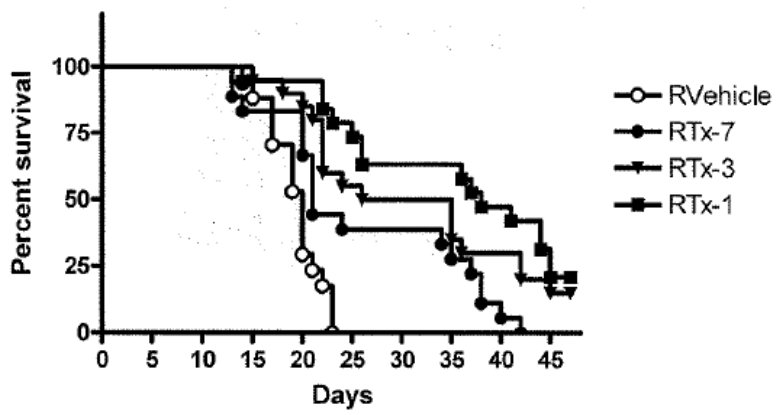


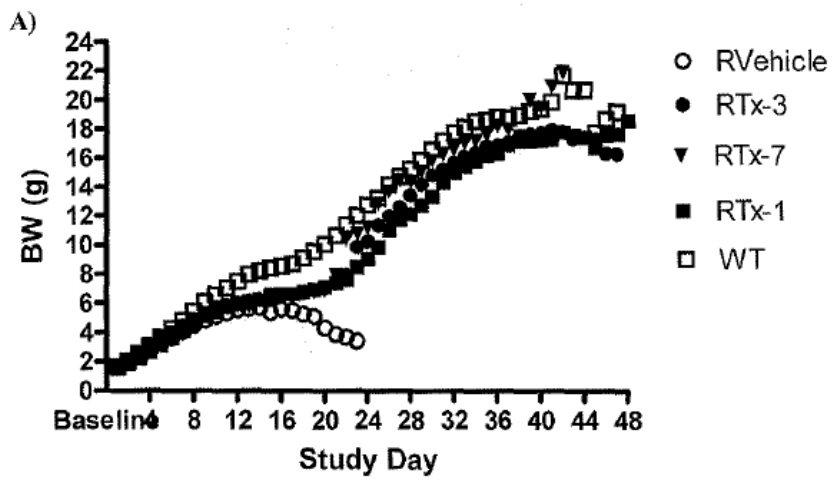
Figure 2 : Survival curves.

		P value			
		Group			
Group	Median Survival (Day)	Rvehicle	RTx-7	RTx-3	RTx-1
Rvehicle	20				
RTx-7	21	0.0054*			
RTx-3	30.5	< 0.0001 *	0.0549		
RTx-1	38	< 0.0001 *	0.0010*	0.2514	

* Significant

Table 2 : Median survival and results of comparisons between survival curves.

At the initiation of treatment (day 12), there was no difference in body weights between RVehicle (average: 5.6±1.6 g) and RTx-1 (average: 6.0±1.2 g), RTx-3 (average: 6.1±1.3 g) and RTx-7 (average: 5.8±1.8 g) groups; the body weights were however significantly different compared to the WT (average: 7.5±1.2 g) animals. The mean body weights of RTx-1 and RVehicle groups were constantly and significantly lower than the body weight of the WT group. The mean body weight of RTx-7 and RTx-3 were significantly lower than the WT group until day 21. Body weights for different groups are presented in the applicant's Figure below.



In conclusion, unusually high prevalence of normal mineralization in the vehicle group was seen in this study. More frequent dosing with ENB-0040 appeared to be more effective in correcting established mineral defects. Treatment with ENB-0040 significantly improved survival rates in all treated groups.

Evaluation in *Akp2*$^{-/-}$ Mice of the Relationship between Dose and Response after 43 Days of Bolus Subcutaneous Injections of ENB-0040 (Study # ALP-PT-11).

The objective of this study was to find the relationship between different doses of ENB-0040 and the therapeutic response.

Akp2$^{-/-}$ mice received SC injection of ENB-0040 or vehicle daily from day 1 to 43 post-partum. The study design is presented in the applicant's Table below.

Group No.	Group description	Dose Level (mg/kg)	*Akp2*$^{-/-}$ N=		WT N=	
			Male	Female	Male	Female
1	Vehicle	0	9	12	0	0
2	Tx-0.5	0.5	8	10	0	0
3	Tx-2.0	2.0	8	12	0	0
4	Tx-8.2	8.2	11	8	0	0
5	WT	No injection	0	0	15	18

WT: wild-type littermates of *Akp2*$^{-/-}$ mice

Bone mineralization was determined for the left foot, rib cage and right pelvic limb by radiography at the end of treatment.

At necropsy, only 10% to 15% of mice treated with the vehicle had normal mineralization of left foot, rib cage or pelvic limb compared to 100% for the WT mice.

The percentage of animals with normal mineralization of the left foot was improved from 10% in vehicle control to 39%, 85% and 100% with 0.5, 2 and 8.2 mg/kg/day of ENB-0040, respectively. The mineralization efficacy of 2.0 and 8.2 mg/kg doses were statistically significant when compared to vehicle ($p<0.0001$). X-Ray analysis of the rib cage specimens showed that the percentage of animals with normal mineralization was slightly increased from 10% in the vehicle group to 30% with 2.0 mg/kg and 24% with 8.2 mg/kg doses of ENB-0040. The increases were not dose proportional and not statistically significant when compared to vehicle. The percentage of animals with normal mineralization of the pelvic limb was improved from 15% in the vehicle group to 55% at 2.0 mg/kg and 53% at 8.2 mg/kg dose of ENB-0040. The mineralization efficacy at 2.0 and 8.2 mg/kg doses were statistically significant when compared to vehicle ($p=0.0187$). At the 0.5 mg/kg dose, the percentage of animals with normal mineralization (11%) was similar to the vehicle-treated animals (15%). Data are presented in the applicant's Table below.

Group	Left Foot		Rib Cage		Right Pelvic Limb	
	Abnormal (%)	Normal (%)	Abnormal (%)	Normal (%)	Abnormal (%)	Normal (%)
Vehicle (N=20)	18 (90)	2 (10)	18 (90)	2 (10)	17 (85)	3 (15)
Tx-0.5 (N=18)	11 (61)	7 (39)	17 (94)	1 (6)	16 (89)	2 (11)
Tx-2.0 (N=20)	3 (15)	17 (85)	14 (70)	6 (30)	9 (45)	11 (55)
Tx-8.2 (N=19)*	0 (0)	19 (100)	13 (76)	4 (24)	9 (47)	10 (53)

* N=17 for rib cage specimens

Table 2: Distribution of radiographs of the feet, rib cages and pelvic limbs.

The survival of mice with each treatment was significantly higher when compared to the vehicle treated group ($p<0.0001$). The differences were also statistically significant when the survival curves of the treated groups were compared between themselves ($p<0.0001$). Median survival was 19, 24 and 30.5 days in the vehicle, Tx-0.5 and Tx-2.0 groups, respectively. Only one of 19 mice in Tx-8.2 died before the end of the study; thus, the median survival could not be calculated for this group. Data are shown in the applicant's Figure below.

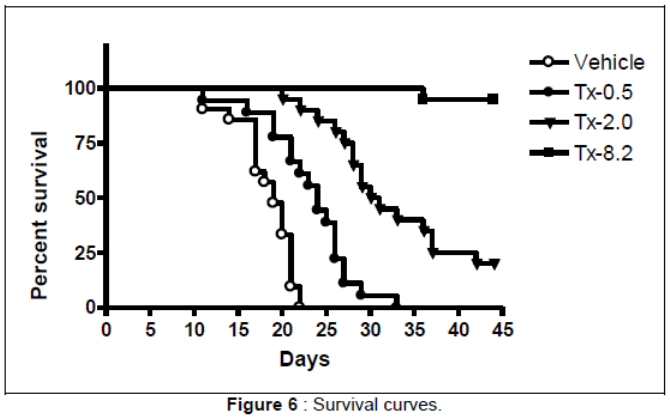


Figure 6 : Survival curves.

In conclusion, ENB-0040 was effective in preventing bone mineralization defects of the feet, rib cages and pelvic limbs in *Akp2*$^{-/-}$ mice. In addition, treatments with ENB-0040 improved survival in all treated groups.

4.2 Secondary Pharmacology

No secondary pharmacology studies were submitted.

4.3 Safety Pharmacology

Central Nervous System:

ENB-0040: A functional Observational Battery (FOB) Neurological in Sprague-Dawley Rats Following a Single Intravenous Injection (Study # 1007-2491):

Methods: The aim of this study was to evaluate the pharmacological effects of asfotase alfa (ENB-0040) on the general behavior of male SD rats, following single IV injection. ENB-0040 and vehicle were administered once intravenously at doses of 0, 3, 30 and 88 mg/kg body weight.

Animals were monitored before dosing, immediately after dosing, 30 minutes after dosing, and, 6 and 24 hours after dosing for functional observation battery tests (motor activity, rearing activity, grip strength, landing foot splay etc.) in an open-field test; neurological abnormalities (cornea reflex, involuntary movement); sensory motor and neuromuscular effects; autonomic effects (lacrimation, salivation, pupil responsiveness, excessive defecation or urination, piloerection); and body temperature and respiration.

Results: Animals in the mid and high dose groups showed reduced motor activity and decreased sensorimotor responsiveness (touch stimuli) characterized by a decreased rearing activity in the open field immediately after the injection and 30 minutes following the treatment with ENB-0040. These effects were not observed at 6 and 24 hours post dosing and were not observed in animals in the 3 mg/kg dose group. No neurological abnormalities (cornea reflex, involuntary movements) were observed in rats following the treatment of ENB-0040 at all doses. Immediately after the injection and 30 minutes following the treatment with ENB-0040 at 30 and 88 mg/kg doses, significant neuromuscular effects such as impaired gait and reduced mobility, reduced extensor thrust reflex, altered landing foot splay and lower grip strength of both fore limbs and hind limbs were noted. These effects were not observed at 6 and 24 hours post dosing. These effects were also not observed in animals in the 3 mg/kg dose group.

Thirty minutes following the treatment with ENB-0040, a statistically significant decrease in body temperature was recorded in the 30 mg/kg (36.7^0C) and 88 mg/kg (36.5^0C) dose groups when compared to the control animals (37.9^0C) and to the pre-dosing observations (37.4^0C to 37.7^0C); and irregular/labored respiration was also observed in some of these animals. Body temperature and respiration were comparable to the pre-dosing observations at 6 and 24 hours post dosing. These changes in body temperature

and respiration were not observed in animals in the 3 mg/kg dose group. No autonomic effects (lacrimation, salivation, pupil responsiveness, excessive defecation or urination, piloerection) were observed following the administration of ENB-0040 at any dose levels.

Respiratory System:

ENB-0040: A Respiratory Safety Pharmacology Study following a Single Intravenous Injection in Conscious Sprague-Dawley Rats (Study # 1007-2501).

Methods: The aim of this study was to assess the potential pharmacological effects of ENB-0040 on the respiratory function of conscious Sprague-Dawley rats following a single IV injection. The test and control articles were administered once intravenously at dose levels of 0, 3, 30 and 90 mg/kg body weight.

The respiratory parameters (tidal volume, respiratory rate and minute volume) were monitored during the first 2 hours after dosing and again at 6 and 24 hours after dosing.

Results: Animals in all dose groups (3, 30 and 90 mg/kg) had a decrease in respiratory rate (dradypneia) in the post-dosing period (up to 2 hours post-dose). The dradypneia was also sporadically observed during the subsequent 6.5-7.5 h post-dosing period. Effects on tidal and/or minute volumes were observed at the 24.5 hours post-dosing in some animals from all ENB-0040 treated groups. The severity of the respiratory effects was generally higher in the high dose group compared to low and mid dose groups.

Cardiovascular System:

The Sponsor did not conduct separate cardiovascular safety pharmacology study with ENB-0040 (asfotase alfa). However, cardiovascular safety pharmacology endpoints (electrocardiogram [ECG], limb leads I, II, III, aVR, aVL, and aVF) were assessed in the repeat-dose (26-week SC) toxicology study in juvenile Cynomolgus monkeys. Asfotase alfa was administered SC to juvenile Cynomolgus monkeys at doses of 0, 0.43, 2.14, and 10 mg/kg/day for a period of 26 weeks followed by 4 weeks recovery period. The ECG was monitored pretest and during Weeks 1 and 26 at 15 minutes, 4, 12 and 24 hours post dose.

ENB-0040 had no effects on ECG parameters in juvenile Cynomolgus monkeys when administered by SC injection at dose levels up to 10 mg/kg.

5 Pharmacokinetics/ADME/Toxicokinetics

5.1 PK/ADME

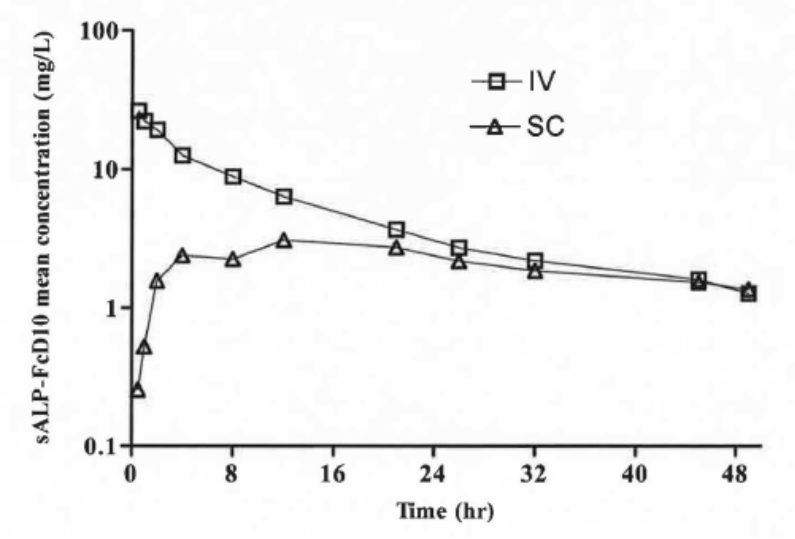
ABSORPTION:

***In vivo* pharmacokinetic study of sALP-FcD10 lot 070629 (non GLP Tox material) administered IV or SC in C57BL/6 mice (Study # ALP-PC-09).**

Methods: A single IV or SC dose of 2 mg/kg sALP-FcD10 (3.08 mL/kg, asfotase alfa) was administered to 36- to 40-week-old C57BL/6 mice (n=12). Blood samples were collected for measurement of plasma sALP-FcD10 concentrations before and 0.5, 1, 2, 4, 6, 8, 12, 21, 26, 32, 45 and 49 h after dosing.

Results: Following IV or SC administration of asfotase alfa, the serum concentration was quantifiable for up to 49 hours post dose. Following intravenous administration, the C_{max} value was 26.39 mg/L. Asfotase alfa was eliminated relatively slowly as suggested by a half-life of 15.6 hours following IV administration.

Following subcutaneous administration, the time to maximum serum concentration (t_{max}) was 12 hours, suggesting relatively slow absorption. The elimination was slow as suggested by half-life value of 31.1 hours following SC administration. After reaching maximum serum concentration (C_{max}), the concentration-time profile declined in parallel to the IV concentration-time curve. The $AUC_{0\text{-}49h}$ was 100 mg.h/L and 258 mg.h/L for the SC and IV doses, respectively. The bioavailability following subcutaneous dosing was calculated to be 57%. Mean concentration to time profile and PK parameters of asfotase alfa in mice after single IV and SC administration are presented in the applicant's Figure and Table below.



11.1 PK Parameters of IV or SC injected sALP-FcD10 in mice

Parameters	Units	IV	SC
Rsqr	NA	0.958	0.975
Tmax	hr	NA	12.000
Cmax	mg/L	26.393	3.071
Cmax/D	mg/L/mg/kg	13.197	1.536
AUC last	mg/L*hr	257.895	100.050
AUC last/D	mg/L*hr/mg/kg	128.947	50.025
AUC inf	mg/L*hr	286.467	160.858
AUC inf/D	mg/L*hr/mg/kg	142.233	80.429
CL	L/hr/kg	0.007	0.012
Half-life	hr	15.606	31.083
Bioavailability	%	NA	56 (39)*

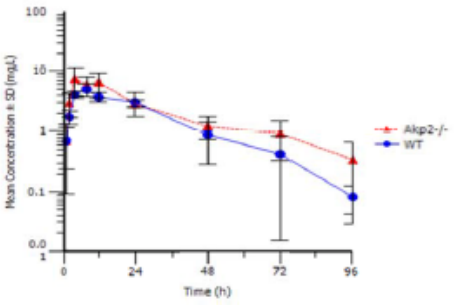
Table 1 : PK parameters of IV and SC sALP-FcD_{10}
*value in bracket represents bioavailability calculated based on AUClast

***In Vivo* Pharmacokinetic Study of Asfotase alfa (cGMP3 Lot PUR012G02) Administered SC to *AKP2*$^{-/-}$ and WT Juvenile Mice (Study # ALP-PC-25).**

Methods: A single SC dose of 8.2 mg/kg asfotase alfa (3.43 mL/kg) was administered to 12-day old *Akp2*$^{-/-}$ and WT mice (n=4/time point/group). Blood samples were collected for pharmacokinetic assessment before and 1, 2, 4, 8, 12, 24, 48, 72 and 96 h after dosing.

Results: Following a single SC dose of 8.2 mg/kg, the serum asfotase alfa concentration was detectable for up to 96 hours post dose in both *Akp2*$^{-/-}$ and WT mice. After reaching t_{max} (4 and 8 hours for *Akp2*$^{-/-}$ and WT mice, respectively), a mono-exponential decline in serum concentration was observed. The systemic exposure was higher in *Akp2*$^{-/-}$ mice compared to WT mice. $AUC_{0\text{-}96h}$ was 213 and 154 mg.h/L for *Akp2*$^{-/-}$ and WT animals, respectively, and C_{max} was 7.51 and 5.03 mg/L, respectively. The clearance (CL/F) in *Akp2*$^{-/-}$ and WT mice was 0.036 and 0.053 L/h/kg, respectively, with a volume of distribution (Vz/F) of 1.34 and 1.07 L/kg, respectively. The PK data are presented in the applicant's Figure and Table below.

Figure 1: Concentration-Time Profiles of asfotase alfa Lot PUR012G02 in *Akp2*$^{-/-}$ and WT Mice after a Single SC Administration at 8.2 mg/kg



n=4/time point/group

Table 17: Pharmacokinetic Parameters of Asfotase Alfa in *Alp2*$^{-/-}$ and WT Mice after a Single SC Administration at 8.2 mg/kg (Report ALP-PC-25, Table 3, page 12)

Strain	CL/F (L/h/kg)	Vz/F (L/kg)	$t_{½}$ (h)	C_{max}[a] (mg/L)	C_{max} /Dose[b]	AUC_{∞} (mg*h/L)	$AUC_{0\text{-}96h}$ (mg*h/L)	$AUC_{0\text{-}96h}$ /Dose[b]	t_{max}[a] (h)
Akp2$^{-/-}$	0.0361	1.34	14.0	7.51	0.916	227	213	25.9	4
WT	0.0527	1.07	25.5	5.03	0.613	155	154	18.8	8

[a] C_{max} and t_{max} were observed values taken directly from the serum concentration-time profiles.
[b] The units of C_{max}/Dose and $AUC_{0\text{-}96h}$/dose are (mg/L)/(mg/kg) and (mg*h/L)/(mg/kg), respectively.

Evaluation of Circulating Concentrations of Asfotase alfa in Fetuses Following 5 Daily SC injection of Asfotase alfa to CD-1 Pregnant Mice (study # ALP-PT-15).

Methods: The objective of this study was to assess if asfotase alfa can cross the blood-placenta barrier (BPB) in mice. Serum concentrations of asfotase alfa were measured in fetuses following 5 daily SC injections of asfotase alfa to CD-1 pregnant mice from gestation days 13-17. Eight pregnant (CD-1) and 7 non-pregnant (C57BL/6) mice were daily injected with asfotase alfa at dose levels of 0, 0.5, 2 or 8.2 mg/kg/day for 5 consecutive days. Blood samples were collected from nonpregnant and pregnant mice and fetuses at a single time point of 21 hours after the 5th dose.

Results: After repeated SC doses to pregnant CD-1 mice, asfotase alfa was quantifiable in both dams and fetuses at all dose levels tested. Dose-normalized concentrations (0.160-0.208 mg/L for dams and 0.116-0.155 mg/L for fetuses) were similar across the doses evaluated suggesting a linear kinetics. Fetuses-to-dam concentration ratios (calculated with the mean values of each group) ranged from 0.727-0.856 for the dose range examined. Immunoglobulin G (IgG) is known to cross the blood-placenta-barrier by FcRn-mediated placental endocytosis. As a fusion protein, asfotase alfa contains Fc portion of human IgG1. Asfotase alfa concentrations were higher in nonpregnant mice compared to pregnant mice with a nonpregnant-to-pregnant mouse ratio ranging from 6.14-2.53 across the doses evaluated. Because the observations were based on a single time point (21 hours after the 5th dose), data are insufficient to determine whether the concentration differences were pregnancy related or due to a difference in strain. The findings are presented in the applicant's Figure and Table below.

Figure 1: Corrected serum concentrations of asfotase alfa following repeated SC administration at various doses in nonpregnant, pregnant and fetal mice

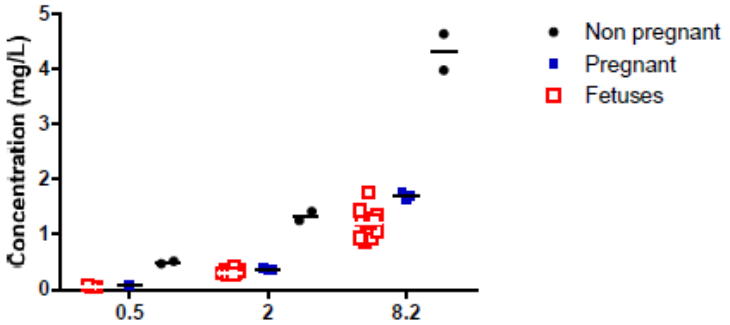


Table 3: Corrected serum concentrations and ratios of asfotase alfa following repeated SC administration at various doses in nonpregnant, pregnant and fetal mice

Daily dose (mg/kg), SC	Nonpregnant[a]		Pregnant[b]		Fetal[c]		Ratio nonpregnant / pregnant[d]	Ratio fetal/ pregnant[d]
	average (individual)	DN[e]	mean (±SD)	DN[e]	mean (±SD)	DN[e]		
0.5	0.491 (0.467, 0.515)	0.982	0.080	0.160	0.058 (0.017)	0.116	6.138	0.727
2.0	1.335 (1.416, 1.253)	0.667	0.362 (0.017)	0.181	0.310 (0.045)	0.155	3.683	0.856
8.2	4.303 (3.974, 4.631)	0.525	1.704 (0.059)	0.208	1.206 (0.259)	0.147	2.525	0.708

[a] n=2
[b] n=1, 3 and 3 at 0.5, 2.0 and 8.2 mg/kg, respectively
[c] n=3, 10 and 11 at 0.5, 2.0 and 8.2 mg/kg, respectively
[d] calculated with the corresponding individual, average (n=2) or mean (n=3-11) concentration value
[e] DN, dose normalized concentration; calculated by mean concentration divided by dose with a unit of (mg/L)/(mg/kg)

Conclusion: Asfotase alfa crossed the BPB in mice at all doses tested (0.5, 2 or 8.2 mg/kg/day).

***In vivo* pharmacokinetic study of ENB-0040 administered IV or SC in rats (Study # PK2008010951).**

Methods: A single IV or SC dose of 3.118 mg/kg ENB-0040 (asfotase alfa) was administered to 6 to 8 weeks old male (n=3) and female (n=3) SD rats. Rats were bled at pre-dose, and at 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 h post-dose for pharmacokinetic assessment.

Results: Following IV administration, ENB-0040 was eliminated relatively slowly with half-life values of 27.3 and 33.8 h in males and females, respectively. The volumes of distribution (V_{ss}) were calculated to be 0.766 and 1.39 L/kg in males and females, respectively. Clearance values were 0.0312 and 0.0492 L/h/kg in males and females, respectively. Total exposure to ENB-0040 (AUC_∞) was 106 mg·h/L in males and 62.1 mg·h/L in females.

Following subcutaneous administration of ENB-0040, the t_{max} was 32 hours for males and 20 hours for females with a median value of 24 hours, suggesting that asfotase alfa was absorbed slowly from the SC injection site. After reaching C_{max}, the SC serum concentration profiles declined in parallel to the IV concentration profiles. The $AUC_{0\text{-}72h}$ was ~1.4 times higher in males compared to females, while there were no sex differences in C_{max} values. Based on the $AUC_{0\text{-}72h}$ from the IV and SC groups, the estimated SC bioavailability was 26.3% and 32.2% for male and female rats, respectively. AUC_∞ values after SC administration were considered to be inaccurate as the extrapolated area exceeds 25%. The findings are presented in the applicant's Figure and Table below.

Figure 6: **Mean (n=3 ±SD) Concentration-time Profiles of Asfotase Alfa in Adult Rats after IV or SC Administration at 3 mg/kg (Report PK2008010951, Figure 1, page 13)**

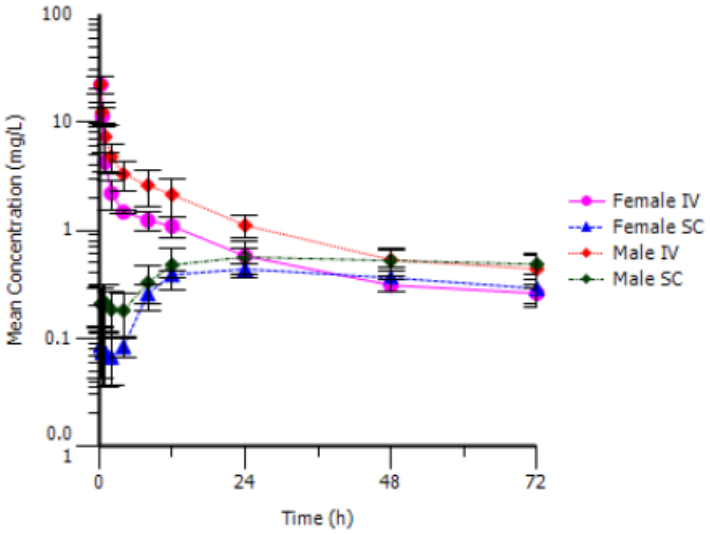


Table 20: **Pharmacokinetic Parameters of Asfotase Alfa in Adult Rats after a Single IV or SC Administration (Report PK2008010951, Section 8.2, page 14)**

Sex	RoA[a]	CL or CL/F (L/h/kg)	V_{ss} or V_{ss}/F (L/kg)	$t_{½}$ (h)	C_{max}[b] (mg/L)	C_{max}/Dose[c]	AUC_{∞} (mg*h/L)	$AUC_{0\text{-}72h}$ (mg*h/L)	$AUC_{0\text{-}72h}$/Dose[c]	t_{max}[b] (h)
M	IV	0.0312	0.766	27.3	22.2	7.40	106	93.1	31.0	0.25
	SC	DNS	DNS	DNS	0.424	0.141	DNS	24.5	8.17	32
F	IV	0.0492	1.39	33.8	22.1	7.36	62.1	53.7	17.9	0.25
	SC	DNS	DNS	DNS	0.342	0.114	DNS	17.3	5.77	20

[a] RoA = route of administration.
[b] C_{max} and t_{max} were observed values taken directly from the serum concentration-time profiles.
[c] The units of C_{max}/Dose and AUC/Dose are (mg/L)/(mg/kg) and (mg*h/L)/(mg/kg), respectively, and dose of 3.0 was used to calculate dose normalized AUC and C_{max}.
DNS = data not shown; because extrapolated area for AUC_{∞} exceeds 25%.

Conclusion: ENB-0040 appears to be absorbed slowly following SC administration. After reaching C_{max}, the SC serum concentrations of asfotase alfa declined in parallel to the IV concentrations.

ENB-0040: A Single Dose Intravenous or Subcutaneous Pharmacokinetic Study in Female Cynomolgus Monkeys (Study # 3008-0133).

Methods: ENB-0040 was administered as a single IV or SC injection (3 mg/kg) to 3-year-old female Cynomolgus Monkeys (n=2). Following administration of ENB-0040, blood samples were collected via the cephalic or femoral vein at pre-dose and at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 h post-dose for PK analysis.

Results: After IV and SC administration, serum concentrations of asfotase alfa were detectable for up to 72 hours (the last sampling time point) post dose. Following IV

administration, the average CL was 0.0193 L/h/kg and the apparent terminal $t_{1/2}$ was 31.4 hours. The average V_{ss} was 0.432 L/kg and the average AUC_{∞} and $AUC_{0\text{-}72h}$ were 158 and 141 mg.h/L, respectively, with an average C_{max} of 68.8 mg/L.

Following subcutaneous administration, ENB-0040 was absorbed relatively slowly as suggested by the average T_{max} of 10.0 h. After reaching C_{max}, the serum concentration profile declined over time in parallel with the concentration curves following IV administration. The $AUC_{0\text{-}72h}$ values for SC and IV groups were 49.0 and 141 mg.h/L, respectively, with an estimated SC bioavailability of 34.8%. The findings are presented in the applicant's Figure and Table below.

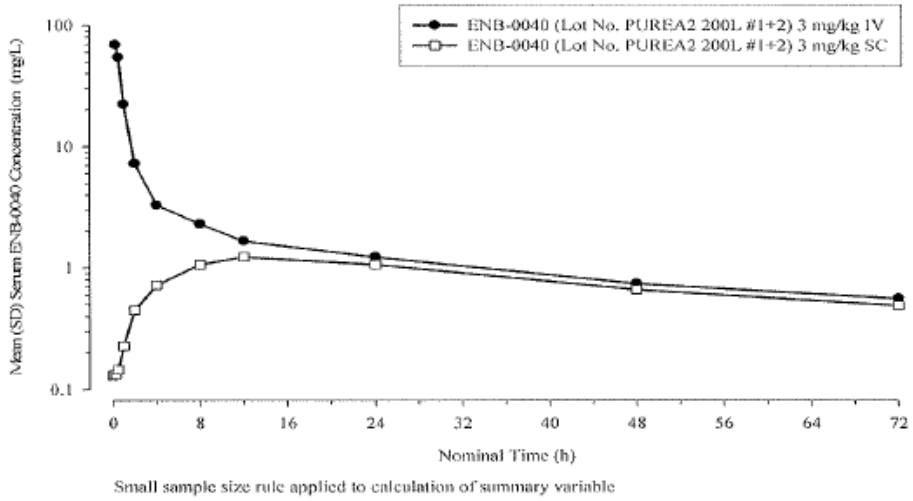


Figure 3: Mean (SD) Serum Concentrations of ENB-0040 in female Cynomolgus monkey after IV or SC administration (uncorrected for baseline)

Table 21: Pharmacokinetic Parameters of Asfotase Alfa in Monkeys after a Single IV or SC Administration at 3 mg/kg (Report 3008-0133, Section 8.3, Table 3, page 16)

RoA[a]	CL or CL/F (L/h/kg)	Vss or Vss/F (L/kg)	$t_{½}$ (h)	C_{max}[b] (mg/L)	C_{max} /Dose[c]	AUC_{∞} (mg*h/L)	$AUC_{0\text{-}72h}$ (mg*h/L)	$AUC_{0\text{-}72h}$ /Dose[c]	t_{max}[b] (h)
IV	0.0193	0.432	31.4	68.8	22.9	158	141	47.0	0.25
SC	DNS	DNS	DNS	1.10	0.367	DNS	49.0	16.3	10

[a] RoA = route of administration.
[b] C_{max} and t_{max} were observed values taken directly from the serum concentration-time profiles.
[c] The units of C_{max}/Dose and $AUC_{0\text{-}72h}$/Dose are (mg/L)/(mg/kg) and (mg*h/L)/(mg/kg), respectively.
DNS = data not shown, because extrapolated area for AUC_{∞} exceeds 25%.

A Toxicokinetic Study of ENB-0040 in the Female Rabbit Following a Single Intravenous Bolus Injection or Subcutaneous Injection (Study # 902235).

Methods: ENB-0040 was administered to female rabbits (n=2) by single intravenous or subcutaneous injections at 5 and 50 mg/kg dose levels. Blood samples (approximately 0.5 mL) were collected at 20 and 40 min, and at 1, 2, 6, 12, 24, 48, 72 and 96 hours after dosing.

Results: After IV administration, serum asfotase alfa concentrations were quantifiable for up to 96 hours (the last sampling time) post dose. Mean serum ENB-0040 concentrations declined in a bi-exponential fashion. Clearance ranged from 0.0540 L/h/kg at 5 mg/kg dose group to 0.0172 L/h/kg at 50 mg/kg dose, suggesting no-linear kinetics. C_{max} and $AUC_{0\text{-}96h}$ increased nonlinearly by about 22-28-fold for a 10-fold increase in dose. V_{ss} value ranged from 0.645 L/kg at 5 mg/kg dose group to 0.0693 L/kg at 50 mg/kg dose group.

Following subcutaneous injection, C_{max} and $AUC_{0\text{-}96h}$ increased in a dose-proportional manner as apparent systemic clearance was similar across doses. At the low (5 mg/kg) and high (50 mg/kg) doses, $AUC_{0\text{-}96h}$ was 25.9 and 280 mg.h/L, respectively, while C_{max} was 0.929 and 11.0 mg/L. The t_{max} was 6-12 hours and the bioavailability was 33.7% following subcutaneous administration. The PK data are presented in the applicant's Table below.

Text Table 2 Summary TK Parameters of ENB-0040 in Female Rabbits

Parameters	Mean (CV%)			
	IV		SC	
	ENB-0040 (Group 1: 5 mg/kg IV)	ENB-0040 (Group 2: 50 mg/kg IV)	ENB-0040 (Group 3: 5 mg/kg SC)	ENB-0040 (Group 4: 50 mg/kg SC)
N	3	3	3	3
$AUC_{(0\text{-}t)}$ (mg•h/L)	102 (23.1)	2896 (4.3)	25.9 (16.5)	280 (32.6)
$AUC_{(0\text{-}\infty)}$ (mg•h/L)	92.70 (NC)[b]	2918 (4.6)	31.2 (15.0)	304 (30.7)
C_{max} (mg/L)	51.4 (1.8)	1137 (6.7)	0.929 (2.9)	11.0 (43.8)
T_{max}[a] (h)	0.37 (0.35, 0.38)	0.42 (0.37, 0.67)	6.03 (6.00, 6.05)	12.00 (6.02, 12.02)
$t_{1/2}$ (h)	29.7 (NC)[b]	33.6 (15.9)	35.9 (16.9)	29.4 (5.1)
CL/(F)[c] (L/h/kg)	0.0540 (NC)[b]	0.0172 (4.5)	0.163 (14.0)	0.175 (28.9)
$V_{ss}/(F)$[d] (L/kg)	0.645 (NC)[b]	0.0693 (13.1)	6.78 (3.2)	6.37 (43.4)

a Median (Min, Max)
b n = 2
c CL for IV dose and CL/F for SC dose
d Vss for IV dose and Vss/F for SC dose
NC = Not calculated IV=intravenous SC = subcutaneous

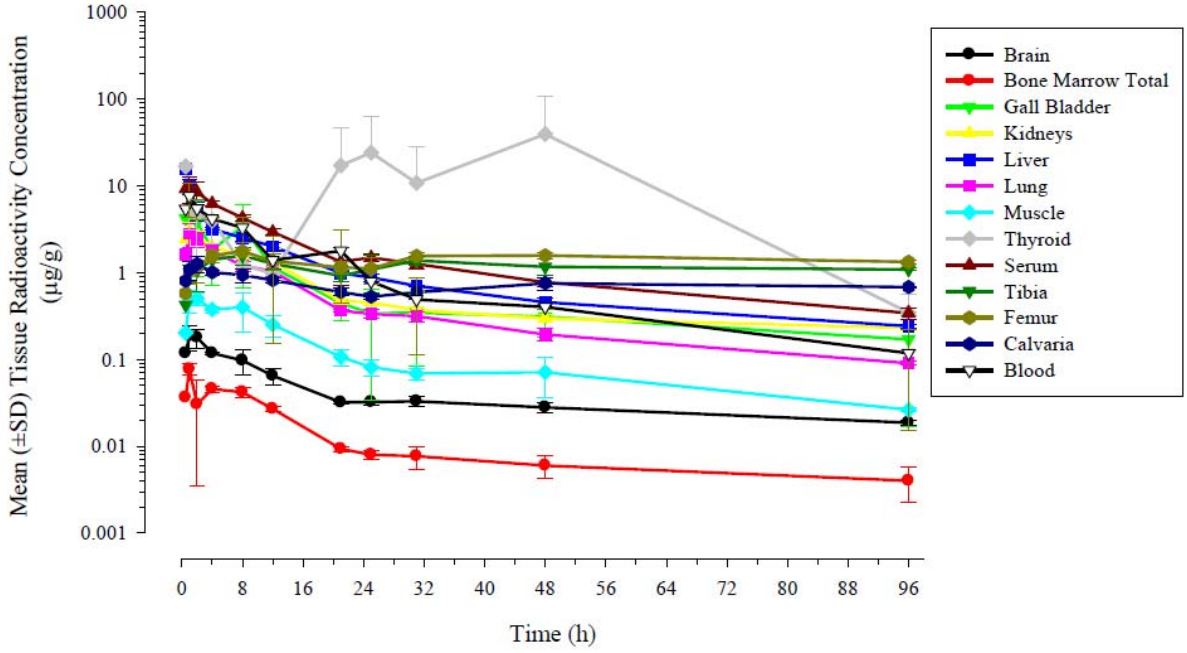
DISTRIBUTION:

***In vivo* Biodistribution Study of [^{125}I] ENB-0040 Administered IV in 129J Mice (Study # ALP-PD-01).**

Methods: A targeted dose of 5 mg/kg ^{125}I-ENB-0040 (380 µCi/kg) was administered as a single IV dose to 33 male 129J mice (5 weeks old). The actual administered dose was 4.1 mg/kg with a radioactivity of 280 µCi/kg. Three mice were sacrificed at each of the following time points: 0.5, 1, 2, 4, 8, 12, 21, 25, 31, 48 and 96 hours post-dose.

Following sacrifice, blood and tissues (thyroid, liver, kidneys, muscle [musculus *gastrocnemius caput laterale],* tibias, femurs, bone marrow, brain, lungs, gall bladder and calvaria) were collected to measure the levels of radioactivity and determine the pharmacokinetics of ^{125}I-ENB-0040 derived radioactivity in each tissue.

Results: Following IV administration of ^{125}I-ENB-0040, blood/serum radioactivity declined in a bi-exponential fashion. Radioactivity in serum/blood declined relatively faster than that in bone tissues (femur and tibia). Among all organs, thyroid, which is known as iodine trapping organ, had the highest radioactivity. Radioactivity concentrations of ^{125}I-ENB-0040 in various tissues at different time points after a single IV administration to mice are presented in the applicant's Figure below.



The volume of distribution (V_{ss}) calculated from blood and serum radioactivity concentrations were 1104 and 981 g/kg, respectively, which was about 1.2-fold greater than the volume of total body water in mice (approximately 725 mL/kg), suggesting distribution of ENB-0040 into peripheral tissues. The half-life ($t_{1/2}$) in bone marrow (71.7h) and femur (262h) was relatively long compared to half-life in kidney, liver and lung (101, 44.1 and 37.6h, respectively). The C_{max} values for blood and serum were 7.63 and 10.1 µg/g, respectively. Among all organs, thyroid had the highest C_{max} values (39. 6 µg/g). Brain had the lowest C_{max} values (0.185 µg/g); suggesting ENB-0040 derived radioactivity limited passage through the blood brain barrier. A slightly higher C_{max} value (0.502 µg/g) was observed in skeletal muscles. Intermediate C_{max} values were observed in richly-perfused organs such as liver, kidney, lung and gall bladder. The t_{max} was between 0.5 to 2h post-dose in most tissues except for long bones (tibia and femur) where t_{max} values were 8h. The ^{125}1-ENB-0040 derived radioactivity was very low in bone marrow (total, pellet and supernatant) when compared to marrow-

deprived long bones. PK parameters for different organs and tissues are presented in the applicant's Tables below.

Tissue	AUC_{∞} (h•µg/g)	CL(/F) (g/h/kg)	$t_{1/2}$ (h)	MRT_{∞} (h)	V_{ss} (/F) (g/kg)	V_z (/F) (g/kg)
Blood	95.1	43.1	30.6	25.6	1104	1900
Serum	163	25.1	35.7	36.0	904	1291
Serum Total	161	25.4	37.7	38.5	981	1385
Bone Marrow Pellet	NA	NA	NA	NA	NA	NA
Bone Marrow SUP	0.886	4630	36.0	28.5	131877	240566
Bone Marrow Total*	1.50	2741	71.7	73.7	202027	283506
Brain*	5.89	697	79.7	96.9	67474	80106
Calvaria	NA	NA	NA	NA	NA	NA
Femurs*	642	6.39	262	382	2442	2412
Gall Bladder	78.9	52.0	61.4	52.2	2713	4599
Kidneys*	86.7	47.3	101	110	5209	6878
Liver	111	37.0	44.1	40.8	1510	2353
Lung	44.5	92.1	37.6	36.0	3318	4991
Muscle	12.1	340	43.0	43.0	14617	21097
Serum Pellet	141	29.1	38.3	42.3	1233	1609
Serum SUP	NA	NA	NA	NA	NA	NA
Thyroid	NA	NA	NA	NA	NA	NA
Tibias	NA	NA	NA	NA	NA	NA

NA = Not Available

*Extrapolation exeeds 25%, included in calculation

Tissue	AUC_{last} (h•µg/g)	±SE	C_0 (µg/g)	C_{max} (µg/g)	±SE	T_{max} (h)	T_{last} (h)	MRT_{last} (h)
Blood	89.9	8.05	5.45	7.63	1.88	1	96	19.0
Serum	146	4.39	9.20	9.48	1.82	1	96	22.6
Serum Total	142	5.72	8.78	10.1	1.62	1	96	23.2
Bone Marrow Pellet	0.289	0.0422	NA	0.0137	0.0112	2	96	46.7
Bone Marrow SUP	0.816	0.0411	NA	0.0673	0.0100	1	96	18.3
Bone Marrow Total	1.08	0.0524	NA	0.0773	0.0068	1	96	25.6
Brain	3.74	0.125	NA	0.185	0.0337	1	96	31.3
Calvaria	69.6	2.57	NA	1.26	0.152	2	96	46.3
Femurs	138	3.46	NA	1.77	0.0270	8	96	47.7
Gall Bladder	63.9	8.26	NA	8.93	1.73	1	96	21.0
Kidneys	53.1	2.32	NA	3.09	0.419	1	96	27.1
Liver	95.5	2.55	NA	15.8	3.29	0.5	96	21.7
Lung	39.6	1.21	NA	2.81	0.510	1	96	21.9
Muscle	10.4	0.857	NA	0.502	0.0469	2	96	25.0
Serum Pellet	122	2.95	NA	7.69	1.94	0.5	96	25.3
Serum SUP	23.3	1.44	NA	2.97	0.833	2	96	9.86
Thyroid	1695	1270	NA	39.6	38.6	48	96	42.5
Tibias	113	5.19	NA	1.58	0.202	8	96	46.6

NA = Not Available

The tissue-to-blood ratios for calvaria, tibia and femur were 0.732, 1.19 and 6.75, respectively. The tissue-to-blood ratios for richly-perfused organs, such as liver, kidneys, lung and gall bladder, ranged from 0.468-1.17. The lowest tissue-to-blood ratio (0.0619) of ^{125}I-ENB-0040 was observed in the brain, suggesting limited penetration of ^{125}I-ENB-0040 into central nervous system. Tissue-to-blood ratio of ^{125}I-ENB-0040 in mice is presented in the applicant's Table below.

Tissue	AUC_{∞} (h•µg/g)	AUC_{last} (h•µg/g)	Ratio[a]
Blood	95.1	89.9	NA
Serum	163	146	1.72
Serum Total	161	142	1.69
Bone Marrow Pellet#	NA	0.289	0.00303
Bone Marrow SUP	0.886	0.816	0.00931
Bone Marrow Total*	1.50	1.08	0.0157
Brain*	5.89	3.74	0.0619
Calvaria#	NA	69.6	0.732
Femurs*	642	138	6.75
Gall Bladder	78.9	63.9	0.830
Kidneys*	86.7	53.1	0.912
Liver	111	95.5	1.17
Lung	44.5	39.6	0.468
Muscle	12.1	10.4	0.127
Serum Pellet	141	122	1.48
Serum SUP#	NA	23.3	0.245
Thyroid#	NA	1695	17.8
Tibias#	NA	113	1.19

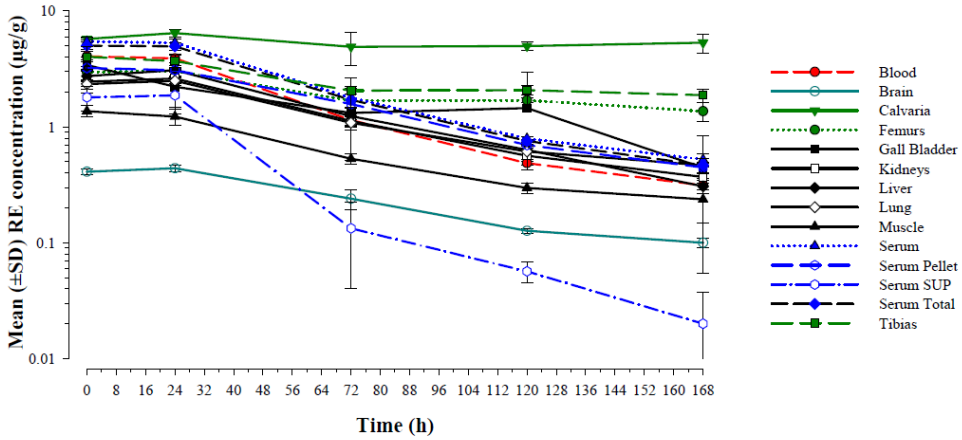
[a] Ratio AUC_{∞} (tissue) / AUC_{∞} (blood)
NA = Not Available
# = Ratio of AUC_{last} (tissue) / AUC_{∞} (blood)
*Extrapolation exeeds 25%, included in calculation

Tissue Distribution and Pharmacokinetics of [^{125}I] Asfotase Alfa in Newborn CD-1 Mice Following Two Weeks of Daily Subcutaneous (SC) Injections of [^{125}I] Asfotase Alfa (Study # ALP-PD-02).

Methods: Fifteen newborn CD-1 mice (1-day-old) were administered ^{125}I-asfotase alfa at a dose of 4.3 mg/kg (98.9 µCi/kg) for 14 days subcutaneously. On Day 15, blood, serum and tissue samples were collected from 3 mice per time-point at the 24h post Day 14 and then at 24, 72, 120 and 168h post Day 15 to measure the levels of radioactivity and determine the pharmacokinetics of ^{125}I-asfotase alfa derived radioactivity in each tissue.

Results: The radioactive equivalent (RE) concentrations of ^{125}I-asfotase alfa in blood and tissues measured 24 h following the last dose on day 14 or 15 were similar. The AUC and C_{max} of ^{125}I-asfotase alfa were higher in the long bones (calvaria, tibia and femur) compared to soft tissues (brain, gallbladder, kidneys, liver, lung, and muscle). Radioactivity concentrations of ^{125}I-asfotase alfa in various tissues at different time points are presented in the applicant's Figure below.

Figure 1 Mean (±SD) RE concentrations of [^{125}I] asfotase alfa in blood, serum, and tissues of newborn mice



SUP=supernatant
"Serum Total" radioactive equivalent (RE) concentrations were calculated by sum of "Serum SUP" and "Serum Pellet" concentrations.

The AUC and C_{max} in bone tissues were similar or somewhat higher than the corresponding values in blood. Brain had the lowest C_{max}, suggesting that the distribution of ^{125}I-asfotase alfa to the brain was limited. The second lowest C_{max} values were observed in the muscle, while the intermediate C_{max} values were observed in well-perfused organs such as liver, kidney, lung and gall bladder. The apparent half-life ($t_{1/2}$) values of ^{125}I-asfotase alfa in soft tissues ranged from 35.1 to 82.5h. The $t_{1/2}$ of ^{125}I-asfotase alfa in bone tissues (tibia, 741h) appeared to be longer than that of soft tissues. Bone tissues (calvaria, tibia and femur) were the only tissues that showed a flat (unchanged) disposition/elimination from 72 to 168 hours post dose, indicating a long persistent exposure of ^{125}I-asfotase alfa in these tissues. PK parameters are presented in the applicant's Tables below.

Table 1 PK parameters of [^{125}I] asfotase alfa in newborn mice

Tissue	AUC_{last} (h•µg/g)	±SE	AUC_{τ} (h•µg/g)	C_{avg} (µg/g)	C_{max} (µg/g)	±SE	T_{max} (h)	T_{last} (h)	MRT_{last} (h)
Blood	274	8.38	93.8	3.91	3.91	0.203	24	168	41.7
Brain	41.1	1.43	10.6	0.440	0.440	0.0173	24	168	57.0
Calvaria	913	48.1	155	6.45	6.45	0.299	24	168	79.7
Femurs	342	17.1	73.2	3.05	3.05	0.197	24	168	69.5
Gall Bladder	250	51.5	53.4	2.22	2.22	0.534	24	168	66.4
Kidneys	215	5.92	62.7	2.61	2.61	0.0876	24	168	50.5
Liver	245	6.11	74.2	3.09	3.09	0.0700	24	168	48.2
Lung	213	5.56	60.2	2.51	2.51	0.0769	24	168	53.2
Muscle	104	4.56	29.5	1.23	1.23	0.112	24	168	53.2
Serum	392	11.6	128	5.33	5.33	0.208	24	168	44.5
Serum Pellet	268	9.59	74.2	3.09	3.09	0.0153	24	168	52.0
Serum SUP	99.5	8.72	45.0	1.87	1.87	0.231	24	168	25.0
Serum Total	368	13.6	119	4.97	4.97	0.219	24	168	44.7
Tibias	421	19.5	89.0	3.71	3.71	0.258	24	168	70.8

NA = Not Available
SUP=Supernatant
"Serum Total" radioactive equivalent (RE) concentrations used for PK calculations, were calculated by sum of "Serum SUP" and "Serum Pellet" concentrations

Tissue	AUC_{∞} (h•µg/g)	CL/F (g/h/kg)	$t_{1/2}$ (h)	MRT_{∞} (h)	V_{ss}/F (g/kg)	V_z/F (g/kg)
Blood	297	46.0	51.3	63.9	2942	3407
Brain	52.1	409.1	76.0	106	43518	44859
Calvaria	NA	27.9	NA	NA	NA	NA
Femurs	NA	59.0	NA	NA	NA	NA
Gall Bladder	NA	81.0	NA	NA	NA	NA
Kidneys	247	68.9	59.8	82.6	5692	5938
Liver	267	58.3	47.7	74.3	4326	4010
Lung	266	71.8	78.7	94.2	6762	8156
Muscle	133	146	82.5	95.8	14018	17425
Serum	433	33.8	54.3	69.2	2336	2643
Serum Pellet	302	58.3	52.8	85.8	4997	4434
Serum SUP	101	96.1	35.1	41.7	4003	4862
Serum Total	403	36.2	51.2	69.1	2503	2675
Tibias*	2431	48.6	741	644	31265	51905

NA = Not Available

*Extrapolation exeeds 25%, included in calculation

SUP=Supernatant

"Serum Total" radioactive equivalent (RE) concentrations used for PK calculations, were calculated by sum of "Serum SUP" and "Serum Pellet" concentrations

The tissue-to-blood ratios for calvaria, tibia and femur were 1.65, 0.948 and 0.780, respectively. The tissue-to-blood ratios for liver, kidneys, lung and gall bladder, ranged from 0.569-0.790. Tissue-to-Blood Ratio of ^{125}I-asfotase alfa in newborn CD1 mice is presented in the applicant's Table below.

Table 3 Tissue partition coefficient (Ri or Ratio)

Tissue	AUC_τ (h•µg/g)	Ratio[a]
Blood	93.8	NA
Brain	10.6	0.113
Calvaria	155	1.65
Femurs	73.2	0.780
Gall Bladder	53.4	0.569
Kidneys	62.7	0.668
Liver	74.2	0.790
Lung	60.2	0.641
Muscle	29.5	0.315
Serum	128	1.36
Serum Pellet	74.2	0.790
Serum SUP	45.0	0.479
Serum Total	119	1.27
Tibias*	89.0	0.948

[a] Ratio AUCτ (tissue) / AUCτ (blood)
NA = Not Available
*Extrapolation exeeds 25%, included in calculation

METABOLISM and EXCRETION:

The applicant did not conduct any metabolism and/or excretion studies for asfotase alfa.

5.2 Toxicokinetics

ENB-0040: A 4-Week Intravenous or Subcutaneous Injection Toxicokinetic Study in Juvenile Sprague-Dawley Rats (Study # 70552).

Methods:
The aim of this study was to determine and compare toxicokinetic profile of ENB-0040 following once weekly intravenous injection and daily subcutaneous injection for 4 weeks to juvenile SD (Sprague-Dawley) rats. ENB-0040 was administered to juvenile rats (22 and 25 days-old) once weekly for four weeks by intravenous injections or once daily for 4 consecutive weeks by subcutaneous injections. The study design is described in the applicant's Table below:

Group Numbers	Route	Group Designation	Frequency	Dose Level (mg/kg/day)	Dose Concentration (mg/mL)	No. of Animals Day of Sacrifice Following last Blood Collection for Toxicokinetics				
						Day 1*		Day 22		Day 28
						Male	Female	Male	Female	Male
1	IV	Control	Weekly	0	0	3	3	3	3	-
2		Low Dose		3	0.6	30	30	6	6	-
3		Mid Dose		30	6.0	30	30	6	6	-
4		High Dose		90	18.0	30	30	6	6	-
5	SC	Control	Daily	0	0	3	-	-	-	3
6		Low Dose		0.84	0.84	30	-	-	-	6
7		Mid Dose		8.4	8.4	30	-	-	-	6
8		High Dose		25.2	25.2	30	-	-	-	6

IV = intravenous injection
SC = subcutaneous injection
* = Animals euthanized by CO_2 asphyxiation followed by cervical dislocation, following last blood collection for toxicokinetics and discarded without further examination.

Animals were monitored for mortality, clinical signs, body weight, and gross and histopathological examinations of the dosing sites and lymph nodes (axillary, inguinal, mandibular and mesenteric) were conducted.

For toxicokinetic analysis, blood samples (approximately 0.2 mL) were collected on Day 1, and on Day 22 (Groups 1 to 4) or Day 28 (Groups 5 to 8 males only). On Day 1, single sample was collected at 0.5 hour post treatment from Group 1 and 24 hours post treatment from Group 5, and from groups 2-4, samples were collected at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 hours post dosing; from Groups 6 to 8, samples were collected at 0.5, 1, 2, 4, 8, 12, 24, 48, 72 and 96 hours post dosing. On Days 22 and 28, single sample was collected at 0.5 hour post treatment from Group 1 and 24 hours post treatment from Group 5, and from groups 2-4, samples were collected at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 hours post dosing; from Groups 6 to 8, samples were collected at 0.5, 1, 2, 4, 8, 12, 24, 48, 72 and 96 hours post dosing.

Results:
There was no treatment-related mortality or change in body weights for animals subjected to ENB-0040 treatment via the intravenous or subcutaneous route.

Treatment-related clinical observations following IV administration included slight to moderate swelling of limbs (includes forepaws or hindpaws) head and muzzle regions, piloerection (body and/or head), slight to moderate decreased activity and lying on cage floor, skin discoloration of the limbs and tail (blue in appearance), and partly closed eyes. The majority of these clinical observations were noted in Groups 3 and 4 in a dose-related fashion while treatment-related clinical signs for Group 2 were limited to piloerection (body) and generally slight swelling of the limbs and/or head, and/or muzzle. There were no treatment-related clinical observations in animals administered ENB-0040 via the subcutaneous route.

There were no treatment-related macroscopic findings associated with the intravenous injection. A treatment-related enlargement of the axillary lymph node was observed in Group 6 (2/6), Group 7 (3/6), and Group 8 (3/6) males that received SC injections of

ENB-0040. Treatment-related microscopic findings following SC administration included minimal to mild perivascular/subcutaneous mononuclear cell infiltrate at the dosing sites in Group 6 (5/6), Group 7 (6/6) and Group 8 (5/6) males, and minimal lymphoid hyperplasia of the axillary lymph node in Group 7 (2/6) and Group 8 (2/6) males.

Following IV injection of 3, 30 and 90 mg/kg ENB-0040 on Day 1 systemic clearances (CL) were 0.0287, 0.0339 and 0.0459 L/h/kg, respectively and elimination half-lives were 18.3, 15.8 and 15.3 h, respectively. The C_{max} (16.2, 298 and 567 mg/L, respectively) was found to increase across doses and was generally dose proportional. AUC_{0-168} were 104, 886, and 1960 mg·h/L, respectively for the 3, 30 and 90 mg/kg dose groups. On Day 22, systemic clearance and elimination half-life of ENB-0040 in the serum was comparable to Day 1. Similar to Day 1, the C_{max} on Day 22 (i.e., 16.2, 147 and 678 mg/L, respectively) was increased across doses and was generally dose proportional. Slightly less than dose-proportional increases were noted for AUC_T (i.e., 76.8, 591 and 2054 mg·h/L, respectively). The accumulation ratio (R_A) on Day 22 (0.667-1.05) indicated that there was no significant accumulation upon multiple weekly IV dosing. Data are presented in the applicant's Tables below:

Table 7-1 PK Parameters of ENB-0040 Serum (IV; Genders Combined)

Treatment = ENB-0040 IV weekly

Serum ENB-0040

Dose mg/kg	Day	AUC_∞ (mg•h/L)	AUC_{last} (mg•h/L)	±SE	AUC_{0-168} (mg•h/L)	CL (L/h/kg)	C_{max} (mg/L)	±SE	MRT_∞ (h)	$t_{1/2}$ (h)	T_{max} (h)	V_{ss} (L/kg)
3	1	104	100	2.21	104	0.0287	16.2	0.652	17.1	18.3	0.25	0.493
30	1	886	869	11.5	886	0.0339	298	9.36	12.1	15.8	0.25	0.408
90	1	1961	1927	75.7	1960	0.0459	567	96.5	10.8	15.3	0.25	0.497

Dose mg/kg	Day	AUC_∞ (mg•h/L)	AUC_{last} (mg•h/L)	±SE	AUC_τ (mg•h/L)	CL_{ss} (L/h/kg)	C_{max} (mg/L)	±SE	MRT_∞ (h)	$t_{1/2}$ (h)	T_{max} (h)	V_{ss} (L/kg)
3	22	76.9	73.1	5.14	76.8	0.0391	16.2	1.04	18.4	19.8	0.25	0.717
30	22	592	567	41.1	591	0.0508	147	13.8	16.8	18.5	0.25	0.853
90	22	2061	1957	87.0	2054	0.0438	678	53.7	14.7	23.2	0.50	0.643

Table 7-3 Accumulation Ratio (R_A) of ENB-0040 in Serum

Day Comparison

Route	Gender	Dose mg/kg	Day	RA	LI
IV	Combined	3	22	0.736	0.736
IV	Combined	30	22	0.667	0.667
IV	Combined	90	22	1.05	1.05
SC	Male	0.84	28	0.339	0.118
SC	Male	8.4	28	0.0539	0.0211
SC	Male	25.2	28	0.0488	0.0210

Following subcutaneous administration of 0.84, 8.4 and 25.2 mg/kg doses on Day 1, systemic clearance (CL/F) of ENB-0040 was similar across doses (0.0473, 0.0591 and 0.0724 L/h/kg, respectively), and the elimination half-life (21.0, 17.8 and 21.4 h, respectively) was relatively consistent across doses. The increases in C_{max} (0.326, 2.94 and 8.00 mg/L, respectively) across doses were slightly less than dose proportional. The increases in AUC_{0-24} were also slightly less than dose proportional (6.17, 55.7, and 149 mg·h/L, respectively). On Day 28, the systemic clearance (CL_{ss}/F) of ENB-0040 in serum increased with dose increases (i.e., 0.401, 2.80 and 3.46 L/h/kg, respectively). This could be due to decreasing systemic bioavailability (higher CL or lower %F) over time due to repeated subcutaneous injections causing either physiological change at the local administration site or increased clearance due to immunomodulation. Elimination half-life was 12.2 and 79.2h at mid and high doses, respectively. The C_{max} was 0.182, 0.215 and 0.532 mg/L, respectively, and the increase across doses was less than dose proportional as with AUC_{τ} (i.e., 2.09, 3.00 and 7.29 mg·h/L, respectively). Data are presented in the applicant's Table below:

Table 7-2 PK Parameters of ENB-0040 Serum (SC Males)

Treatment = ENB-0040 SC daily dose												
Serum ENB-0040												
Dose mg/kg	Day	AUC_{∞} (mg•h/L)	AUC_{last} (mg•h/L)	±SE	AUC_{0-24} (mg•h/L)	CL/F (L/h/kg)	C_{max} (mg/L)	±SE	MRT_{∞} (h)	$t_{1/2}$ (h)	T_{max} (h)	Vss/F (L/kg)
0.84	1	17.8	16.6	1.63	6.17	0.0473	0.326	0.0874	40.0	21.0	12.00	1.89
8.4	1	142	137	5.48	55.7	0.0591	2.94	0.416	34.7	17.8	8.00	2.05
25.2	1	348	333	33.0	149	0.0724	8.00	1.47	33.3	21.4	12.00	2.41
Dose mg/kg	Day	AUC_{∞} (mg•h/L)	AUC_{last} (mg•h/L)	±SE	AUC_{τ} (mg•h/L)	CL_{ss}/F (L/h/kg)	C_{max} (mg/L)	±SE	MRT_{∞} (h)	$t_{1/2}$ (h)	T_{max} (h)	Vss/F (L/kg)
0.84	28	NC	6.00	5.77	2.09	0.401	0.182	0.173	NC	NC	12.00	NC
8.4	28	7.05	6.85	4.28	3.00	2.80	0.215	0.166	45.4	12.2	8.00	127
25.2	28	17.3	13.8	5.93	7.29	3.46	0.532	0.242	45.1	79.2	2.00	156

NC: Not calculated

The absolute subcutaneous bioavailability (%F) across doses on Day 28 was markedly lower than those on Day 1 (2.3-25% vs. 55.1–58.1%). Data are presented in the applicant's Table below:

Table 7-4 Absolute Bioavailability (%F) of ENB-0040 in Serum (Males)

Day	Dose mg/kg	F %
1	0.840	58.1
1	8.40	56.7
1	25.2	55.1
22-28	0.840	24.9
22-28	8.40	3.66
22-28	25.2	2.30

No gender-specific differences in PK parameters of ENB-0040 in serum were observed.

Other TK studies were included in the toxicology section.

6 General Toxicology

6.1 Single-Dose Toxicity

sALP-FcD10: An Intravenous Injection and Infusion Maximum Tolerated Dose Toxicity Study in Juvenile Cynomolgus Monkeys (Study # 2007-0693).

Key study findings: sALP-FcD10 (asfotase alfa) was administered up to six times to the same monkey by IV injection or infusion. A dose escalation design was used and each dose was followed by at least a 7-day washout period between treatments. The dose levels administered were 5 or 15 mg/kg by IV bolus injection and 45, 90, or 180 mg/kg by IV infusion (the latter over 3, 6 or 12 minutes, respectively). After the 180 mg/kg IV infusion, a final IV bolus injection of 45 mg/kg was administered to the main study animals (n=2) on day 46. No test article-related changes in clinical signs, body weight, and food intake were observed at any dose levels. There were no deaths. An expected marked dose-proportional increase in alkaline phosphatase was observed in all animals throughout the study due to the presence of circulating test article. Transient increases in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in serum were observed in three animals during the study. Thus, single IV administration of asfotase alfa at doses up to 180 mg/kg was well-tolerated in juvenile Cynomolgus monkeys.

Study no.: 2007-0693
Volume #, and page #: Electronic submission
Conducting laboratory and location: (b) (4)
GLP compliance: No
Drug, lot #, and % purity: 070629; purity: 97%.

Methods

Doses: 5, 15, 45, 90 and 180 mg/kg body weight
Species/strain: Cynomolgus monkey
Number/sex/group or time point (main study): 3 animal/sex/group
Route, formulation, volume, and infusion rate: Intravenous, test article was formulated in 25 mM sodium PO_4 pH 7.4, 150 mM NaCl
Satellite groups used for toxicokinetics or recovery: None
Age: Approximately 12 months-old
Weight: 1.5 to 1.7 kg for both males and females
Sampling times: N/A
Unique study design or methodology (if any): none

Observation and Times:

Mortality:	Mortality check was performed twice daily.
Clinical signs:	Clinical observations were performed once daily before pre-treatment period, and twice daily during the dosing period.

Body weight:	All animals were weighed once at arrival and at least once during the week preceding the first dosing (Day 1) and one day before each dose.
Toxicokinetic:	Blood samples (approximately 0.5 mL) were collected from all toxicokinetic animals at pre-dose and at 0.5, 1, 2, 4, 8, 24, and 48 h post dose.
Urine Collection:	Urine was collected from all animals as follows, for possible inorganic pyrophosphate (PPi) and creatinine analysis: Day 1: Prior dosing (6 hours prior to dosing to within 30 minutes prior to dosing (-6 to 0h)) and at 1 (0-1), 8 (1-8), and 24 (8-24) hour post dose.
Clinical Pathology:	Clinical pathology investigations (hematology and clinical chemistry) were performed for all main study animals once during the pre-treatment period, and once a day after each dose. In order to verify if there was any sort of recovery following the last infusion at 180 mg/kg, blood samples were also collected on main study animals one day before the sixth dose (Day 45 or 16 days after the 180 mg/kg dose).

Results:

Mortality: All animals survived to the end of the study.

Clinical signs: No adverse treatment-related clinical signs were observed following each treatment.

Body weights: No changes in body weight.

Toxicokinetic:

The washout period between treatments was insufficient due to the presence of baseline levels on Day 1 and measurable concentration of ENB-0040 was noted at predose samples from subsequent periods. To correct for possible accumulation over dosing periods, a curve stripping methodology was used to remove the carryover of ENB-0040 from previous treatments and noncompartmental PK parameters were then calculated using concentration-time profiles free of carryover concentrations. For the IV injections mean AUC_{∞} values ranged from 797 to 2950 mg.h/L and mean C_{max} values ranged from 65 to 396 mg/L over the dose range studied. For the IV infusions, mean AUC_{∞} ranged from 9410 to 48400 mg.h/L and C_{max} ranged from 1230 to 7720 mg/L over the dose range studied. Mean $t_{1/2}$ values of ENB-0040 appeared to decrease with increasing dose levels. Although systemic clearance of ENB-0040 was relatively consistent across dose levels, the 90 mg/kg dose group appeared to be a pharmacokinetic outlier with a substantially lower clearance when compared to the other dose levels (approximately five-fold). No obvious gender related trends were noted. PK parameters of ENB-0040 are presented in the applicant's Table below.

Mean	Serum ENB-0040				
	Bolus 5 mg/kg	Bolus 15 mg/kg	Infusion 45 mg/kg	Infusion 90 mg/kg	Infusion 180 mg/kg
N	2	2	2	2	2
AUC_{last} (mg•h/L)	763	2860	8950	47000	33500
AUC_{∞} (mg•h/L)	797	2950	9410	48400	36000
C_{max} (mg/L)	64.9	306	1230	7720	3830
T_{max}[a] (h)	0.75 (0.50, 1.00)	0.50 (0.50, 0.50)	0.55 (0.55, 0.55)	2.10 (2.10, 2.10)	0.95 (0.70, 1.20)
$t_{1/2}$ (h)	40.4	30.6	30.0	19.4	20.9
CL (L/h/kg)	0.00633	0.00509	0.00504	0.00188	0.00507
V_{ss} (L/kg)	0.255	0.166	0.0972	0.0168	0.0638
AUC_{∞}/Dose (kg•h/L)	159	197	209	538	200
C_{max}/Dose (kg/L)	13.0	20.4	27.3	85.8	21.3
[a] Median (Min, Max) NC = Not calculated					

Hematology:
Slight decreases in erythrocytes (RBC), hemoglobin (HGB) and hematocrit (HCT) and slight increases in red cell distribution width (RDW), hemoglobin distribution width (HDW), reticulocytes (RETIC) and eosinophil count (EOS, absolute and relative) were observed in males and females as dose levels increased. Since the changes were modest in amplitude, consistent with multiple blood sampling, and within normal biological variation in Cynomolgus monkeys, they were not considered to be toxicologically significant.

Clinical Chemistry:
A marked dose proportional increase in alkaline phosphatase was observed in all monkeys throughout the study but this was related to the presence of the test article in the bloodstream of the animals after each dosing. Increases in alanine aminotransferase and aspartate aminotransferase were also observed in three animals during the study but the toxicological significance of this finding is uncertain. The ALT and AST levels returned to normal 16 days after the treatment at the highest dose level, 180 mg/kg. Data are presented in the applicant's Tables below:

TABLE NO. 5 CLINICAL CHEMISTRY STUDY NO. 2007-0693
MALES

DAY 1 : sALP-FcD10 (5 mg/kg)
DAY 8 : sALP-FcD10 (15 mg/kg)
DAY 15 : sALP-FcD10 (45 mg/kg)
DAY 22 : sALP-FcD10 (90 mg/kg)
DAY 29 : sALP-FcD10 (180 mg/kg)
DAY 46 : sALP-FcD10 (45 mg/kg)

ANIMAL NO.	BIL-T µmol/L	Ca mmol/L	PHOS mmol/L	AST U/L	ALT U/L	ALP U/L	GLUC mmol/L	CHOL mmol/L	TRIG mmol/L
				PRE-TREATMENT					
1001A	3.7	2.78	2.58	44	53	397	3.6	3.52	0.73
1002A	3.9	2.57	2.38	56	46	439	2.9	3.31	0.42
				DAY 2					
1001A	1.9	2.75	2.05	75	176	20980 *	4.4	3.29	0.84
1002A	2.4	2.51	2.34	61	58	19300 *	3.8	3.47	0.37
				DAY 9					
1001A	2.9	2.69	2.45	91	204	63525 *	4.6	3.40	0.72
1002A	3.2	2.51	2.64	87	74	51100 *	3.6	3.69	0.35
				DAY 16					
1001A	1.9	2.78	2.30	80	179	197112*	4.2	4.23	0.38
1002A	2.1	2.54	2.48	79	92	221720*	4.8	3.63	0.34
				DAY 23					
1001A	2.7	2.63	2.20	146	330	381537*	4.1	3.45	0.61
1002A H1	2.9	2.45	2.45	105	92	277823*	3.3	3.96	0.39
				DAY 30					
1001A	2.4	2.70	2.36	162	267	720000*	3.9	3.55	0.49
1002A	3.2	2.42	2.11	105	81	539400*	2.8	3.84	0.38
				DAY 45					
1001A	<1.7	2.82	1.79	35	51	1031	4.2	3.08	0.67
1002A H1	<1.7	2.64	1.99	56	48	1089	4.3	3.39	0.66

H1: Slight hemolysis
* : Result over linearity

TABLE NO. 5 CLINICAL CHEMISTRY FEMALES STUDY NO. 2007-0693

DAY 1 : sALP-FcD10 (5 mg/kg)
DAY 8 : sALP-FcD10 (15 mg/kg)
DAY 15 : sALP-FcD10 (45 mg/kg)
DAY 22 : sALP-FcD10 (90 mg/kg)
DAY 29 : sALP-FcD10 (180 mg/kg)
DAY 46 : sALP-FcD10 (45 mg/kg)

ANIMAL NO.	BIL-T µmol/L	Ca mmol/L	PHOS mmol/L	AST U/L	ALT U/L	ALP U/L	GLUC mmol/L	CHOL mmol/L	TRIG mmol/L
				PRE-TREATMENT					
1501A	2.6	3.01	2.47	49	27	514	4.9	4.32	0.58
1502A	4.1	2.68	2.74	45	46	438	3.3	3.83	0.49
				DAY 2					
1501A	<1.7	2.92	2.23	63	55	22840 *	4.2	3.35	0.51
1502A	<1.7	2.52	1.60	34	43	19320 *	3.4	3.55	0.41
				DAY 9					
1501A	1.8	2.86	2.47	75	70	66225 *	5.9	3.76	0.45
1502A	2.0	2.73	2.73	38	51	59800 *	4.1	4.05	0.36
				DAY 16					
1501A	2.1	2.87	2.60	117	125	172855*	3.6	4.15	0.37
1502A	1.8	2.70	2.56	40	52	299920*	3.7	4.34	0.36
				DAY 23					
1501A	<1.7	2.78	2.26	121	117	348257*	5.4	3.74	0.38
1502A	2.8	2.59	2.31	42	51	325682*	2.8	3.77	0.38
				DAY 30					
1501A	<1.7	2.83	2.39	165	61	743400*	5.7	3.52	0.36
1502A	3.2	2.58	2.26	57	53	382200*	2.1	4.13	0.46
				DAY 45					
1501A H1	<1.7	2.89	1.79	55	21	198	8.3	3.57	0.46
1502A	<1.7	2.78	2.32	32	37	539	3.9	3.74	0.28

H1: Slight hemolysis
* : Result over linearity

6.2 Repeat-Dose Toxicity

1. Study title: sALP-FcD10: A Maximum Tolerated Dose Intravenous Toxicity Study in Juvenile Sprague-Dawley Rats.

Study no.:	70385
Study report location:	Electronic submission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	July 31, 2007
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	070629, purity: 97.8%

Key Study Findings

sALP-Fc10 (asfotase alfa) was administered intravenously to 23 to 24 days-old SD rats once a week for 4 weeks at dose levels of 10, 30, 90 and 180 mg/kg. There were no deaths. sALP-Fc10 had no significant effects on body weight, organ weight and coagulation parameters. Rats in the 90 and 180 mg/kg dose groups showed acute injection site reactions and reduced motor activity and a slight swelling of the limbs, pinnae and muzzle. Skin discolorations (red or blue in appearance) were noted in the extremities. Rats in the high dose group showed excessive scratching, piloerection and hyperpnea. Animals in the 10 and 30 mg/kg dose groups did not show any clinical signs on the first dosing. However, animals in these dose groups showed the similar types of clinical signs as of the high dose group on Days 8, 15, and 22. As expected, a dose proportional increase in alkaline phosphatase activity was observed in all animals throughout the study due to the presence of circulating test article. Slight decreases in platelets and increases in reticulocytes were noted in the 30, 90, and 180 mg/kg dose groups. Animals in the 90 and 180 mg/kg dose groups showed minimal to mild erosion/ulceration in the stomach that was observed macroscopically and microscopically. Based on clinical signs noted in the 180 mg/kg/week dose group, this dose was considered to be too high for subsequent studies.

Methods

Doses:	10, 30, 90 and 180 mg/kg/week
Frequency of dosing:	Weekly
Route of administration:	IV
Dose volume:	5 mL/kg
Formulation/Vehicle:	A solution of 25 mM sodium PO_4 pH 7.4, 150 mM NaCl was used as the vehicle.
Species/Strain:	Sprague-Dawley (SD) Rats
Number/Sex/Group:	3 males and 3 females/group
Age:	23-24 days-old
Weight:	50 to 60g for both males and females
Satellite groups:	None
Unique study design:	None
Deviation from study protocol:	None

Observations and Results

Mortality

Animals were observed twice daily for mortality.

No deaths or moribundity occurred during the experimental period.

Clinical Signs

All animals were observed once daily during the pretreatment period and twice daily on the dosing days.

During the first dosing, no clinical signs were observed in the 10 and 30 mg/kg dose groups. However, following the first dosing, animals in the two high dose groups showed decreased activity, slight swelling of limbs, pinna and muzzle with skin discoloration (red or blue in appearance) at the extremities. Animals in the 10 and 30 mg/kg dose groups showed swelling of limbs, pinna and muzzle with skin discoloration (red or blue in appearance) at the extremities following the second, third and fourth dosing (Days 8, 15 and 22). In animals in the 90 and 180 mg/kg dose groups, the clinical signs of reduced motor activity, piloerection, hyperpnea and swelling of limbs, pinna and muzzle with skin coloration became more evident as dosing progressed from the first dosing occasion to the fourth. These clinical signs were acute, and the severity increased as the dose level increased and as dosing progressed; however, the clinical signs were transient. All clinical signs appeared within 50 minutes after administration of test article, some animals recovered within thirty minutes to 2 hours. The majority of the animals recovered completely by the next morning.

Body Weights

All animals were weighed during the pretreatment (day -10), prior to dosing on Day 1, and weekly during dosing period.

Males in the high dose group (180 mg/kg) had 10% lower body weight compared to low dose males (10 mg/kg).

Hematology

Hematology, coagulation, clinical chemistry was performed on all animals at termination.

There was no treatment-related alteration in coagulation parameters. However, a treatment-related decrease in platelet counts was noted in the males and females. There was an increase in the percentage of reticulocytes (RETIC) in the majority of treated groups with the exception of the males treated at the lowest dose. Minor changes in white cell parameters were apparent in males. These included a slight

increase in total white blood cell counts at the two high dose levels and corresponding increases in neutrophils (NEUT), monocytes (MONO) and large unstained cells (LUC).

Clinical Chemistry

Serum samples for clinical chemistry were collected at termination.

For the majority of treated animals the level of alkaline phosphatase (ALP) in the blood was higher than could be quantified by the analytical instrument. Dilution of the samples, below acceptable precision, did not yield any meaningful results. Results that were available for the low dose females were dramatically higher than the background range. These results were expected as the test article is an active modified ALP. In comparison with available historical reference range mean value, a decrease in mean creatinine (CRE) values in males and females from all the treatment groups was considered to be treatment-related. Data are presented in the applicant's Tables below:

TABLE 4 ITR STUDY NO. 70385

CLINICAL CHEMISTRY
SUMMARY OF MEANS
DAY 23
MALES

GROUP 1: zALP - FcD10 (10 mg/kg/day)
GROUP 2: zALP - FcD10 (30 mg/kg/day)
GROUP 3: zALP - FcD10 (90 mg/kg/day)
GROUP 4: zALP - FcD10 (180 mg/kg/day)

GROUP NO.		ALT U/L	AST U/L	ALP U/L	BILT µmol/L	CHOL mmol/L	TRIG mmol/L	GLU mmol/L	TP g/L	ALB g/L
1	MEAN	38	149	NA	NA	1.94	0.61	6.3	56	39
	SD	7.5	18.8	NA	NA	0.757	0.015	0.86	1.5	1.2
	N	3	3	0	0	3	3	3	3	3
2	MEAN	35	146	NA	2.0	1.60	0.38	5.7	56	37
	SD	0.6	36.4	NA	0.3	0.139	0.115	0.78	2.5	1.2
	N	3	3	0	2	3	3	3	3	3
3	MEAN	38	149	NA	1.9	2.09	0.65	5.0	53	35
	SD	6.7	14.5	NA	NA	0.413	0.221	0.32	0.6	1.2
	N	3	3	0	1	3	3	3	3	3
4	MEAN	41	145	NA	2.0	2.20	0.47	5.7	53	35
	SD	4.4	31.6	NA	NA	0.485	0.096	1.10	4.7	3.5
	N	3	3	0	1	3	3	3	3	3

TABLE 4 ITR STUDY NO. 70385

CLINICAL CHEMISTRY
SUMMARY OF MEANS
DAY 23
MALES

GROUP 1: zALP - FcD10 (10 mg/kg/day)
GROUP 2: zALP - FcD10 (30 mg/kg/day)
GROUP 3: zALP - FcD10 (90 mg/kg/day)
GROUP 4: zALP - FcD10 (180 mg/kg/day)

GROUP NO.		GLOB g/L	AG	UREA mmol/L	CRE µmol/L	CA mmol/L	PHOS mmol/L	NA mmol/L	K mmol/L	CL mmol/L
1	MEAN	16	2.5	4.1	14	2.50	3.12	146	4.8	102
	SD	2.1	0.40	0.62	2.3	0.012	0.165	0.6	0.46	0.0
	N	3	3	3	3	3	3	3	3	3
2	MEAN	18	2.0	3.9	14	2.47	3.25	146	4.9	104
	SD	1.5	0.12	0.61	0.6	0.123	0.460	1.2	0.75	3.1
	N	3	3	3	3	3	3	3	3	3
3	MEAN	17	2.0	4.4	12	2.53	3.18	146	4.9	103
	SD	0.6	0.12	0.79	0.6	0.031	0.270	1.0	0.35	1.5
	N	3	3	3	3	3	3	3	3	3
4	MEAN	17	2.1	5.1	14	2.48	3.11	146	4.2	104
	SD	1.5	0.15	0.49	1.2	0.040	0.284	1.0	0.26	0.6

TABLE 4 ITR STUDY NO. 70385

CLINICAL CHEMISTRY
SUMMARY OF MEANS
DAY 23
FEMALES

GROUP 1: sALP - FcD10 (10 mg/kg/day)
GROUP 2: sALP - FcD10 (30 mg/kg/day)
GROUP 3: sALP - FcD10 (90 mg/kg/day)
GROUP 4: sALP - FcD10 (180 mg/kg/day)

GROUP NO.		ALT U/L	AST U/L	ALP U/L	BILT µmol/L	CHOL mmol/L	TRIG mmol/L	GLU mmol/L	TP g/L	ALB g/L
1	MEAN	31	131	13423	1.9	1.74	0.31	6.4	57	39
	SD	7.2	9.9	1326.1	NA	0.514	0.137	1.16	2.5	2.1
	N	3	3	3	1	3	3	3	3	3
2	MEAN	31	130	NA	1.8	2.53	0.41	7.3	60	41
	SD	1.2	14.8	NA	NA	0.737	0.116	1.20	3.1	1.5
	N	3	3	0	1	3	3	3	3	3
3	MEAN	30	215	NA	1.9	1.64	0.33	5.6	56	38
	SD	7.4	142.7	NA	0.1	0.440	0.110	0.40	3.2	2.1
	N	3	3	0	3	3	3	3	3	3
4	MEAN	34	128	NA	1.9	1.85	0.42	6.3	56	39
	SD	8.5	39.1	NA	NA	0.284	0.179	0.95	2.5	1.5
	N	3	3	0	1	3	3	3	3	3

TABLE 4 ITR STUDY NO. 7038

CLINICAL CHEMISTRY
SUMMARY OF MEANS
DAY 23
FEMALES

GROUP 1: sALP - FcD10 (10 mg/kg/day)
GROUP 2: sALP - FcD10 (30 mg/kg/day)
GROUP 3: sALP - FcD10 (90 mg/kg/day)
GROUP 4: sALP - FcD10 (180 mg/kg/day)

GROUP NO.		GLOB g/L	AG	UREA mmol/L	CRE µmol/L	CA mmol/L	PHOS mmol/L	NA mmol/L	K mmol/L	CL mmol/L
1	MEAN	18	2.2	5.4	14	2.60	2.82	144	4.4	104
	SD	1.0	0.15	0.17	1.5	0.085	0.257	2.1	0.45	1.0
	N	3	3	3	3	3	3	3	3	3
2	MEAN	18	2.3	5.0	16	2.68	2.84	146	4.3	103
	SD	1.5	0.10	0.30	3.1	0.067	0.031	0.0	0.31	2.1
	N	3	3	3	3	3	3	3	3	3
3	MEAN	18	2.1	4.5	15	2.59	2.90	145	4.8	105
	SD	2.0	0.21	0.70	3.5	0.040	0.119	1.0	0.23	0.6
	N	3	3	3	3	3	3	3	3	3
4	MEAN	17	2.3	4.2	15	2.62	2.88	147	4.3	105
	SD	1.0	0.06	0.35	1.0	0.038	0.151	1.7	0.35	1.5
	N	3	3	3	3	3	3	3	3	3

Gross Pathology

Necropsies included examination of the external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, including viscera.

Macroscopic examination revealed dark focus/area and/or depressed area of the glandular stomach in 3 of 6 animals (2 males/1 females) and 4 of 6 animals (2 males/2 females) in the 90 and 180 mg/kg dose groups, respectively.

Organ Weights

Designated organs (indicated in the Table below in the histopathology section marked as “√”) from all animals were weighed at scheduled necropsy.

Asfotase alfa treatment had no effects on organ weights.

Histopathology

Adequate Battery: Yes

Peer Review: No

Histological Findings

Representative sections of the collected organs were fixed in 10% formalin except for the eye (Davidson's fixative). The testes were fixed in Bouin's solution. The list of tissues and organs collected is shown in the applicant's Table below.

ORGANS/TISSUES	Retain (•)	Weigh (√)	Examine (€)
Adrenals	•	√	€
Animal identification	•		
Aorta (thoracic)	•		€
Blood			
Bone marrow smears (3)	•		
Brain	•	√	€
Cecum	•		
Colon	•		
Epididymides	•d		
Esophagus	•		
Eyes	•a		
Femur + marrow	•		
Heart	•	√	€
Kidneys	•	√	€
Liver (2 lobes)	•	√	€
Lungs + bronchi (2 Lobes)	•bc	√	€
Lymph node, mandibular	•		
Lymph node, mesenteric	•		
Mammary gland (inguinal)	•		
Optic nerves	•a		
Ovaries	•	√	€
Pancreas	•		
Pituitary	•	√	
Prostate	•		
Rectum	•		
Salivary gland (mandibular)	•		
Sciatic nerve	•		
Seminal vesicles	•		
Skeletal muscle	•		
Skin + subcutis (inguinal)	•		

ORGANS/TISSUES	Retain (•)	Weigh (√)	Examine (€)
Small intestine, duodenum	•		
Small intestine, jejunum	•		
Small intestine, ileum	•		
Spinal cord (cervical)	•		
Spleen	•	√	€
Sternum + marrow	•		
Stomach	•		€
Testes	•d	√	€
Thymus	•	√	
Thyroid lobes + parathyroids	•	√	
Tongue	•		
Trachea	•c		
Urinary bladder	•		
Uterus	•		
Vagina	•		
Abnormal findings	√		€
Additional Tissues presented below			
)	•		
Dosing Site (Tail Vein)	•		€

a	Davidson's fluid (euthanised animal only)
b	Lungs were infused with 10% neutral buffered formalin (euthanised animal only)
c	Lungs weighed with trachea
d	Bouin's fluid (euthanised animal only)

Notes
Paired organs, weighed together
Parathyroids, mammary gland and optic nerves will only be examined histologically if present in routine sections

Treatment-related changes were observed in the stomach. Minimal to mild erosion/ulcer of the glandular stomach, occasionally associated with submucosal edema, was noted in 3 of 6 animals (2 males,1 females) and 4 of 6 (2 males,2 females) animals in the 90

and 180 mg/kg dose groups, respectively. These findings correlated to dark focus/area and/or depressed area of the glandular stomach noted grossly.

Dosing Solution Analysis

Dosing formulations were measured using three different assays: ELISA, enzyme and spectrophotometry (absorbance at 280 nm).

All dosing formulations were within 10% of their nominal concentration.

2. Study title: A 4-Week (Once Weekly) Intravenous Injection Toxicity Study of ENB-0040 in the Juvenile Albino Rat Followed by a 28-Day Recovery Period.

Study no.:	670314
Study report location:	Electronic submission
Conducting laboratory and location:	(b) (4) Quebec, Canada
Date of study initiation:	October 22, 2007
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	CON025F02, purity: 99%

Key Study Findings

ENB-0040 was administered intravenously to male and female SD rats at dose levels of 0, 2.6, 26 and 77 mg/kg once every 7 days for a period of 4 weeks followed by a 4 weeks recovery period. ENB-0040 had no effects on ophthalmology, urinalysis, bone formation marker (osteocalcin), crown-rump length, organ weights, gross pathology, and radiology or microscopy examination. ENB-0040-related clinical signs observed at all dose groups includes partly closed eyes, decreased muscle tone, lying on the side, hunched posture, cold to touch, uncoordinated movements, decreased activity, abnormal gait and/or blue, red and/or firm swollen hindpaws and/or forepaws during cage-side observations at 5, 15, 30 and/or 60 minutes post dose. These observations were transient and did not occur on non-dosing days or during the recovery period. Males in the 90 mg/kg/dose group showed slight decreases in body weight and food consumption with slightly smaller tibiae and femurs. Serum phosphorus levels were slightly increased in males in the 90 mg/kg/dose group. Elevated serum alkaline phosphatase levels were likely attributed to circulating levels of ENB-0040. Injection site reactions were transient in the 3 and 30 mg/kg/dose groups, and were not considered to be adverse. In the 90 mg/kg/dose group, this reaction was more severe and accompanied by decreases in body weight gain, reduced food consumption, and potentially some bone growth. The no observed adverse effect level (NOAEL) was 30 mg/kg/dose.

Methods

Doses:	Actual doses: 0, 2.6, 26 and 77 mg/kg (Targeted doses: 0, 3, 30 and 90 mg/kg)
Frequency of dosing:	Weekly
Route of administration:	IV
Dose volume:	5 mL/kg
Formulation/Vehicle:	A solution of 25 mM sodium PO_4 pH 7.4, 150 mM NaCl was used as the vehicle.
Species/Strain:	Crl:CD(SD) rats
Number/Sex/Group:	10/sex/dose group in the main study and 5/sex/dose group in the recovery group.
Age:	21 days old
Weight:	Males: 41 to 57g, Females: 38 to 54g
Satellite groups:	No
Unique study design:	No
Deviation from study protocol:	Minor changes in sample collection and analysis, which had no impact on the overall findings of the study.

Observations and Results

Mortality

All animals were observed twice daily for mortality.

One male in the 90 mg/kg dose group (Animal No. 4015) was found dead on study Day 25. No histopathological abnormalities were noticed. The cause of death was undetermined. All remaining animals survived until the end of the study.

Clinical Signs

All animals were observed for clinical signs on dosing days (prior to dosing) and approximately 5, 15, 30 and 60 minutes following dosing.

All animals treated with ENB-0040 showed acute clinical signs such as partly closed eyes, decreased muscle tone, lying on the side, hunched posture, cold to touch, incoordination, decreased activity, abnormal gait and/or blue, red and/or firm swollen hindpaws and/or forepaws. These changes were observed as early as Day 1 for the 30 and 90 mg/kg/dose groups from 5 minutes post dose to up to 60 minutes post dose. Red skin and/or firm swollen hindpaws and/or forepaws in males were observed infrequently on days 8 and 22 starting at 5 to 60 minutes post dose for the 3 mg/kg/dose group. The severity of swollen forepaws and/or hindpaws and/or incoordination were higher in the 30 and 90 mg/kg/dose groups on Days 1 or 22 at 15 to 30 minutes post dose. In general, the incidences and severity were less at the 60 minutes post dose observations. After each weekly dose, the clinical signs were diminished approximately

24 hours after dosing. These observations were transient and did not occur on non-dosing days or during the recovery period.

Body Weights

Individual body weights were measured once during the acclimation period (at randomization) and weekly throughout the study.

There were no significant effects on body weight during the study. However, a trend for slight decrease (-7.4, -10.4 and -12.1%) of absolute body weight gains was noted in males treated at 3, 30 and 90 mg/kg/doses, respectively between Day 29 and until the end of the recovery period. The effect on bone size (femur and tibia) correlated with decrease in body weights. Body weight data are presented in the applicant's Table below.

Table 3 Group Mean Body Weights (g)

Males

Group 1 - Vehicle Control
Group 2 - ENB-0040 3 mg/kg/dose
Group 3 - ENB-0040 30 mg/kg/dose
Group 4 - ENB-0040 90 mg/kg/dose

Group	Summary Information	Random	Day 1 (21 PP)	8	15	22	29	36	43	50	56
1	Mean	35.05	49.8	92.1	151.3	213.6	232.5	348.8	418.6	468.8	493.2
	SD	3.10	4.9	7.7	13.1	20.3	20.2	30.6	38.9	45.6	46.5
	N	15	15	15	15	15	15	5	5	5	5
2	Mean	35.25	50.5	94.8	154.3	214.1	232.0	332.0	394.0	439.2	466.4
	SD	2.34	3.1	4.7	6.4	10.1	12.8	16.4	20.0	21.7	28.6
	N	15	15	15	15	15	15	5	5	5	5
3	Mean	34.69	49.7	92.3	148.9	208.3	226.7	325.0	387.8	431.6	454.6
	SD	2.30	3.0	6.1	10.1	11.9	12.5	19.3	19.3	23.1	27.9
	N	15	15	15	15	15	15	5	5	5	5
4	Mean	35.12	49.3	91.8	148.5	206.4	221.5	313.3	375.3	415.5	438.0
	SD	3.15	4.5	7.7	15.9	24.9	27.7	32.9	30.5	39.1	32.5
	N	15	15	15	15	15	14	4	4	4	4

Significantly different from control group (group 1) value: A - P≤0.05 B - P≤0.01 C - P≤0.001 (Dunnett)
D - P≤0.05 E - P≤0.01 F - P≤0.001 (Dunn)

Table 3 Group Mean Body Weights (g)

Females

Group 1 - Vehicle Control
Group 2 - ENB-0040 3 mg/kg/dose
Group 3 - ENB-0040 30 mg/kg/dose
Group 4 - ENB-0040 90 mg/kg/dose

Group	Summary Information	Day Random	1 (21 PP)	8	15	22	29	36	43	50	56
1	Mean	33.85	47.7	85.5	129.5	157.3	159.9	222.6	250.2	264.4	273.4
	SD	2.94	3.7	6.7	10.8	12.9	14.4	11.4	9.4	11.2	6.3
	N	15	15	15	15	15	15	5	5	5	5
2	Mean	32.76	46.6	84.4	128.4	156.9	160.3	211.2	237.2	250.8	262.6
	SD	3.44	4.4	6.9	12.6	15.9	16.3	17.5	21.7	26.7	20.7
	N	15	15	15	15	15	15	5	5	5	5
3	Mean	33.35	47.7	85.9	131.7	161.2	163.3	223.8	255.0	272.8	285.6
	SD	2.94	3.6	7.1	10.9	14.2	15.5	18.4	24.2	26.4	30.7
	N	15	15	15	15	15	15	5	5	5	5
4	Mean	33.70	47.4	85.4	128.9	160.6	162.0	223.2	246.4	258.2	270.4
	SD	2.83	3.9	6.0	9.3	13.2	15.3	16.7	17.6	24.3	27.4
	N	15	15	15	15	15	15	5	5	5	5

Significantly different from control group (group 1) value: A - $P \leq 0.05$ B - $P \leq 0.01$ C - $P \leq 0.001$ (Dunnett)
D - $P \leq 0.05$ E - $P \leq 0.01$ F - $P \leq 0.001$ (Dunn)

Feed Consumption

Food consumption was measured weekly from weaning. Food consumption was recorded on a cage basis during Week 1 post-weaning and individually thereafter.

There were no significant effects on food consumption during the study. However, throughout the study, a trend for slightly decreased food consumption was noted in males in the 3, 30 and 90 mg/kg/dose groups, which was correlated with the decreased body weights.

Ophthalmoscopy

Ophthalmoscopy examination was conducted on all main study and recovery animals at the end of the treatment period and the end of the recovery period.

No ocular abnormality was observed.

Hematology

Hematology and serum chemistry were evaluated in samples collected from all animals at necropsy at the end of the treatment phase and the recovery phase.

There were no significant test article-related alterations in hematology and coagulation parameters. However, animals in the 90 mg/kg/dose group showed slight decreases in absolute neutrophils (up to -26%), monocytes (up to -11%), and/or eosinophils (up to -23%) compared to the control group. Additionally, slight increases in lymphocytes (up to 12%), platelets (up to 14%) and absolute reticulocytes (up to 23%) were observed compared to the control group. At the end of the recovery period, these slight changes were still generally apparent in the animals treated with 90 mg/kg.

Clinical Chemistry

Serum samples were collected at termination for evaluation of clinical chemistry.

At the end of the treatment, blood samples were also collected to determine bone turnover markers (osteocalcin and C-telopeptide).

There were no test article-related alterations in serum chemistry parameters. However, there were drug-related increases in alkaline phosphatase of up to 23.2, 141.1 and 331.9% in 3, 30 and 90 mg/kg dose groups, respectively, compared to controls. Slight statistically significant increases in phosphorus (15.4%) was observed in males in the 90 mg/kg dose group during Week 4, associated with non-significant increases in serum total calcium (3.2%). At the end of the recovery period, these changes returned to control values.

The bone turnover markers (osteocalcin and C-telopeptide) remained unchanged in ENB-0040 treated animals as compared to controls.

ENB-0040 concentrations in blood serum were similar on Days 16 and 23. Serum drug levels were not proportional to dose. At 30 and 90 mg/kg dose, the serum levels of males were slightly higher than the females. Data are presented in the applicant's Table below.

Groups	Nominal Dose Level (mg/kg/dose)	Nominal Concentration (mg/mL)	Mean Serum Levels (µg/mL) (24 hours post dose)		Mean Alkaline Phosphatase (U/L)	
			Males Day 16/23	Females Day 16/23	Males (Day 29)	Females (Day 29)
1/ Vehicle Control	0	0	0.27/0.23	0.22/0.20	221.2	153.4
2/ Low Dose	3	0.6	0.95/0.96	0.82/0.77	238.5	189.0
3/ Mid Dose	30	6	4.7/4.4	4.1/3.6	525.4	369.9
4/ High Dose	90	18	7.9/8.8	6.9/6.7	955.3	656.3

Urinalysis

Urine samples were collected from each main and recovery animal on study Day 29 and at the end of the recovery phase from each recovery animal.

The urinalysis parameters were unaffected by the administration of ENB-0040.

Gross Pathology

Necropsies included, examination of the external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, including viscera.

There were no ENB-0040-related macroscopic findings in rats at any dose levels at the end of the dosing and recovery periods.

Organ Weights

For each main study and recovery animals euthanized at scheduled necropsy, the following organs were dissected and weighed:

adrenal glands
brain
heart
kidneys
liver
ovaries/testes
pituitary
prostate
spleen
thymus
thyroid lobes and parathyroid glands
uterus

At the end of the treatment period, there were no ENB-0040-related effects on organ weights expressed either as absolute values or ratios to body or brain weights. At the end of the recovery phase, significant inter-group differences that reached statistical significance were seen for a few organs. They included a decrease in liver weight, expressed as absolute and ratio to brain weight, for males in the 30 and 90 mg/kg/dose groups and an increase of brain weight relative to body weight for males treated with 90 mg/kg/dose. Due to a lack of histopathological findings, a lack of consistency with respect to gender, and the small number of animals, these changes were not considered to be an adverse.

Histopathology

Adequate Battery: Yes

Peer Review: No

Histological Findings:

Representative sections of collected organs were fixed in 10% formalin except for the eye (Davidson's fixative) and testis (Bouin's solution). The list of tissues and organs collected is shown below as provided by the applicant.

aorta (entire, thoraco-abdominal)
bone and marrow (sternum)
brain (cerebrum, cerebellum, midbrain and medulla oblongata)
cecum
colon
duodenum
epididymides
esophagus
eyes
harderian glands
heart (including aortic arch)
ileum
injection site(s)
jejunum
joint, femoro-tibial (left)
joint, tarsus (left)
kidneys
lacrimal glands
liver (sample of 2 lobes)
lungs
lymph nodes (mandibular, unilateral; mesenteric)
mammary gland (inguinal)
optic nerves
ovaries
pancreas
pituitary
prostate
rectum
salivary gland
sciatic nerve
seminal vesicles
skeletal muscle
skin (inguinal)
spinal cord (cervical)
spleen
stomach
tendon (Achilles, left)
testes
thymus
thyroid lobes (and parathyroids)
tongue
trachea
urinary bladder
uterus (horns, body and cervix)
vagina

No ENB-0040-related histopathological findings were noted at the end of the study or the recovery period.

Anti-drug antibodies determination:

Blood samples were collected from the jugular vein from all main study and recovery animals on study Day -1, week 5 and at termination (via abdominal aorta).

One animal had anti-drug antibodies before exposure to ENB-0040 and 2 animals from the control group had anti-drug antibodies at Day 29. Eight animals presented anti-drug antibodies after exposure to ENB-0040. Overall, the levels of anti-drug antibodies present in positive animals were low (with titers ranging from 5 to 11). Of all the animals presenting anti-drug antibodies at Day 28, no animal was positive after recovery period (Day 57). There was 1 high dose female showing antibody titers after the recovery period (Day 57) which were not present on Days -1 and 28.

Bone Mineral Density Measurement:

Bone mineral density (BMD) and bone mineral content (BMC) were determined by dual energy X-ray absorptiometry (DXA). Single scans were performed on study Day -1 (including 1 spare litter) and Day 28 from the main study and recovery animals and on study Days 14 and 56 from the recovery animals.

The whole body analysis showed that there were no statistically significant differences in DXA bone densitometry parameters between controls and treated groups during the course of the study. At the end of the recovery period, slight decreases (4-16%) in area and/or bone mineral content of treated males relative to controls were noted. DXA analysis of lumbar showed no statistically significant effect on bone densitometry parameters. DXA analysis of the femur of females did not show any changes in densitometry parameters. However, femurs of males showed slight (not dose dependent) decreases in area and bone mineral content compared to controls during the course of the study. The percent differences for treated animals relative to controls ranged between 1-11% and 15-23%, respectively at the end of treatment and recovery periods. These results parallel the body weight effect in the males seen at the end of recovery. Data are presented in the applicant's Tables below.

Table 11 Group Mean Bone Densitometry Values by Dual Energy X-Ray Absorptiometry

Main Study
Day 28
Males

Group 1 - Vehicle Control
Group 2 - ENB-0040 3 mg/kg/dose
Group 3 - ENB-0040 30 mg/kg/dose
Group 4 - ENB-0040 90 mg/kg/dose

		Whole Body			Lumbar Spine (L1-L4)			Right Femur - Global		
Group	Summary Information	Area cm^2	BMC g	BMD g/cm^2	Area cm^2	BMC g	BMD g/cm^2	Area cm^2	BMC g	BMD g/cm^2
1	Mean	54.613	6.603	0.1209	1.585	0.219	0.1382	1.140	0.185	0.1622
	SD	4.051	0.562	0.0061	0.109	0.026	0.0094	0.074	0.018	0.0094
	N	15	15	15	15	15	15	15	15	15
2	Mean	53.768	6.503	0.1208	1.578	0.223	0.1418	1.135	0.185	0.1629
	SD	2.473	0.557	0.0062	0.057	0.015	0.0107	0.068	0.014	0.0077
	N	15	15	15	15	15	15	15	15	15
3	Mean	53.298	6.287	0.1179	1.560	0.219	0.1402	1.119	0.181	0.1615
	SD	2.761	0.519	0.0064	0.122	0.028	0.0102	0.050	0.019	0.0124
	N	15	15	15	15	15	15	15	15	15
4	Mean	51.681	6.195	0.1197	1.530	0.210	0.1365	1.117	0.178	0.1597
	SD	3.831	0.630	0.0057	0.114	0.031	0.0132	0.105	0.021	0.0107
	N	13	13	13	13	13	13	13	13	13

Table 11 Group Mean Bone Densitometry Values by Dual Energy X-Ray Absorptiometry

Recovery Period
Day 56
Males

Group 1 - Vehicle Control
Group 2 - ENB-0040 3 mg/kg/dose
Group 3 - ENB-0040 30 mg/kg/dose
Group 4 - ENB-0040 90 mg/kg/dose

		Whole Body			Lumbar Spine (L1-L4)			Right Femur - Global		
Group	Summary Information	Area cm^2	BMC g	BMD g/cm^2	Area cm^2	BMC g	BMD g/cm^2	Area cm^2	BMC g	BMD g/cm^2
1	Mean	87.742	13.620	0.1551	2.292	0.531	0.2319	1.818	0.487	0.2678
	SD	7.867	1.344	0.0023	0.144	0.031	0.0100	0.082	0.024	0.0042
	N	5	5	5	5	5	5	5	5	5
2	Mean	81.722	12.649	0.1547	2.272	0.526	0.2313	1.726	0.458	0.2653
	SD	3.199	0.789	0.0047	0.071	0.032	0.0103	0.043	0.016	0.0076
	N	5	5	5	5	5	5	5	5	5
3	Mean	79.077	12.141	0.154	2.269	0.517	0.228	1.735	0.460	0.2647
	SD	2.496	0.481	0.007	0.153	0.055	0.018	0.068	0.044	0.0184
	N	5	5	5	5	5	5	5	5	5
4	Mean	79.062	11.865	0.1500	2.242	0.490	0.2179	1.707	0.432	0.2531
	SD	5.073	0.835	0.0011	0.167	0.074	0.0184	0.083	0.018	0.0048
	N	4	4	4	4	4	4	4	4	4

Dosing Solution Analysis

Duplicate samples (0.2 mL) were collected from each dose formulation including control on the day of each preparation for concentration verification.

All results were within 10% of their nominal concentration.

3. Study title: A 26-week Intravenous Injection Toxicity Study of ENB-0040 in the Juvenile Albino Rat Followed by a 28-day Recovery Period.

Study no.:	670315
Study report location:	Electronic submission
Conducting laboratory and location:	(b) (4) Quebec, Canada.
Date of study initiation:	August 13, 2008
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	PUR012G01, FIL094G02, PUR012G02; purity: 98.7 to 99.4%

Key Study Findings

ENB-0040 was administered intravenously to male and female SD rats at 1, 3 and 13 mg/kg daily for a period of 26 weeks followed by a 28-day recovery period. ENB-0040-related transient clinical signs include red and/or firm swollen hindpaws and/or forepaws and swollen muzzle observed up to 1 hour post dose throughout the dosing period and most consistently in the 13 mg/kg/day group. The transient clinical signs were completely reversed during the recovery period. There were no toxicologically meaningful effects on body weight, food consumption, ophthalmology, hematology,

clinical chemistry and urinalysis. ENB-0040 had no effects on bone turnover markers, bone densitometry or bone geometry parameters (assessed by DXA and pQCT), behavioral performance and physical development of the animals. Similarly, ENB-0040 did not have any effects on estrous cycle, organ weights and body weight, macroscopic and microscopic findings, ectopic calcification, and male reproductive assessments. All treated animals showed elevated serum alkaline phosphatase levels, which was related to the circulating levels of ENB-0040. The NOAEL was considered to be 13 mg/kg/day with corresponding AUC_{last} and C_{max} values of 406 mg.h/L and 331 mg/L for both male and female combined, respectively.

Methods

Doses:	0, 1, 3 and 13 mg/kg
Frequency of dosing:	Once daily
Route of administration:	IV
Dose volume:	5 mL/kg
Formulation/Vehicle:	A solution of 25 mM sodium PO_4 pH 7.4, 150 mM NaCl was used as the vehicle.
Species/Strain:	Sprague-Dawley Crl:CD(SD) rats
Number/Sex/Group:	Main study 12/sex/dose group; TK: 3/sex in vehicle and 9/sex in low, mid and high dose groups
Age:	21 days old
Weight:	Males: 41-59g; Females: 38-56g
Satellite groups:	Yes, TK: 3 animals/sex in the vehicle and 9 animals/sex in low, mid and high dose groups
Unique study design:	No
Deviation from study protocol:	Minor changes in sample collection and analysis, which had no impact on the overall findings of the study.

Observations and Results

Mortality

All animals were observed twice daily for mortality.

During the dosing period, one control male (No. 1008 on Day 23), one male treated with 1 mg/kg/day (No. 2009 on Day 62), one female treated with 3 mg/kg/day (No. 3506 on Day 62) and one male given 13 mg/kg/day (No. 4009 on Day 89) dose were pre-terminally euthanized due to the deteriorating condition of their injection sites. Histopathological examination showed perivascular/vascular inflammation and/or perivascular necrosis at the injection site, which were most likely due to injection procedure and not related to ENB-0040. Additionally, two control females (No. 1513 on Day 96 and No. 1522 on Day 45) and two females treated with 1 mg/kg/day (No. 2510 on Day 112 and No. 2515 on Day 22) were found dead. The cause of death remained undetermined due to lack of clinical sigs and pathological findings. During the recovery

phase, one female given 3 mg/kg/day was found dead (No. 3516 on Day 211). Hemorrhage present in the skeletal muscle of the axillary region was regarded as the cause of death and was likely attributed to the experimental procedure.

Clinical Signs

The animals were observed for clinical signs twice daily prior to dosing and 4 times daily on the dosing days (before dosing, and approximately15 min, 1 and 4 to 6 hours after the end of dosing).

ENB-0040-related acute clinical signs were observed in all treated groups. These included red and/or firm swollen hindpaws and/or forepaws and swollen muzzle throughout the dosing period, most consistently in the 13 mg/kg/day dose group. Partially closed eyes were noted in all treated groups but were more pronounced in the 13 mg/kg/day dose group. Above clinical sigs were generally observed as early as Day 1 at 15 minutes to 1 hour following dosing. In general, the incidences and severity were greater in the 3 and 13 mg/kg/day groups at 15 minutes and 1 hour post dosing compared to the vehicle treated animals. These observations were transient during the dosing period and did not occur at all during the recovery period.

Body Weights

Individual body weights were measured for all pups once during the acclimation period (at randomization), twice a week starting on Day 21 *post-partum* (Day 1) and weekly starting on Day 21 of the treatment period. In addition, each main study/recovery animal was weighed (fasted) before scheduled necropsy.

No test article-related changes were noted in the body weights.

Feed Consumption

Food consumption was measured weekly from weaning. Food consumption was recorded on a cage basis during Week 1 post-weaning and individually thereafter.

Food consumption was unaffected by the test article administration.

Ophthalmoscopy

Ophthalmoscopy examination was conducted at the end of the treatment period.

No ocular abnormality was observed.

Hematology

Hematology and serum chemistry were evaluated in samples collected from all animals at necropsy during the treatment and recovery phases.
There were no test article-related alterations in hematology parameters.

Clinical Chemistry

Serum samples for clinical chemistry evaluations were collected at necropsy.

There were no test article-related alterations in serum chemistry parameters at any doses. However, ENB-0040 was associated with statistically significant dose-related increases in alkaline phosphatase of up to 33, 78 and 295-fold in the 1, 3 and 13 mg/kg/day groups, respectively, during Week 13 and up to 13, 33 and 126-fold in the 1, 3 and 13 mg/kg/day groups, respectively, at the end of the dosing period, relative to controls. Considering the nature of ENB-0040 (recombinant soluble form of tissue nonspecific alkaline phosphatase), these increases were considered attributable to circulating levels of ENB-0040. At the end of the recovery period, these values returned to normal reference ranges. ALP values are presented in the Table below, adopted from the applicant's submission.

Alkaline Phosphatase (U/L)				
ENB-0040 (mg/kg/day		**13 weeks**	**26 weeks**	**Recovery period (Week 30)**
0	**Male**	131.6 ±28.5	61.9±17.3	73.3±38.2
	Female	90.4±37.6	34.1±12.3	25.4±8.1
1	**Male**	3999.4±433.2*	732.2±152.7*	67.3±20.2
	Female	2979.2±409.9*	455.7±82.7	39.4±11.4
3	**Male**	9147±2058**	1910.4±230**	69.7±12.4
	Female	7075.6±1030.2**	1141.4±204.5**	36.8±15.9
13	**Male**	36617±3839.1**	6556.4±589.7	103.3±26.9
	Female	26688.2±3506.8**	4309.8±794.4**	37.8±12.5

*=p≤0.05; **=p≤0.001

Urinalysis

Urinalysis was conducted at the end of dosing and recovery periods.

No ENB-0040-related changes in urinary parameters were observed.

Gross Pathology

Necropsies included, examination of the external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, including viscera.

There were no ENB-0040-related macroscopic findings at the end of the dosing and recovery periods.

Organ Weights

For each main study and recovery animal euthanized at scheduled necropsy, the following organs were dissected and weighed:

adrenal glands
brain
heart
kidneys
liver
ovaries/testes
pituitary
prostate
spleen
thymus
thyroid lobes and parathyroid glands
uterus

There were no ENB-0040-related effects on organ weights at any time. A statistically significant decrease in liver weights (only when expressed as ratio to body weight) was noted in females received 1 mg/kg/day at the end of the recovery period.

Histopathology

Adequate Battery: Yes

Peer Review: No

Histological Findings:

Representative sections of collected organs were fixed in 10% formalin except for the eye (Davidson's fixative) and testis (Bouin's solution). The list of tissues and organs collected is shown below.

aorta (entire, thoraco-abdominal)
bone and marrow (sternum)
brain (cerebrum, cerebellum, midbrain and medulla oblongata)
cecum
colon
duodenum
epididymides
esophagus
eyes
harderian glands
heart (including aortic arch)
ileum
injection site(s)
jejunum
joint, femoro-tibial (left)
joint, tarsus (left)
kidneys
lacrimal glands
liver (sample of 2 lobes)
lungs
lymph nodes (mandibular, unilateral; mesenteric)
mammary gland (inguinal)
optic nerves
ovaries
pancreas
pituitary
prostate
rectum
salivary gland
sciatic nerve
seminal vesicles
skeletal muscle
skin (inguinal)
spinal cord (cervical)
spleen
stomach
tendon (Achilles, left)
testes
thymus
thyroid lobes (and parathyroids)
tongue
trachea
urinary bladder
uterus (horns, body and cervix)
vagina

There were no ENB-0040-related histopathological findings during the main study period. Perivascular/vascular inflammation, generally graded minimal to slight in severity, was seen at the injection sites of both vehicle and ENB-0040-treated rats, and was considered to be procedure related.

There were no ENB-0040-related histopathological findings during the recovery period. In the liver, focal hepatocellular vacuolation, graded minimal to slight in severity, was noted in one male and three females treated with the 13 mg/kg/day dose and in one female in the 3 mg/kg/day group. This observation was incidental as it was present in some control animals and was not uncommon in laboratory rats.

Special Evaluation

Bone Mineral Density Measurement:

Dual energy X-ray absorptiometry (DXA) scans were used to measure bone mineral density (BMD) and bone mineral content (BMC), and the area of the whole body, lumbar spine (L1-L4) and of the whole right femur from main study and recovery animals.

Single scans were acquired once on Day -1, during Weeks 4, 13 and 26 (end of the treatment period) and again at the end of the recovery period (Week 30).

Peripheral quantitative computed tomography (pQCT) was used to measure bone density on the right proximal tibia of main study and recovery animals once on Day -1 and during Weeks 4, 13 and 26 (end of the treatment period) and again at the end of the recovery period (Week 30/31).

No statistically significant or biologically meaningful effects on whole body bone densitometry parameters (area, BMC and BMD) were noted in ENB-0040-treated animals (both males and females) compared to vehicle controls over the study period (treatment and recovery). At the end of the treatment or recovery period, ENB-0040 had no statistically significant or biologically meaningful effects on bone densitometry parameters of lumbar spine (L1-L4 combined) or femur in all treated animals compared to vehicle controls.

Peripheral quantitative computed tomography (pQCT) results showed no treatment related effects at the tibia metaphysis for either males or females over the course of study.

Male Reproductive Assessments

Male animals from the main study and recovery groups were euthanized at the end of the study and left cauda and vas deferens were removed for male reproductive assessments. Following parameters were determined:

- Sperm motility
- Sperm counts and
- Sperm morphology.

In the main study, there were no ENB-0040-related effects on sperm motility, sperm concentration and sperm morphology. The group mean values of the sperm motility and sperm concentration were slightly decreased in animals given ENB-0040 at 13 mg/kg/day compared to the control group. These decreases did not reach statistical significance and there were no microscopic findings in the testis or epididymis correlating with these differences.

At end of the recovery study, there were no ENB-0040-related effects upon sperm motility, sperm concentration or on sperm morphology.

Estrous cycle

The estrous cycles of all females from the main study were assessed during Weeks 8, 9 and 10 of the treatment period (Weeks 11 to 13 *post-partum*) by examination of the vaginal lavage.

ENB-0040 had no effects on estrous cycles.

Behavioral Performance

Main and recovery study animals were tested for functional observational battery (FOB) before daily dosing during Weeks 14 and 26 of the treatment period. Animals were placed in a 2 foot square Plexiglas placed on a raised platform. Following FOB tests were performed:

Observations in Home Cage:
body position
convulsions, twitches and tremors
bizarre/stereotypic behavior

Removal from Home Cage:
ease of removal
vocalization

Observations in Arena:
rearing
gait
bizarre/stereotypic behavior
palpebral closure
piloerection
tremors, twitches, convulsions
olfactory response
locomotor activity level
arousal
grooming
defecation/urination

Handling Observations:
lacrimation
pupil size
salivation
urinary staining
diarrhea
body tone
extensor thrust
respiratory rate/pattern
corneal reflex
pinna reflex
toe pinch
tail pinch
visual placing

On Surface:
auricular startle
air righting reflex

On Top of Box:
positional passivity

Animals were also tested for grip strength, hind limb splay (landing foot spread), body temperature, motor activity, auditory startle habituation and water maize (Cincinnati).

There were no ENB-0040-related effects on the functional observational battery parameters during the study. The motor activity, auditory startle habituation response and water maize were unaffected by administration of ENB-0040.

Toxicokinetics

For toxicokinetic analysis, blood samples (about 0.3 mL) were collected on Day 1 at 0.5h post treatment, during Week 4 at 5, 30 and 60 minutes post treatment from 1, 3 and 13 mg/kg/day dose groups (3 animals/sex/time point), and during Weeks 4, 17 and 26 at 5 minutes post treatment from the control group 1 (3 animals/sex). Additionally, samples were collected from toxicokinetic animals from 1, 3 and 13 mg/kg/day dose groups (3 animals/sex/time point) on the following occasions: during Week 17 at 5, 10, 30, 60 minutes and 2, 4, 5, 6 and 8 hours post treatment and on Day 182 at 5, 10, 30, 60 minutes and 5, 12, 24, 48 and 72 hours post treatment.

For all dose levels, the $t_{1/2}$ decreased from Week 1 (First Dose) to Week 4 and then significantly increased at Week 17. On Week 26 (Last Dose), the $t_{1/2}$ decreased to reach similar range as the Week 1 (First Dose) values. CL increased from Week 1 (First Dose) to Week 4 and then significantly decreased at Week 17 (except for 1 mg/kg). On Week 26 (Last Dose), CL displayed a noticeable increase across doses compared to Week 1(First Dose). Data are presented in the applicant's Table below.

Text Table 9 **Mean (± CV) Week 1 (First Dose), Week 4, Week 17 and Week 26 TK Compartmental Parameters of ENB-0040 Serum**

Dose	Time	AUC (mg*h/L)	CL (L/h)	C_{max} (mg/L)	$t_{1/2}$ (h)	V_{ss} (L)	BW (kg)
1 mg/kg	Week 1 (First Dose)	26.0 (3.4)	0.00202 (6.3)	6.07 (33.7)	18.3 (57.3)	0.0537 (61.3)	0.0524 (6.4)
	Week 4	30.7 (7.3)	0.00616 (11.9)	8.01 (15.1)	15.3 (7.1)	0.136 (12.8)	0.189 (15.4)
	Week 17	75.8 (47.7)	0.0062 (29.9)	8.83 (40.1)	34.0 (39.4)	0.280 (26.7)	0.427 (32.0)
	Week 26 (Last Dose)	16.1 (31.3)	0.0318 (35.1)	8.37 (62.9)	7.99 (36.1)	0.335 (30.4)	0.483 (33.8)
3 mg/kg	Week 1 (First Dose)	58.4 (33.5)	0.00126 (60.4)	19.3 (27.8)	32.1 (47.5)	0.0474 (75.9)	0.0491 (9.1)
	Week 4	88.6 (9.0)	0.00631 (14.5)	25.5 (29.5)	14.8 (11.4)	0.134 (11.2)	0.185 (13.1)
	Week 17	284 (48.1)	0.00502 (30.5)	42.7 (44.1)	39.6 (40.8)	0.265 (27.4)	0.433 (32.1)
	Week 26 (Last Dose)	61.9 38.7)	0.0262 (23.3)	42.3 (46.2)	8.85 (39.3)	0.312 (27.8)	0.510 (30.5)
13 mg/kg	Week 1 (First Dose)	364 (3.7)	0.00175 (9.5)	115 (11.2)	16.0 (2.5)	0.0402 (7.8)	0.0488 (7.9)
	Week 4	401 (10.6)	0.00607 (13.1)	147 (30.9)	14.7 (10.3)	0.128 (8.7)	0.186 (11.0)
	Week 17	1370 (33.6)	0.00434 (23.1)	302 (27.5)	41.7 (29.4)	0.252 (25.2)	0.441 (29.8)
	Week 26 (Last Dose)	379 (26.9)	0.0178 (27.6)	281 (35.2)	11.6 (24.4)	0.289 (26.3)	0.509 (32.4)

* $AUC_{0\text{-}24}$ except on Day 1 where AUC_{∞} was calculated,
Values represent mean (%CV),

Following IV administration of 1, 3 and 13 mg/kg/day doses on Week 26 (last dose), systemic clearance (CL) was 0.0814, 0.0702 and 0.0393 L/h/kg, respectively and the elimination half-life was 29.6, 31.0 and 42.7 h respectively. CL decreased with increasing dose. C_{max} (genders combined) was 10.7, 45.4 and 331 mg/L for Groups 2-4, respectively, while AUC_{last} (genders combined) was 17.2, 60.0 and 406 mg.h/L for Groups 2-4, respectively. C_{max} and AUC_{last} increased in a more than dose-proportional manner with increasing dose due to the decreased CL. V_{ss} also decreased as a function of dose, from 1.53 L/kg in Group 2 to 0.506 L/kg in Group 4. Data are presented in the applicant's Table below.

Text Table 8 NCA TK Parameters of ENB-0040 Serum (Week 26 (Last Dose))

Group	AUC_{0-24} (mg·h/L)	AUC_{last} (mg·h/L)	±	SE	CL (L/h/kg)	C_{max} (mg/L)	±	SE	$t_{1/2}$ (h)	t_{max} (h)	V_{ss} (L/kg)
Treatment = ENB-0040 (Group 2 :1 mg/kg)											
Serum ENB-0040											
2- Combined	12.3	17.2	±	1.45	0.0814	10.7	±	0.933	29.6	0.08	1.53
2- Female	9.78	14.4	±	2.02	0.102	8.85	±	0.802	NC	0.08	NC
2- Male	14.8	20.0	±	1.17	0.0675	12.5	±	0.574	42.0	0.08	1.34
Treatment = ENB-0040 (Group 3 :3 mg/kg)											
Serum ENB-0040											
3- Combined	42.8	60.0	±	4.54	0.0702	45.4	±	4.79	31.0	0.08	1.35
3- Female	31.7	45.2	±	3.66	0.0946	35.0	±	0.299	29.0	0.08	1.85
3- Male	53.8	74.8	±	3.61	0.0557	55.7	±	2.80	32.4	0.08	1.06
Treatment = ENB-0040 (Group 4 :13 mg/kg)											
Serum ENB-0040											
4- Combined	331	406	±	36.5	0.0393	331	±	13.0	42.7	0.08	0.506
4- Female	239	297	±	17.2	0.0543	305	±	13.2	40.0	0.08	0.694
4- Male	422	516	±	42.9	0.0308	357	±	4.47	44.5	0.08	0.399

Nominal Times instead of Actual Times

NC = Not calculated

4. Study title: ENB-0040: A 4-Week Intravenous Injection Toxicity Study in Juvenile Cynomolgus Monkeys Followed by A 28-Day Recovery Period.

Study no.:	1007-1503
Study report location:	Electronic submission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	November 9, 2007
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	PURE A2 (1 + 2), purity: 99%

Key Study Findings

ENB-0040 was administered intravenously to male and female juvenile Cynomolgus monkeys at dose levels of 0, 5, 15 and 45 mg/kg once every 7 days for a period of 4 weeks followed by a 28-day recovery period. There were no mortalities. Body weight, food intake, hematology, blood chemistry, urinalysis, ophthalmology, electrocardiography, organ weight, and macroscopic and microscopic parameters were unaffected in all dose groups. A slight to pronounced dose related increase in alkaline phosphatase was observed in all test article treated animals throughout the treatment period, and was due to the presence of the drug in the bloodstream of the animals after each dose. The NOAEL was considered to be 45 mg/kg with corresponding AUC_{last} and C_{max} values on Day 22 were 2670 mg.h/L and 691 mg/L, respectively.

Methods

Doses:	0, 5, 15 and 45 mg/kg
Frequency of dosing:	Once weekly
Route of administration:	IV
Dose volume:	4 mL/kg
Formulation/Vehicle:	A solution of 25 mM sodium PO_4 pH 7.4, 150 mM NaCl was used as the vehicle.
Species/Strain:	Cynomolgus monkeys
Number/Sex/Group:	3/sex/dose in main study and 2/sex/dose in recovery groups
Age:	11 and 13 months old
Weight:	Males: 1.2 to1.8 kg; Females: 1.2 to 1.9 kg
Satellite groups:	None
Unique study design:	None
Deviation from study protocol:	No.

Observations and Results

Mortality

All animals were observed twice daily for mortality.

All animals survived until the end of the study.

Clinical Signs

Cage-side observations were performed at least once daily. A detailed clinical examination was performed once at arrival, once during the week prior to initiation of dosing and weekly thereafter. A detailed clinical examination was also performed on the day of necropsy.

No adverse treatment-related clinical signs were observed during the study.

Radiographic Assessment of Bone Development

Any treatment-related effect on bone development was evaluated once during the pre-treatment and at the end of the treatment and recovery periods using X-Ray imaging. The following parameters were evaluated:

- Calvarium (sagittal or coronal sutures) to assess potential abnormalities of suture closure
- Mandible for teeth development
- Lumbar spine to assess potential vertebral fusion
- Femorotibial joint and tarsus to assess ectopic mineralization of joints

Body measurements were performed once during the pre-treatment period and at the end of the treatment and recovery periods. These include measurements of the circumference of the cranium, approximate length of the humerus, forearm (elbow to the wrist), tibia and pelvic limb (from the hip to the ankle).

Bone development in juvenile monkeys was not affected by ENB-0040 at any dose levels. At the end of the treatment and recovery periods, there were no differences for cranial circumference, humerus, forearm, tibia or pelvic limb lengths.

Body Weights

All animals were weighed once at arrival, once before randomization, once during the week preceding the start of treatment, one day before the start of dosing and weekly thereafter.

No test article-related changes were noted in the body weight in either the dosing or the recovery phases.

Feed Consumption

Food consumption was recorded daily.

Food consumption was unaffected by test article administration.

Ophthalmoscopy

Ophthalmoscopy examination was conducted once prior to the initiation of treatment and at the end of the treatment (Days 27 or 28).

No ocular abnormality was observed.

ECG

Electrocardiograms (leads I, II and III, and augmented leads aVR, aVL and aVF) was performed for all animals once during the pre-treatment period and at the end of the treatment period (Day 26 or 27).

No treatment-related changes in the ECG parameters were observed in any animals at any dose levels.

Hematology

Blood samples were collected from femoral vein for the assessment of hematology, coagulation and clinical chemistry parameters once during the pre-treatment period, at the end of week 4 of the study (Day 29), and at the end of the recovery period.
There were no test article-related alterations in hematology and coagulation parameters either in pre-dosing, dosing and recovery phases.

Clinical Chemistry

Blood samples for clinical chemistry evaluation were collected near the end of Weeks 1, 2, 3, 5, 6, 7. For these evaluations, the animals were deprived of food overnight.

There were no test article-related alterations in serum chemistry parameters at any doses. However, a slight to pronounced dose related increase in alkaline phosphatase (ALP) was observed in all treated animals throughout the treatment period (often achieving statistical significance). The exception to this was for females in Week 4, when the increases were apparent, but did not exhibit the dose-related trend. Alkaline phosphatase levels were generally more comparable to control values by the end of the recovery period. Since the test article is recombinant soluble form of tissue nonspecific alkaline phosphatase, this increase was due to the presence of the drug in the bloodstream of the animals after each dose, and thus the increases were considered to be non-adverse.

Bone Mineral Density (BMD) Measurement and Biochemical Markers of Bone Turnover

BMD was measured ex vivo by Dual Energy X-ray Absorptiometry (DXA) and Peripheral Quantitative Computed Tomography (pQCT) at the end of the study in treated and control animals.

A blood sample (approximately 1 mL) was collected from all main study and recovery animals in the morning (between 8h30 to 9h30) prior to initiation of treatment (Day 1), and on Days 29 and 57 for the evaluation of the markers of bone turnover (osteocalcin and C-telopeptide).

There were no statistically significant differences for bone densitometry (pQCT and DXA) or serum bone turnover markers between control and treatment groups.

Urinalysis

Urinalysis was conducted at the end of dosing and recovery periods.

No ENB-0040-related changes in urinary parameters were observed.

Gross Pathology

The necropsies included, examination of the external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, including viscera.

There were no ENB-0040-related macroscopic findings at any dose levels at the end of the dosing and recovery periods.

Organ Weights

Designated organs (listed below) from all animals were weighed at scheduled necropsy.

Adrenals	Pituitary
Brain	Prostate
Heart	Spleen
Kidneys	Testes
Liver	Thymus
Lungs	Thyroids (with Parathyroids)
Ovaries	Uterus

ENB-0040 had no effects on organ weights.

Histopathology

Adequate Battery: Yes

Peer Review: No

Histological Findings:

Representative sections of collected organs were fixed in 10% formalin except for the eye (Davidson's fixative) and testis (Bouin's solution). The list of tissues and organs collected is shown in the applicant's Table below.

Abnormal tissues/lesions	Pancreas
Achilles tendon (left)	Pituitary
Adrenals	Prostate
Animal Identification *	Rectum
Aorta (thoracic and abdominal)	Right Femur and Tibia[a]
Brain	Salivary Gland (mandibular)
Calvarium (sagittal and coronal sutures) [@]	Sciatic Nerve
Cecum	Seminal Vesicles
Colon	Skeletal Muscle
Epididymides	Skin & Subcutis (thoracic)
Esophagus	Small Intestine, Duodenum
Eyes	Small Intestine, Ileum
Femur & Marrow	Small Intestine, Jejunum
Gallbladder	Spinal Cord (cervical)
Heart **	Spleen
Injection Site (s)	Sternum & Marrow
Kidneys	Stomach
Left tarsus	Testes
Left femorotibial joint	Thymus
Liver (2 lobes)	Thyroids & Parathyroids#
Lungs (2 lobes with bronchi) ***	Tongue
Lymph Node, Mandibular	Trachea
Lymph Node, Mesenteric	Urinary Bladder
Mammary Gland (thoracic)	Uterus
Mandibule [a]	Vagina
Optic Nerves #	Vertebrae (L1-L2) and (L3 to L5)[a] with intervertebral
Ovaries	disk

* Fixation and preservation only.
** Section including both ventricles and atria, septum with papillary muscle.
*** Lungs were infused with formalin.
# Parathyroids and optic nerves were examined histologically only if present in routine sections.
[@] Should avoid damaging the sutures during calvarium dissection for brain extraction.
[a] Refer to the bone retention section below.

No treatment-related histopathological changes were noticed.

Anti-drug antibodies determination:

Blood samples (approximately 1.5 mL) were collected from all animals once during the pre-treatment period and at the end of the treatment (Day 29) and recovery periods for immunogenicity assessment.

Anti-drug antibodies were detected in a total of seven animals (2602B, 3003C, 3504D, 4002B, 4003C, 4502B, 4503C) and most of the positive results were observed at the end of the recovery period. Overall, the levels of anti-drug antibodies present in positive samples were low, except for two animals with titers of 335 (3504D, Day 29) and 68 (4503C, Day 57).

Toxicokinetics

For toxicokinetic analysis, blood samples were collected on Days 1 and 22 (last treatment, week 4), at predose, and at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 hours after dosing.

On Day 1, the rate of clearance (CL) generally decreased as a function of dose (0.0370, 0.0240 and 0.0141 L/h/kg, at low, mid and high doses, respectively), whereas elimination half-life was relatively consistent across doses (i.e., 28.2, 26.4, and 28.4 h, respectively). On Day 22, CL_{ss} generally decreased as a function of dose (i.e., 0.0556, 0.0304 and 0.0177 L/h/kg, respectively), whereas elimination half-life was relatively consistent across doses (i.e., 39.4, 34.9, and 23.0 h, respectively). This decrease across doses could be due to saturation of elimination processes from the serum.

On Day 1, the C_{max} was 68.1, 245 and 726 mg/L for the 5, 15 and 45 mg/kg dose levels, respectively. The T_{max} was up to 1h. On Day 22, the C_{max} was 53.7, 236 and 691 mg/L for the 5, 15 and 45 mg/kg dose levels, respectively, and this increase across doses was generally dose proportional. The R_A on Day 22 for the 5, 15 and 45 mg/kg dose levels was 0.760, 0.841 and 0.832, respectively, suggesting that levels of ENB-0040 in serum following once weekly administration remained relatively constant. No gender-specific differences in PK parameters of ENB-0040 were observed. The TK data are presented in the applicant's Table below

Mean (CV%)	Serum ENB-0040		
	Group 2 ENB-0040 (5 mg/kg)	Group 3 ENB-0040 (15 mg/kg)	Group 4 ENB-0040 (45 mg/kg)
	Day 1	Day 1	Day 1
N	10	10	10
AUC_{last} (mg•h/L)	142 (31.2)	606 (12.7)	3190 (20.4)
AUC_{∞} (mg•h/L)	143 (27.4)[b]	632 (12.2)	3290 (20.3)
CL (L/h/kg)	0.0370 (22.6)[b]	0.0240 (12.1)	0.0141 (17.0)
C_{max} (mg/L)	68.1 (16.1)	245 (16.1)	726 (21.5)
C_0 (mg/L)	91.5 (42.6)	260 (29.9)	851 (44.0)
$t_{1/2}$ (h)	28.2 (22.6)[b]	26.4 (20.6)	28.4 (23.8)
T_{max}[a] (h)	0.25 (0.25, 0.50)	0.26 (0.25, 0.67)	0.38 (0.25, 1.00)
V_{ss} (L/kg)	0.609 (40.4)[b]	0.274 (30.1)	0.130 (29.8)

[a] Median (Min, Max)

[b] n=9, Animal 2602B not included in calculation of summary statistics

Mean (CV%)	Serum ENB-0040		
	Group 2 ENB-0040 (5 mg/kg)	Group 3 ENB-0040 (15 mg/kg)	Group 4 ENB-0040 (45 mg/kg)
	Day 22	Day 22	Day 22
N	10	10	10
AUC_{last} (mg•h/L)	105 (33.2)	524 (24.6)	2670 (20.6)
AUC_{τ} (mg•h/L)	106 (32.6)	524 (24.5)	2670 (20.5)
CL_{ss} (L/h/kg)	0.0556 (54.3)	0.0304 (27.3)	0.0177 (26.6)
C_{max} (mg/L)	53.7 (17.5)	236 (27.1)	691 (12.2)
C_0 (mg/L)	75.3 (14.5)	329 (82.1)	732 (18.7)
$t_{1/2}$ (h)	39.4 (53.6)[b]	34.9 (60.4)[c]	23.0 (89.4)[d]
T_{max}[a] (h)	0.25 (0.25, 0.28)	0.25 (0.25, 0.52)	0.28 (0.25, 1.00)
V_{ss} (L/kg)	0.887 (47.0)[b]	0.301 (55.3)[c]	0.100 (52.4)[d]

[a] Median (Min, Max)

[b] n=9, Animal 2004D not included in calculation of summary statistics

[c] n=7, Animal 3003C, 3004D, 3505E not included in calculation of summary statistics

[d] n=6, Animal 4001A, 4003C, 4004D, 4505E not included in calculation of summary statistics

Dosing Solution Analysis

In order to verify the concentration of the test article in the dosing formulations, duplicate samples (2 x 0.1 mL) from each dosing formulation, including control, were taken from formulations intended for use on Days 1, 8, 15 and 22 (last treatment).

All dosing formulations were within 10% of their nominal concentration (from 90.5 to 99.6 %), with 10% deviation from the nominal being the validated accuracy of the assay.

5. Study title: A 6-month Multiple Dose Toxicity Bone Study of ENB-0040 in the Juvenile Monkey Followed by a 4-week Recovery Period.

Study no.:	670388
Study report location:	Electronic submission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	February 7, 2008
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Lot # PUR012F01, purity: 99.2%

Key Study Findings

ENB-0040 was administered SC to male and female juvenile Cynomolgus monkeys daily for a period of 6 months at dose levels of 0, 0.43, 2.14 and 10 mg/kg/day. ENB-0040-related clinical signs include skin scab, dryness and redness of the skin at one or several injection sites. There were no drug-related effects on mortalities, body weight, food intake, hematology, blood chemistry, urinalysis, ophthalmology, electrocardiography, organ weight, and macroscopic and microscopic observations in all dosage groups. The biochemical markers of bone turnover, bone densitometry or bone geometry parameters, assessed by Dual Energy X-ray Absorptiometry (DXA) and Peripheral Quantitative Computed Tomography (pQCT) were not affected by ENB-0040 administration in all dose groups. Elevated serum alkaline phosphatase levels observed were likely attributed to circulating levels of ENB-0040. The NOEL was considered to be 10 mg/kg/day with mean AUC_{last} and C_{max} of 303 mg.h/L and 6.68 mg/L, respectively.

Methods

Doses:	0, 0.43, 2.14 and 10 mg/kg/day
Frequency of dosing:	Once daily
Route of administration:	SC
Dose volume:	0.25 mL/kg
Formulation/Vehicle:	A solution of 25 mM sodium PO_4 pH 7.4, 150 mM NaCl was used as the vehicle.
Species/Strain:	Cynomolgus monkeys
Number/Sex/Group:	3/sex/dose in main study and 2/sex/dose in recovery study
Age:	Approximately 12 months old
Weight:	1.4 to 1.8 kg for both males and females.
Satellite groups:	No
Unique study design:	No
Deviation from study protocol:	Minor changes in sample collection and analysis, which had no impact on the overall findings of the study.

Observations and Results

Mortality

The animals were observed for mortality twice daily.

There was no test article-related mortality. On Day 179, Animal No. 151 (control group) was euthanized *in extremis* because its head was caught in the cage.

Clinical Signs

The animals were observed for clinical signs twice daily (prior to dosing and 15 to 30 min after dosing). A detailed clinical examination was conducted once weekly.

Animals in all treated groups showed skin scabs, skin dryness and/or skin redness at one or several injection sites with higher incidence in the high dose group. These findings at the injection sites were not observed in the control group. Only one control male (Animal No. 103) had skin scab at one injection site at the end of the treatment period and was considered incidental. At the end of the recovery period, these clinical observations recovered partially to completely.

Body Weights

Individual body weights were measured for all animals on the day of randomization and weekly starting in Week -1, and extending through the treatment and recovery periods.

There were no toxicologically significant effects on body weights and body weight gains during the treatment and recovery periods.

Feed Consumption

Appetite was generally assessed once daily (generally in the p.m.) for all animals by visual inspection (documented as whether the animal ate or not) during the last week of acclimation and throughout the treatment and recovery periods.

There were no effects on appetence during the study.

Ophthalmoscopy

Ophthalmoscopy examination was conducted once prior to the initiation of treatment and during Weeks 12 and 26.

No ocular abnormality was observed.

ECG

Electrocardiograms (leads I, II and III, and augmented leads aVR, aVL and aVF) were performed for all animals once during the pre-treatment period and again during Weeks 1 and 25 (predose [for Week 25 only], approximately 15 minutes, 4, 12 and 24 hours post treatment) of the treatment period, and during Week 29 for the recovery animals. A quantitative measurement of heart rate, PR, QRS, QT and RR intervals and a qualitative assessment of the trace for rhythm and abnormalities were performed.

No treatment-related changes were observed on ECG parameters in any animals at any dose levels.

Hematology

Blood samples were collected from femoral vein for hematology, coagulation and clinical chemistry from all animals once during the pre-treatment period, and during Weeks 13, 26 and during Week 30 from the recovery animals.

Administration of ENB-0040 at dose levels of 0.43, 2.14 or 10 mg/kg/day was not associated with any toxicologically or biologically significant changes in hematology parameters when compared to the control animals.

Clinical Chemistry

There were no toxicologically significant effects on serum chemistry parameters. Treatment with ENB-0040 was associated with dose-related increases, on occasion attaining statistical significance, in alkaline phosphatase of up to 3, 4 and 34-fold of the control values during Week 13 and up to 4, 6 and 35-fold of the control values during Week 26 in the 0.43, 2.14 and 10 mg/kg/dose groups, respectively. Considering the nature of ENB-0040 (recombinant soluble form of tissue nonspecific alkaline phosphatase), these increases were considered to be related to the circulating levels of ENB-0040. ALP values are presented in the Table below which is adopted from the applicant's submission.

Alkaline Phosphatase (U/L)				
ENB-0040 (mg/kg/day		**Week 13 (n=5)**	**Week 26 (n=5)**	**Recovery period (Week 30) (n=2)**
0	**Male**	654.8 ±101.4	713.8±159.7	865 and 811
	Female	590.6±112.5	580.6±94.7	579 and 680
0.43	**Male**	2228.6±303.7	2288.8±538.2	2552 and 3337
	Female	1949.6±184.7	2048±454.8	2186 and 1763
2.14	**Male**	2743±1046.9	4116.4±2161.2*	1416 and 1875
	Female	2655.4±1452.7	3040.2±1924.8	696 and 278
10	**Male**	36617±3839.1**	22888±6011.9**	641 and 1063
	Female	19860±5096.6**	20602±3695**	890 and 589

Urinalysis

Urinalysis was conducted at the end of dosing and recovery periods.

The urinalysis parameters evaluated were unaffected by the administration of ENB-0040.

Gross Pathology

All animals were fasted overnight before scheduled necropsy. Terminal body weights were recorded prior to necropsy. The necropsies included, examination of the external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, including viscera.

There were no ENB-0040-related macroscopic findings in monkeys at any dose levels at the end of the dosing and recovery periods.

Organ Weights

Designated organs (listed below) from all animals were weighed at scheduled necropsy.

Adrenals	Pituitary
Brain	Prostate
Heart	Spleen
Kidneys	Testes
Liver	Thymus
Lungs	Thyroids (with Parathyroids)
Ovaries	Uterus

There were no ENB-0040-related effects on organ weights.

Histopathology

Adequate Battery: Yes

Peer Review: No

Histological Findings:

Representative sections of collected organs were fixed in 10% formalin except for the eye (Davidson's fixative) and testis (Bouin's solution). The list of tissues and organs collected is shown in the applicant's Table below.

abnormalities
animal identification
adrenals
aorta (thoracic)
bone and marrow (sternum)
bone-calvarium (with suture lines)
brain (forebrain, midbrain, cerebellum and medulla oblongata)
cecum
colon
duodenum
epididymides
esophagus
eyes
femurs
gallbladder
heart (including section of aorta)
humerus
ileum
injection site(s)
jejunum
joint, femorotibial (right)
kidneys
liver (sample of 2 lobes)
lungs (sample of 2 lobes)
lymph nodes (mandibular, unilateral; mesenteric; axillary, bilateral)
mammary gland (thoracic)
optic nerves
ovaries
pancreas
pituitary
prostate
rectum
rib
salivary gland (mandibular, unilateral)
sciatic nerve
seminal vesicles
skeletal muscle
skin (ventral thoracic)
spinal cord (cervical)
spleen
stomach
testes
thymus
thyroid lobes (and parathyroids)
tongue
trachea
urinary bladder
uterus (body and cervix)
vagina
vertebrae

Histopathological evaluation revealed ENB-0040-related microscopic findings at the injection sites. These consisted of minimal to moderate granulomatous inflammation in dermis and subcutis and minimal to moderate perivascular mononuclear cell infiltration at ≥0.43 mg/kg/dose. Granulomatous inflammation in dermis and subcutis was observed in one or more of the injection sites at 0.43, 2.14 and 10 mg/kg/dose in males and at 0.43 and 10 mg/kg/dose in females. In general, the incidence and severity of this finding increased with dose. Perivascular mononuclear cell infiltration was observed at all doses but was without dose-relationship.

Following 4 weeks of recovery period, granulomatous inflammation and mononuclear cell infiltration were reversed in animals treated with 0.43 and 2.14 mg/kg/dose, but remained in some sites with reduced severity (minimal) at 10 mg/kg/dose in both males and females.

Biochemical Markers of Bone Turnovers

Whole blood (2 mL) samples were collected prior to initiation of treatment, and on Weeks 13 and 26 to determine bone formation marker (osteocalcin) and bone resorption markers (C-telopeptide and N-telopeptide). In general, the levels of bone turnover markers and individual variability at the end of Week 26 were compared with the values at the end of the Week 13 and pretreatment period.

ENB-0040 treatment of young Cynomolgus monkeys had no statistically significant or biologically meaningful effects on bone formation and bone resorption markers (C-Telopeptide and N-Telopeptide).

Bone Mineral Density (BMD) Measurement

Bone mineral density (BMD) and bone mineral composition (BMC) of all animals were determined by Dual Energy X-ray Absorptiometry (DXA) and Peripheral Quantitative Computed Tomography (pQCT) once prior to treatment, once during Weeks 13 and 23 of the treatment and at Week 30 for recovery animals. BMC and BMD were assessed on whole body, lumbar spine (L1-L4) and right proximal femur and right proximal tibia (metaphysis and diaphysis).

No statistically significant or biologically meaningful effects on whole body, lumbar (L1-L4) and proximal femur bone densitometry parameters (area, BMC and BMD) were noted in ENB-0040-treated animals (both males and females) compared to vehicle controls during the treatment and recovery periods. No effect was noted on the tibia metaphysis and diaphysis of ENB-0040-treated males or females. However, tibia metaphysis analysis showed a slight increase in BMD in both males and females treated with ENB-0040. Due to the small group size, inconsistency between males and females and lack of a dose response, these differences were not considered to be of biological significance.

Anti-drug antibodies determination:

For anti-ENB-0040 antibody determination, blood samples (approximately 1.0 mL) were collected from all animals once during the pre-treatment period and during Weeks 4, 8, 12, 16, 20 and 24, and during Week 29 from the recovery animals.

Anti-drug antibodies were not detected in the control group (all time points) and samples collected from animals before treatment (all groups). During Week 4, no anti-ENB-0040 antibodies were detected in Group 2 (0.43 mg/kg/dose) but 30% of the Group 3 animals (2.14 mg/kg/dose) showed a positive signal. Exposure to ENB-0040 was immunogenic in at least 50% of the Group 2 (0.43 mg/kg/dose) and Group 3 (2.14 mg/kg/dose) animals during Week 8, 12, 16, 20 and 24 of the treatment period. No anti-ENB-0040 antibody positive samples were observed in Group 4 (10 mg/kg/dose). Because of the presence of high circulating drug levels in this group, interference of the assay by the test article was the likely cause (except pre-treatment and recovery time points).

Toxicokinetics

For toxicokinetic analysis, blood samples were collected on Days 1 and 182 (Week 26) at predose and 0.5, 1, 2, 4, 8, 12, 24, 48, 72 and 96 hours post dose. Additionally, during Week 4, blood samples were collected at predose and at 0.5, 1, 2, 4, 8, 12 and 24 hours post dose. During Weeks 12, 16 and 20 samples were collected at 6 and 24 hours post dose.

On Day 1, following SC administration of ENB-0040 at 0.43, 2.14 and 10 mg/kg/day, the C_{max} (0.0718, 0.446, and 2.66 mg/L, respectively) and AUC_{last} (1.38, 8.50 and 50.0 mg.h/L, respectively) increased as a function of dose and the increases were generally more than dose proportional. Data are presented in the applicant's Table below.

PK Parameters of ENB-0040 Serum (Day 1; Genders Combined)

Mean (CV%)	Serum ENB-0040		
	Day 1		
Dose (mg/kg)	0.43 mg/kg	2.14 mg/kg	10 mg/kg
N	10	10	10
AUC_{last} (mg•h/L)	1.38 (44.0)	8.50 (20.8)	50.0 (28.6)
AUC_{0-24} (mg•h/L)	1.38 (44.0)	8.50 (20.8)	50.0 (28.6)
AUC_{0-24}/Dose (kg•h/L)	3.20 (44.0)	3.97 (20.8)	5.00 (28.6)
CL/F (L/h/kg)	NC (NC)[b]	NC (NC)[b]	NC (NC)[b]
C_{max} (mg/L)	0.0718 (49.0)	0.446 (20.4)	2.66 (30.6)
C_{max}/Dose (kg/L)	0.174 (39.2)	0.208 (20.4)	0.266 (30.6)
T_{max}[a] (h)	8.00 (4.00, 24.00)	12.00 (4.00, 24.00)	12.00 (4.00, 24.00)

[a] Median (Min, Max)
[b] n = 0
Nominal Times instead of Actual Times
NC = Not calculated

On Week 4, following SC administration of ENB-0040 at 0.43, 2.14 and 10 mg/kg/day, the AUC_{last} (5.22, 5.81 and 132 mg.h/L, respectively) and C_{max} (0.265, 0.338 and 6.62 mg/L, respectively) appeared to increase as a function of dose. For the low and high doses, the increases of C_{max} and AUC were dose proportional. However, the systemic clearance (CL_{ss}/F) of mid dose (1.08 L/h/kg) was higher when compared to the low (CLss/F: 0.138 L/h/kg) and high (CL_{ss}/F: 0.137 L/h/kg) doses. These differences were statistically significant. Data are presented in the applicant's Table below.

PK Parameters of ENB-0040 Serum (Week 4; Genders Combined)

Mean (CV%)	Serum ENB-0040		
	Week 4		
Dose (mg/kg)	0.43 mg/kg	2.14 mg/kg	10 mg/kg
N	10	10	10
AUC_{last} (mg•h/L)	5.22 (53.2)	5.81 (94.1)	132 (77.2)
AUC_{τ} (mg•h/L)	5.22 (53.2)	5.91 (91.5)	132 (77.2)
AUC_{τ}/Dose (kg•h/L)	12.1 (53.2)	2.76 (91.6)	13.2 (77.2)
CL_{ss}/F (L/h/kg)	0.138 (105.0)	1.08 (142.0)	0.137 (91.1)
C_{max} (mg/L)	0.265 (48.3)	0.338 (77.3)	6.62 (70.4)
C_{max}/Dose (kg/L)	0.616 (48.3)	0.158 (77.3)	0.662 (70.4)
T_{max} [a] (h)	4.00 (0.00, 12.00)	6.00 (1.00, 12.00)	8.00 (1.00, 12.00)
[a] Median (Min, Max) Nominal Times instead of Actual Times			

On Week 26, following SC administration of ENB-0040 at 0.43, 2.14 and 10 mg/kg/day, the AUC_{last} (21.5, 35.1 and 303 mg.h/L, respectively) and C_{max} (0.361, 0.796 and 6.68 mg/L, respectively) appeared to increase as a function of dose. For the low and high doses, the increases of C_{max} and AUC_{τ} were dose proportional. Systemic clearance (CL_{ss}/F: 0.238 L/h/kg) following the mid dose was higher when compared to those of low and high doses (0.0651 and 0.0845 L/h/kg, respectively). The C_{max} on Week 26 was achieved within 2 to 5h post dose, earlier than in Week 4. The $t_{1/2}$ (45.3-63.0 h) of ENB-0040 across the three doses was similar. Overall, no gender-specific differences in PK parameters of ENB-0040 were observed. Data are presented in the applicant's Table below

PK Parameters of ENB-0040 Serum (Week 26 Genders Combined)

Mean (CV%)	Serum ENB-0040		
	Week 26		
Dose (mg/kg)	0.43	2.14	10
N	10	10	10
AUC_{last} (mg•h/L)	21.5 (40.5)	35.1 (61.4)	303 (27.3)
AUC_{τ} (mg•h/L)	7.23 (31.0)	14.7 (51.5)	124 (20.1)
AUC_{τ}/Dose (kg•h/L)	16.8 (31.0)	6.87 (51.5)	12.4 (20.1)
CL_{ss}/F (L/h/kg)	0.0651 (32.2)	0.238 (114.5)	0.0845 (24.6)
C_{max} (mg/L)	0.361 (34.2)	0.796 (44.6)	6.68 (34.3)
C_{max}/Dose (kg/L)	0.840 (34.2)	0.372 (44.6)	0.668 (34.3)
$t_{1/2}$ (h)	61.2 (65.9)[b]	63.0 (48.7)[c]	45.3 (37.6)
T_{max}[a] (h)	3.00 (0.00, 12.00)	2.00 (0.50, 12.00)	5.00 (1.00, 8.00)

[a] Median (Min, Max)
[b] n=5, Animal 204, 205, 252, 254, 255 not included in calculation of summary statistics
[c] n=6, Animal 303, 351, 353, 356 not included in calculation of summary statistics

Dosing Solution Analysis

Duplicate samples (0.3 mL) were collected from each dose formulation, including control, on the day of each preparation for concentration verification. Samples were stored refrigerated (approximately 4^0C) and protected from light.

All dosing formulations were found to be within 10% from their nominal concentration (from 91.4 to 101.7%), and all dose formulations were considered stable.

7 Genetic Toxicology

No genetic toxicology studies were submitted.

8 Carcinogenicity

Carcinogenicity studies of asfotase alfa were not conducted. Asfotase alfa is a large protein, and not expected to be carcinogenic or induce cellular proliferation, because it has no known anabolic effects.

Reproductive and Developmental Toxicology

9.1 Fertility and Early Embryonic Development

Study title: A pilot intravenous injection study of ENB-0040 in male rats (Dose range selection).

Study no.:	902230
Study report location:	Electronic submission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	August 10, 2010
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Lot # 169466, Purity: 99.6%

Key Study Findings

ENB-0040 was administered IV to male rats daily for 15 days at dose levels of 0, 25 and 50 mg/kg/day. Transient clinical signs, such as red skin and soft swelling of the muzzle and fore and hind limbs, abnormal gait, decreased activity, lying on side and decreased body weight gain (-13% and -29% at 25 and 50 mg/kg/day, respectively) were observed in ENB-0040-treated animals. No effects were noted on organ weights or macroscopic findings. Based on the above findings, these doses were considered for the pivotal study.

Methods

Doses:	0, 25 and 50 mg/kg/day
Frequency of dosing:	Daily
Dose volume:	5 mL/kg
Route of administration:	IV (Tail vein)
Formulation/Vehicle:	25 mM sodium phosphate, pH 7.4, 150 mM sodium chloride
Species/Strain:	Crl:CD[SD] rats
Number/Sex/Group:	10 males/dose group
Satellite groups:	None
Study design:	

Text Table 1 Study Design

Group Number/ Identification	Dose Level (mg/kg/day)	Number of Animals Males
1/ Vehicle Control	0	10
2/ ENB-0040	25	10
3/ ENB-0040	50	10

Deviation from study protocol:	None

Observations and Results

Mortality

All animals were observed twice daily for mortality.

All rats survived until the scheduled termination of the study.

Clinical Signs

All animals were observed twice daily for clinical signs. A detailed examination was performed daily, prior to dosing and approximately 15 minutes, 1 and 4 hours following dosing.

Test article-related clinical signs noted were red skin and soft swelling of the muzzle and the fore and hind limbs, including the paws in the 25 and 50 mg/kg/day dose groups. These transient findings tended to be more common post dosing, and lasted generally from 1 to 4 hours. Some males had abnormal gait (2/10 and 4/10, respectively), decreased activity (7/10 and 10/10, respectively), lying on side (1/10 and 2/10, respectively) and/or uncoordination (0/10 and 2/10, respectively) at 25 and/or 50 mg/kg/day following dosing, most commonly during the first few days of treatment. A few animals were noted to have black skin on the tail (1/10 and 2/10, respectively) and red skin on the pinnae (all animals in test article groups).

Body Weight

Individual body weights were measured for all animals starting the last week of pretreatment, on the day of randomization and twice weekly throughout the treatment period. In addition, each animal was weighed (fasted) before scheduled necropsy.

Body weight gains were decreased in a dose dependent fashion. Animals in the 25 and 50 mg/kg/day groups showed -13% and -29% weight gains compared to controls, respectively, between Days -6 to 13. Data are presented in the applicant's Table below.

Table 3 Group Mean Body Weight Gains (g)

Males

Group 1 - Vehicle Control
Group 2 - ENB-0040 25 mg/kg/day
Group 3 - ENB-0040 50 mg/kg/day

			Day				
	Summary	From:	-6	-2	2	6	9
Group	Information	To:	-2	2	6	9	13
1	Mean		26.0	12.7	5.5	3.8	8.8
	SD		5.79	5.54	4.20	4.83	4.13
	N		10	10	10	10	10
2	Mean		24.6	10.0	0.1	9.3	5.2
	SD		5.42	8.01	5.88	4.72	4.66
	N		10	10	10	10	10
3	Mean		22.2	6.9	1.0	3.3	6.9
	SD		11.24	7.08	5.96	6.45	7.32
	N		10	10	10	10	10

Significantly different from control group (group 1) value: A - $P \leq 0.05$ B - $P \leq 0.01$ C - $P \leq 0.001$ (Dunnett)
D - $P \leq 0.05$ E - $P \leq 0.01$ F - $P \leq 0.001$ (Dunn)

Feed Consumption

Individual food consumption for all animals was measured weekly commencing on Day -10/-9 of the pre-treatment period and throughout the treatment period.

A slight decrease (-4%) in food consumption was noted for animals in the 50 mg/kg/day dose group in the first week of treatment (Days -2 to 6).

Toxicokinetics

Toxicokinetics was not performed.

Dosing Solution Analysis

Duplicate samples (2.5 mL) were collected from each dose formulation including control on each day of preparation, and one sample/group from the Day 1 preparation**s** was analyzed.

Measured concentrations of ENB-0040 in the dose formulations deviated from the theoretical concentrations by a maximum of 7.7%.

Gross Pathology

On completion of the treatment period, a complete gross pathology examination was performed.

Macroscopic examination revealed no test article-related findings.

Organ Weights

The following organs were dissected free of fat and weighed:

Adrenal glands	Brain	Epididymis	Heart
Kidneys	Liver	Lungs	Testes
Pituitary	Prostate	Seminal vesicle	Spleen
Thymus	Thyroid and	Parathyroids	

Paired organs were weighed together, and organ weight ratios, relative to brain weights and body weights, were calculated.

On completion of the necropsy of each animal, the following tissues and organs were retained in neutral buffered 10% formalin for fixation and preservation except for eyes, optic nerves (fixed in Davidson's fluid) and epididymis, testes (fixed in modified Davidson's fluid).

Aorta	Bone marrow smear	Bone marrow, femur
Adrenal gland	Bone marrow, sternum	Bone, femur
Bone, sternum	Brain	Epididymis
Esophagus	Eyes	Harderian gland
Gut-lymphoid tissue	Heart	Injection site
Kidneys	Lacrimal gland	Large intestine
Larynx	Liver	Lung
Lymph node, mandibular	Lymph node, mesenteric	Mammary gland
Muscle, skeletal	Nasal cavities	Nerve, optic
Nerve, sciatic	Pancreas	Parathyroid
Pituitary	Prostate	Skin
Salivary gland	Seminal vesicle	Small intestine
Spinal cord	Spleen	Stomach
Testis	Thymus	Thyroid
Tongue	Trachea	Urinary bladder

There were no ENB-0040-related differences in organ weights (absolute, relative to body weight and relative to brain weight).

Histopathology

Tissues were retained in fixative (organ weight section) for possible future examination. But the tissues were not examined as there were no changes in the organ weights and gross pathology findings.

Study title: An Intravenous Injection study of Fertility and Early Embryonic Development with ENB-0400 in Rats.

Study no.:	902231
Study report location:	Electronic submission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	February 21, 2012
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Lot # 259248, purity: 98.9%

Key Study Findings

Male and female Sprague-Dawley rats (25/dose group) received ENB-0040 intravenously at doses of 0, 10, 25 and 50 mg/kg/day. The males were dosed for 63 days, up until the day before necropsy. Males were dosed for a minimum of 28 days before mating, and females were dosed for 14 days prior to mating period to the 7th day of gestation. Mean body weights of male rats at 50 mg/kg/day dose group were lower than controls from Day 28 until termination (Day 63). In females, body weight gain was unaffected. Mating and fertility indices were unaffected. The fertility indices were 88, 96, 100 and 100% in 0, 10, 25 and 50 mg/kg/day dose groups, respectively. There were no ENB-0040-related effects on the number of corpora lutea, implantation sites, live embryos, dead embryos and resorptions, or on the pre and post implantation losses. The organ weights and male reproductive parameters (including sperm motility, morphology and concentration) were unaffected by treatment. The parental no-observed-adverse-effect level (NOAEL) was considered to be 25 and 50 mg/kg/day for males and females, respectively. The no-observed-effect-level (NOEL) for the fertility and early embryo-fetal development was considered to be 50 mg/kg/day.

Methods

Doses:	0, 10, 25 and 50 mg/kg/day
Frequency of dosing:	Once daily
Dose volume:	5 mL/kg
Route of administration:	IV
Formulation/Vehicle:	25 mM sodium phosphate pH 7.4, 150 mM sodium chloride
Species/Strain:	Sprague-Dawley rats, Crl:CD(SD)
Number/Sex/Group:	25/sex/dose group
Satellite groups:	None
Study design:	

Experimental Design

Group No.	Dose Level (mg/kg/day)	Dose Volume (mL/kg)	Dose Concentration (mg/mL)	No. of Animals	
				Males	Females
1/Vehicle Control	0	5	0	25	25
2/ENB-0040	10	5	2	25	25
3/ENB-0040	25	5	5	25	25
4/ENB-0040	50	5	10	25	25

Deviation from study protocol: None

Observations and Results

Mortality

Animals were observed twice daily for mortality and moribundity.

No treatment-related deaths were observed. One male in control group (#1024) was euthanized on Day 49 due to abnormal clinical signs including tremors, labored breathing, incoordination, lying on side, cold to touch and pale skin in both hind limbs. Another male in the 10 mg/kg/day dose group (# 2025) was euthanized on Day 37 due to obstruction in the mouth and swollen paws.

Clinical Signs

Cage side clinical observations were performed once daily. A detailed clinical observation was performed on the day of randomization, the first 3 days of treatment (prior to dosing and at 15 minutes, 1 hour and 4 hours following dosing), and then weekly thereafter.

Treatment related clinical signs, such as, blue skin in the hind and forepaws and/or muzzle were noted in males and females in the 50 mg/kg/day dose group on the 1st week of dosing. Males in 25 and 50 mg/kg/day dose groups showed swollen left and right periorbital area on the 1st week of dosing. Females in the 50 mg/kg/day dose group showed decreased activity on Day 1. Animals in all dose groups showed sporadic swelling of fore/hind limbs/paws and lower jaw/muzzle and/or mouth throughout the treatment period.

Body Weight

Animals were weighed individually once during the last week of the pre-study period, twice weekly during treatment (including Day 14 for females and Day 28 for males) and at least weekly for males during the post mating period and at necropsy. Mated females were weighed on Days 0, 3, 7, 10 and 13 postcoitum (pc).

During the premating period, mean body weight gains in males at 50 mg/kg/day were significantly (P ≤0.01) lower than controls from Days 4 and 7. Overall, males in the 50 mg/kg/day group showed significantly decreased body weight gain from Days 1 to 63. The mean body weights (g) of different groups are presented in the applicant's Tables below.

Table 2 Summary of Body Weights (g)

Males

Group 1 - Vehicle Control
Group 2 - ENB-0040 10 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Summary Information	Day -7	1	4	7	10	14	17
1	Mean	368.8	410.6	409.9	421.3	426.6	437.6	443.4
	SD	15.2	21.6	21.7	23.3	23.4	25.3	27.0
	N	25	25	25	25	25	25	25
2	Mean	369.3	408.4	406.5	416.2	422.0	430.2	434.7
	SD	15.4	21.0	22.3	24.9	25.4	27.5	27.8
	N	25	25	25	25	25	25	25
3	Mean	369.4	410.0	408.9	419.0	425.6	434.8	439.2
	SD	15.0	20.6	22.6	23.0	24.7	28.2	30.8
	N	25	25	25	25	25	25	25
4	Mean	369.2	406.7	405.6	411.7	416.7	423.1	429.2
	SD	16.5	23.3	25.1	25.7	26.9	28.1	28.9
	N	25	25	25	25	25	25	25

Significantly different from control group (Group 1) value: A - P ≤ 0.05 B - P ≤ 0.01 C - P ≤ 0.001 (Dunnett)
D - P ≤ 0.05 E - P ≤ 0.01 F - P ≤ 0.001 (Dunn)

Table 2 Summary of Body Weights (g)

Males

Group 1 - Vehicle Control
Group 2 - ENB-0040 10 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Summary Information	Day 21	24	28	31	35	38	42
1	Mean	454.5	462.5	472.1	471.8	482.6	492.8	500.4
	SD	26.9	28.0	29.5	30.8	31.2	31.7	33.0
	N	25	25	25	25	25	25	25
2	Mean	445.8	453.0	460.2	461.2	467.2	473.0	480.0
	SD	29.0	29.7	30.6	31.8	31.5	31.7	32.6
	N	25	25	25	25	25	24	24
3	Mean	448.8	453.9	461.7	458.6	471.7	480.4	488.9
	SD	32.6	33.5	35.5	37.4	39.1	39.4	38.3
	N	25	25	25	25	25	25	25
4	Mean	433.8	438.8	444.8 B	443.1 B	454.3 A	460.7 B	468.1 B
	SD	30.0	31.6	32.9	32.9	34.0	35.7	36.9
	N	25	25	25	25	25	25	25

Significantly different from control group (Group 1) value: A - $P \leq 0.05$ B - $P \leq 0.01$ C - $P \leq 0.001$ (Dunnett)
D - $P \leq 0.05$ E - $P \leq 0.01$ F - $P \leq 0.001$ (Dunn)

Table 2 Summary of Body Weights (g)

Males

Group 1 - Vehicle Control
Group 2 - ENB-0040 10 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Summary Information	Day 45	49	52	56	59	63
1	Mean	505.4	514.1	525.8	525.9	527.5	535.1
	SD	36.5	40.4	36.0	40.7	42.4	46.0
	N	25	25	24	24	24	24
2	Mean	485.2	495.7	500.6	507.2	510.5	515.5
	SD	31.2	34.0	33.4	34.6	34.6	35.2
	N	24	24	24	24	24	24
3	Mean	492.8	503.0	508.4	515.2	518.5	522.8
	SD	38.3	40.1	41.9	42.6	44.2	44.7
	N	25	25	25	25	25	25
4	Mean	470.6 B	480.6 B	483.6 C	489.6 B	491.7 B	497.1 B
	SD	38.8	41.1	40.2	42.6	41.7	43.4
	N	25	25	25	25	25	25

Significantly different from control group (Group 1) value: A - $P \leq 0.05$ B - $P \leq 0.01$ C - $P \leq 0.001$ (Dunnett)
D - $P \leq 0.05$ E - $P \leq 0.01$ F - $P \leq 0.001$ (Dunn)

In females, the body weights remained comparable to controls throughout the premating and gestation periods, except at Day 0 postcoitum (pc) when the mean body weights were significantly ($P \leq 0.05$) greater than controls for females in the 50 mg/kg/day dose group.

Feed Consumption

Food consumption was measured weekly. Food consumption of mated females was measured on Days 0 to 3, 3 to 7, 7 to 10 and 10 to 13 postcoitum (pc).

There were no effects on food consumption during the premating and gestation periods.

Toxicokinetics

Toxicokinetics was not performed.

Dosing Solution Analysis

Duplicate samples were collected from each dose formulation on the days of preparation for Weeks 1, 4 and last, for possible concentration variation.
All study samples analyzed had mean sample concentrations within the acceptance criteria of ± 10% (individual values within ± 15%) of their theoretical concentrations.

Necropsy

Fertility Parameters (Mating/Fertility Index, Corpora Lutea, Preimplantation Loss, etc.)

Male rats were euthanized at the end of the study, and subjected to a complete necropsy examination. Assessment of the male reproductive system was also evaluated (sperm motility, concentration and morphology).

Female rats were euthanized on Day 13 postcoitum (pc) and subjected to a complete necropsy examination which included evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues and an ovarian/uterine examination. Uteri and ovaries from all females were retained in neutral buffered 10% formalin for possible future evaluation. Laparotomies were performed, and the presence or absence of gestation, and distribution and the numbers of corpora lutea, early-stage dead embryos and fetuses (implantation scars, placental residue, absorbed embryos), late-stage dead embryos and fetuses (macerated fetuses, dead fetuses), and surviving fetuses were recorded.

The number of days in estrus, number of cycles seen and average cycle length (4.4, 4.2, 4.5 and 4.2 days for Groups 1, 2, 3 and 4, respectively) were unaffected by treatment.

The mean number of days to mating, the mating index, fertility and conception rates were unaffected by the treatment. The number of pregnant females was 22, 24, 25 and 25 for Groups 1, 2, 3 and 4, respectively. Data are presented in the applicant's Table below.

Table 12 Summary of Parental Performance

Group 1 - Vehicle Control
Group 2 - ENB-0040 10 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Number Placed for Mating		Number Mating	Mean (SD) Day to Mating	Number Females Pregnant	Mating Index (%)	Fertility Index (%)	Conception Rate (%)
	Males	Females						
1	25	25	25	2.4 1.2 (N = 24)	22	100.0	88.0	88.0
2	25	25	25	3.1 2.9 (N = 25)	24	100.0	96.0	96.0
3	25	25	25	2.6 1.3 (N = 25)	25	100.0	100.0	100.0
4	25	25	25	3.5 3.5 (N = 24)	25	100.0	100.0	100.0

Significantly different from control group (Group 1) value: D - P ≤ 0.05 E - P ≤ 0.01 F - P ≤ 0.001 (Dunn - day to mating only)
* - P ≤ 0.05 ** - P ≤ 0.01 *** - P ≤ 0.001 (Fisher's)

There were no test item-related effects on the mean number of corpora lutea (17.8, 16.7, 17.2 and 18.0), number of implantation sites (16.0, 15.5, 15.8 and 16.3), live embryos (14.9, 14.5, 15.0 and 15.3), dead embryos (0, 0, 0 and 0), resorptions (1.1, 1.0, 0.8 and 1.0) and pre- (9.43, 6.98, 7.84 and 9.27) or post-implantation losses (7.77, 6.22, 5.13 and 6.10) for Groups 1, 2, 3 and 4, respectively. Summary of ovarian and uterine findings are presented in the applicant's Tables below.

Table 13 Summary of Ovarian and Uterine Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 10 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Summary Information	Total Number of Corpora Lutea	Total Number of Implantation Sites	Number of Live Embryos	Number of Dead Embryos
1	Mean	17.8	16.0	14.9	0.0
	SD	2.3	2.5	2.9	0.0
	N	21	21	21	21
2	Mean	16.7	15.5	14.5	0.0
	SD	2.1	2.2	2.0	0.2
	N	24	24	24	24
3	Mean	17.2	15.8	15.0	0.0
	SD	3.0	2.4	2.8	0.0
	N	25	25	25	25
4	Mean	18.0	16.3	15.3	0.0
	SD	2.3	2.4	2.6	0.0
	N	24	24	24	24

Significantly different from control group (Group 1) value: D - P ≤ 0.05 E - P ≤ 0.01 F - P ≤ 0.001 (Dunn)

Table 13 Summary of Ovarian and Uterine Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 10 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Summary Information	Number of Early Resorptions	Sum of Early Resorptions and Dead Embryos	Preimplantation Loss %	Post Implantation Loss %
1	Mean	1.1	1.1	9.43	7.77
	SD	0.9	0.9	11.41	6.74
	N	21	21	21	21
2	Mean	1.0	1.0	6.98	6.22
	SD	1.1	1.1	5.68	6.57
	N	24	24	24	24
3	Mean	0.8	0.8	7.84	5.13
	SD	0.9	0.9	6.48	6.35
	N	25	25	25	25
4	Mean	1.0	1.0	9.27	6.10
	SD	1.3	1.3	9.51	8.24
	N	24	24	24	24

Significantly different from control group (Group 1) value: D - $P \leq 0.05$ E - $P \leq 0.01$ F - $P \leq 0.001$ (Dunn)

In male rats, no ENB-0040-related effects were noted on sperm motility, sperm concentration or sperm morphology.

9.2 Embryonic Fetal Development

Study title: A Dose Range-finding Embryo-fetal Development Study of ENB-0040 by Intravenous Injection in Rats.

Study no.:	902233
Study report location:	Electronic Submission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	October 13, 2010
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Lot # 169466, Purity: 99.2%

Key Study Findings

In this dose range-finding study, four groups of pregnant Crl:SD female rats (7 females/group) received IV ENB-0040 at dose levels of 0, 13, 25 and 50 mg/kg/day from day 6 to day 19 of gestation. Treatment related transient clinical signs noted in all treated groups included swelling of the fore/hind limbs/paws and muzzle, and red skin in these same areas. There was no ENB-0040-related effect on body weight gains. ENB-0040 had no effects on the number of implantations, live or dead fetuses, resorptions, post implantation loss or gravid uterine weights. Based on the above findings, the doses selected for the definitive embryo fetal development study were 13, 25 and 50 mg/kg/day.

Methods

Doses:	0,13, 25 and 50 mg/kg/day
Frequency of dosing:	Daily
Dose volume:	5 mL/kg
Route of administration:	Intravenous
Formulation/Vehicle:	25 mM sodium phosphate pH 7.4, 150 mM sodium chloride
Species/Strain:	Crl:CD®(SD) rats
Number/Sex/Group:	7 females/dose group
Satellite groups:	No

Study design:

Text Table 1 Study Design

Group Number/Identification	Dose Level (mg/kg/day)	Dose Volume (mL/kg)	Number of Animals Females
1/Vehicle Control	0	5	7
2/ENB-0040	13	5	7
3/ENB-0040	25	5	7
5/ENB-0040	50	5	7

Deviation from study protocol: None

Observations and Results

Mortality

All animals were observed twice daily for mortality and still birth.

There was no unscheduled mortality.

Clinical Signs

All animals were observed twice daily for clinical signs. In addition, a detailed examination was performed on gestation days 6 to 9 prior to dose administration and generally at 15 minutes, 1 and 4 hours post dose.

Transient clinical signs were noted in all treated groups and considered to be ENB-0040 related. These include swelling of the fore/hind limbs/paws and muzzle, and red skin in the same areas. These observations were primarily noted between 15 minutes and 1 hour after dosing, and diminished by 4 hours after dosing. In several occasions, partly closed eyes were noted in two animals in the 50 mg/kg/day dose group. This symptom disappeared completely by 4 hours post dose.

Body Weight

Individual body weights were measured for all animals on Days 0, 3, 6, 9, 12, 15, 18 and 21 of gestation.

ENB-0040 had no effects on the body weight gain.

Feed Consumption

Individual food consumption for all animals was measured on Days 3 to 6, 6 to 9, 9 to 12, 12 to 15, 15 to 18 and 18 to 21 of gestation.

Food consumption was slightly decreased in the 50 mg/kg/day dose group between gestation days 6 and 9 (following the first 3 days of treatment) but was otherwise unaffected by ENB-0040.

Dosing Solution Analysis

Duplicate samples were collected in containers from each dose formulation on the days of preparation for Weeks 1, 4 and last, for concentration verification.

All samples analyzed were within the acceptance criteria of ±10% (individual values within ±15%) of their nominal concentrations.

Necropsy

On Day 21 of gestation, dams were euthanized and the dams and fetuses were examined. After a complete gross pathological examination, the reproductive tract was dissected out, the ovaries removed and the corpora lutea counted. The gravid uterus was weighed, the uterine contents (including the placentas) were examined and the number and position of live and dead fetuses and early and late resorptions were recorded. The uterus of any animal judged to be non-pregnant was stained with 10% (v/v) aqueous ammonium sulfide solution and examined for implantation sites.
Each fetus was weighed, given a detailed external examination, the sex recorded and euthanized. One fetus/sex/litter was randomly selected and the heart (with the aorta), kidneys and placenta were placed in 10% neutral buffered formalin for possible future histological examination. One fetus/sex/litter was randomly selected and was preserved whole in formalin for possible future examination. Any offspring abnormalities (major external malformations or minor external anomalies) were recorded.

No ENB-0040 related gross findings were noted. There was no still birth, and the rate of pregnancy was 100% for all groups. There were no ENB-0040 related effects on the numbers of implantations, live or dead fetuses, resorptions, the post implantation loss or the gravid uterine weights. Pre-implantation loss appeared to be increased in the 50 mg/kg/day dose group; however, this was due to two animals (Nos. 4503 and 4506) with high values for pre-implantation loss. Data are presented in the applicant's Tables below.

Table 7 Summary of Uterine Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 13 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Summary Information	Total Number of Corpora Lutea	Total Implantation Sites	Male Fetuses	Female Fetuses	Sex Ratio (% Males)
1	Mean	14.9	13.6	7.7	5.4	58.79
	SD	2.7	1.5	1.3	1.1	6.25
	N	7	7	7	7	7
2	Mean	14.7	13.3	5.7	7.3	44.94
	SD	2.4	2.2	1.4	2.4	13.17
	N	7	7	7	7	7
3	Mean	16.1	14.7	7.3	6.7	52.27
	SD	2.0	1.4	2.5	2.7	16.49
	N	7	7	7	7	7
4	Mean	16.6	13.4	6.9	6.6	48.69
	SD	2.9	4.4	2.7	2.2	14.33
	N	7	7	7	7	7

Significantly different from control group (group 1) value: a - $P \leq 0.05$ b - $P \leq 0.01$ c - $P \leq 0.001$ (Wilcoxon)

Table 7 Summary of Uterine Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 13 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Summary Information	Live Fetuses	Dead Fetuses	Early Resorptions	Late Resorptions	Sum of Resorptions
1	Mean	13.1	0.0	0.4	0.0	0.4
	SD	1.8	0.0	1.1	0.0	1.1
	N	7	7	7	7	7
2	Mean	13.0	0.0	0.3	0.0	0.3
	SD	2.0	0.0	0.5	0.0	0.5
	N	7	7	7	7	7
3	Mean	14.0	0.0	0.7	0.0	0.7
	SD	2.0	0.0	1.1	0.0	1.1
	N	7	7	7	7	7
4	Mean	13.4	0.0	0.0	0.0	0.0
	SD	4.4	0.0	0.0	0.0	0.0
	N	7	7	7	7	7

Significantly different from control group (group 1) value: a - $P \leq 0.05$ b - $P \leq 0.01$ c - $P \leq 0.001$ (Wilcoxon)

Table 7 Summary of Uterine Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 13 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Summary Information	Preimplantation Loss (%)	Post Implantation Loss (%)	Gravid Uterus Weight (g)
1	Mean	7.59	3.06	103.0
	SD	8.14	8.09	12.4
	N	7	7	7
2	Mean	9.53	1.91	101.0
	SD	8.98	3.27	11.5
	N	7	7	7
3	Mean	8.43	5.06	110.0
	SD	5.29	7.94	15.3
	N	7	7	7
4	Mean	20.04	0.00	103.7
	SD	20.90	0.00	30.6
	N	7	7	7

Significantly different from control group (group 1) value: a - $P \leq 0.05$ b - $P \leq 0.01$ c - $P \leq 0.001$ (Wilcoxon)

Fetal weights were not affected by the administration of ENB-0040 to the maternal animals.

There were no major fetal malformations in any group. One fetus (No. 2505/3) from a dam in the 13 mg/kg/day dose group was noted to have subcutaneous edema of the cranium, a minor anomaly. No fetuses were noted to have any evidence of ectopic calcification. Data are presented in the applicant's Table below.

Table 9 Summary of Fetal External Findings - Major Malformations and Minor Anomalies

Group 1 - Vehicle Control
Group 2 - ENB-0040 13 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

	Group 1		2		3		4	
	L/E	F/E	L/E	F/E	L/E	F/E	L/E	F/E
External	7	92	7	91	7	98	7	94
	L/A	F/A	L/A	F/A	L/A	F/A	L/A	F/A
Major Malformations (Total)	0	0	0	0	0	0	0	0
Minor External Anomalies (Total)	0	0	1	1	0	0	0	0
Cranium								
Subcutaneous edema	0	0	1	1	0	0	0	0

L/E = Litters examined L/A = Litters affected
F/E = Fetuses examined F/A = Fetuses affected

Study title: An Embryo-fetal Development Study of ENB-0040 by Intravenous Injection in Rats.

Study no.:	902234
Study report location:	Electronic submission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	March 3, 2011
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Lot # 169466; Purity: 99.2%

Key Study Findings

Four groups of pregnant Crl:SD female rats (25 females/group) received IV ENB-0040 at dose levels of 0, 13, 25 and 50 mg/kg/day from day 6 to day 19 of gestation. Animals in all dose groups showed transient clinical signs. Animals in the 25 and 50 mg/kg/day dose groups showed decreased activity, partly closed eyes and irregular respiratory rates up to 1 hour post-dose, but were recovered by 4 hours post-dose. Based on these findings, the maternal no-observed-effect level (NOEL) was considered to be 13 mg/kg/day. There was no evidence of fetotoxicity, embryolethality or teratogenicity attributed to ENB-0040. The NOAEL for embryo-fetal development was considered to be 50 mg/kg/day.

Methods

Doses:	0, 13, 25, 50 mg/kg/day
Frequency of dosing:	Daily
Dose volume:	5 mL/kg
Route of administration:	IV
Formulation/Vehicle:	25 mM sodium phosphate pH 7.4, 150 mM sodium chloride
Species/Strain:	Crl:CD(SD) rats
Number/Sex/Group:	25 females/dose group
Satellite groups:	No
Study design:	

Experimental Design

Group No.	Test Material	Dosage Level (mg/kg)	Concentration (mg/mL)	Dosage Volume (mL/kg)	Animal No. Main study
1	Vehicle Control	0	0	5	25
2	ENB-0040	13	2.6	5	25
3	ENB-0040	25	5	5	25
4	ENB-0040	50	10	5	25

Deviation from study protocol: None

Observations and Results

Mortality

All animals were observed twice daily for mortality and still birth.

No deaths occurred before the time of scheduled termination.

Clinical Signs

All animals were observed twice daily for clinical signs. In addition, a detailed examination was performed on gestation days 6 to 9 prior to dose administration and generally at 15 minutes, 1 and 4 hours post dose.

Test article-related clinical signs include red skin on the fore and hindpaws, red skin on the pinnae, swollen soft fore and hindpaws and swollen soft muzzle in all treated groups, observed from 15 minutes up to 4 hours post-dose, at which point most of the swelling had disappeared or significantly decreased. Animals in the 25 and 50 mg/kg/day dose groups showed decreased activity, partly closed eyes and irregular respiratory rates for up to 1 hour post-dose, but they recovered by 4 hours post-dose. At 50 mg/kg/day blue skin on the fore and hindpaws and transient incoordination were observed up to 1 hour post-dose, but generally recovered at 4 hours post-dose.

Body Weight

Body weights were measured on Days 0, 3, 6, 9, 12, 15, 18 and 21 of gestation.

No effects on body weights and body weight gains were observed.

Feed Consumption

Food consumption was measured on Days 3 to 6, 6 to 9, 9 to 12, 12 to 15, 15 to 18 and 18 to 21 of gestation.

No ENB-0040-related effect was noted on food consumption.

Toxicokinetics

No toxicokinetics data were provided.

Dosing Solution Analysis

Duplicate samples were collected from each dose formulation on the days of preparation for Weeks 1, 4 and last, for concentration verification.

All study samples analyzed were within the acceptance criteria of ±10% (individual values within ±15%) of their theoretical concentrations.

Necropsy

Cesarean Section Data (Implantation Sites, Pre- and Post-Implantation Loss, etc.)

Animals were euthanized on Day 21 of gestation and subjected to a complete necropsy examination, which included evaluations of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain and thoracic, abdominal, and pelvic cavities with their associated organs and tissues. The gravid uterus was weighed. The uterus was opened and the contents were examined. The fetuses were removed from the uterus and placed in individual containers. The rats were examined for the number and distribution of corpora lutea, implantation sites, size, color or shape of the placentae, live and dead fetuses and early and late resorptions. Uteri of non-pregnant animals were stained with 10% (v/v) ammonium sulfide solution and examined for implantation sites.

No treatment-related gross findings were noted in any group. One female in the high dose group was not pregnant. The pregnancy rate was 100% in control, low and mid dose groups, and 96% in the high dose group. No effects were observed on the number of corpora lutea, implantation sites, male fetuses, female fetuses, live fetuses, dead fetuses, or resorptions. Pre- and post-implantation losses and gravid uterus weights for the treatment groups were comparable to controls. Data are presented in the applicant's Tables below.

Table 7 Summary of Ovarian and Uterine Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 13 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Summary Information	Total Number of Corpora Lutea	Total Implantation Sites	Male Fetuses	Female Fetuses	Sex Ratio (% Males)
1	Mean	15.2	12.8	5.7	6.4	48.26
	SD	2.6	3.5	2.7	2.6	20.02
	N	25	25	25	25	25
2	Mean	14.9	13.0	6.8	5.6	55.12
	SD	2.1	1.9	1.8	1.7	12.35
	N	25	25	25	25	25
3	Mean	14.9	12.6	6.2	5.7	52.42
	SD	2.5	2.4	1.9	1.8	13.97
	N	25	25	25	25	25
4	Mean	15.5	14.2	7.1	6.2	53.73
	SD	2.3	1.9	2.0	2.2	13.99
	N	24	24	24	24	24

Significantly different from control group (group 1) value: a - $P \leq 0.05$ b - $P \leq 0.01$ c - $P \leq 0.001$ (Wilcoxon)

Table 7 Summary of Ovarian and Uterine Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 13 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Summary Information	Live Fetuses	Dead Fetuses	Early Resorptions	Late Resorptions	Sum of Resorptions
1	Mean	12.1	0.0	0.7	0.0	0.7
	SD	3.7	0.0	1.3	0.0	1.3
	N	25	25	25	25	25
2	Mean	12.4	0.0	0.6	0.0	0.6
	SD	1.9	0.0	0.9	0.0	0.9
	N	25	25	25	25	25
3	Mean	12.0	0.0	0.6	0.0	0.6
	SD	2.3	0.0	0.9	0.0	0.9
	N	25	25	25	25	25
4	Mean	13.3	0.0	0.9	0.0	0.9
	SD	2.3	0.0	1.6	0.0	1.6
	N	24	24	24	24	24

Significantly different from control group (group 1) value: a - $P \leq 0.05$ b - $P \leq 0.01$ c - $P \leq 0.001$ (Wilcoxon)

Table 7 Summary of Ovarian and Uterine Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 13 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Summary Information	Pre Implantation Loss (%)	Post Implantation Loss (%)	Gravid Uterus Weight (g)
1	Mean	15.85	5.78	96.3
	SD	20.41	10.93	26.3
	N	25	25	25
2	Mean	12.26	4.14	101.1
	SD	10.39	6.27	13.9
	N	25	25	25
3	Mean	15.72	4.68	96.2
	SD	10.78	6.60	17.2
	N	25	25	25
4	Mean	7.75	6.33	104.9
	SD	8.03	10.63	15.8
	N	24	24	24

Significantly different from control group (group 1) value: a - $P \leq 0.05$ b - $P \leq 0.01$ c - $P \leq 0.001$ (Wilcoxon)

Offspring (Malformations, Variations, etc.)

Fetuses were examined for sex and external abnormalities including evidence of ectopic calcification. The body weight of each fetus was recorded. Approximately one-half of the fetuses in each litter were examined for visceral abnormalities by using a modification of the micro-dissection technique. The remaining fetuses (approximately one-half of the fetuses in each litter, plus one abnormal fetus (No. 3515-5) were examined for skeletal abnormalities after staining with alizarin red S.

There was no ENB-0040 related effect on fetal weight, and no external and visceral anomalies were noted. No skeletal anomalies related to ENB-0040 administration were noted. ENB-0040 administration was not associated with any major malformations in fetuses. One fetus (No. 3515-5, 25 mg/kg/day) was noted to have short/fused digits of forepaws. One other fetus (No. 3515-10, 25 mg/kg/day) was noted to have situs inversus, and one fetus (No. 2525-11, 13 mg/kg/day) was noted to have the accessory lung lobe absent. However, these findings were not considered to be treatment-related.

Study title: A Dosage Range-finding Embryo-fetal Development Study of ENB-0040 by Intravenous Injection in Rabbits.

Study no.:	902236
Study report location:	Electronic submission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	October 20, 2010
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Lot # 169466; Purity: 99.2%

Key Study Findings

In this dose range-finding study, ENB-0040 was administered intravenously to pregnant rabbits at dose levels of 6, 13, 25 and 50 mg/kg/day on Days 7 to 19 of gestation. Reduced body weight gain was observed at doses ≥25 mg/kg/day. There was no evidence of teratogenicity. Based on the results of this study, the doses selected for the definitive embryo-fetal development study were 13, 25 and 50 mg/kg/day.

Methods

Doses:	0, 6, 13, 25 and 50 mg/kg/day
Frequency of dosing:	Once daily
Dose volume:	2 mL/kg
Route of administration:	IV
Formulation/Vehicle:	25 mM sodium phosphate, 150 mM sodium chloride, pH 7.4
Species/Strain:	New Zealand White rabbits (*Oryctolagus cuniculus*)
Number/Sex/Group:	5 females/dose group
Satellite groups:	No

Study design:

Text Table 1 Study Design

Group No.	Test Material	Dosage Level (mg/kg)	Concentration (mg/mL)	Dosage Volume (mL/kg)	Animal No. Study Animal Nos.
1	Vehicle Control	0	0	2	1501-1505
2	ENB-0040	6	3	2	2501-2505
3	ENB-0040	13	6.5	2	3501-3505
4	ENB-0040	25	12.5	2	4501-4505
5	ENB-0040	50	25	2	5501-5505

Deviation from study protocol: No.

Observations and Results

Mortality

All animals were observed twice daily for mortality and still birth.

There was no mortality or still birth in any group.

Clinical Signs

All animals were observed twice daily for clinical signs. In addition, detailed examinations were performed once pretreatment, prior to dosing, and approximately 15 minutes, 1 and 4 hours post dosing from days 7 to 11 of gestation.

Clinical observations (skin red/blue, skin scabs, fur staining and thin fur cover) were observed in animals across all dose groups including the control, and were considered either incidental or secondary to the experimental procedures. Decreased fecal output, soft feces and reduced appetite were noted on occasion in a few animals from each treated group between Days 11 and 20 of gestation.

Body Weight

Individual body weights were measured on Days 0, 5, 7, 10, 13, 16, 20, 23, 26 and 29 of gestation. There were slight, statistically insignificant reductions in body weight gain at 25 mg/kg/day throughout the treatment period and at 50 mg/kg/day from gestation Day 16 to 29. Summary of body weight changes (kg) is presented the applicant's Table below.

Table 5 Summary of Corrected Body Weights and Body Weight Changes (kg)

Group 1 - Vehicle Control
Group 2 - ENB-0040 6 mg/kg/day
Group 3 - ENB-0040 13 mg/kg/day
Group 4 - ENB-0040 25 mg/kg/day
Group 5 - ENB-0040 50 mg/kg/day

Group	Summary Information	Corrected Body Weight Day 29 of Gestation	Corrected Body Weight Change Day 7-29 of Gestation
1	Mean	3.3320	0.0570
	SD	0.0986	0.1980
	N	4	4
2	Mean	3.1656	-0.0944
	SD	0.2284	0.1165
	N	5	5
3	Mean	3.2073	-0.1428
	SD	0.2467	0.1358
	N	4	4
4	Mean	3.1188	-0.0612
	SD	0.2469	0.1376
	N	5	5
5	Mean	3.1408	-0.1192
	SD	0.1337	0.1212
	N	5	5

Significantly different from control group (group 1) value: A - $P \leq 0.05$ B - $P \leq 0.01$ C - $P \leq 0.001$ (Dunnett)
D - $P \leq 0.05$ E - $P \leq 0.01$ F - $P \leq 0.001$ (Dunn)

Feed Consumption

Individual food consumption was measured daily from gestation Day 5 till the end of the study.

There were no changes in food intake.

Toxicokinetics

For toxicokinetic analysis, blood samples (approximately 0.5 mL) were collected from an auricular artery. On Day 7 of gestation, blood samples were collected from each animal before dosing. On Day 19 of gestation, blood samples were collected at 0 and 6 hours after dosing from control animals (Group 1) and at 0, 0.5, 1.5, 3, 6, 12, 24, 72 and 144 hours after dosing from ENB-0040 treated groups (Groups 2, 3, 4 and 5).

Following multiple intravenous administrations, systemic clearance (CL) values decreased from 0.136 to 0.0264 L/h/kg for 6 to 50 mg/kg dose levels. This suggests some non-linearities in CL at higher dose levels. Total volume of distribution (V_{ss}) decreased as dose increased, with V_{ss} values ranging from 0.0421 to 0.176 L/kg. AUC_{0-}

$_{24}$ ranged from 64.59 to 1964 mg.h/L from the low to the high dose level, while C_{max} values ranged from 36.2 to 785 mg/L. The C_{max} and AUC_{0-24} increased in a more than dose proportional manner from 6 mg/kg to 50 mg/kg dose levels. The T_{max} was observed at the first sampled time point (30 minutes post dose) in all cases. Summary of the TK parameters is presented in the applicant's Table below.

Text Table 3 Summary TK Parameters of ENB-0040 in Gravid Female Rabbits

Mean (% CV)				
Parameters	**ENB-0040 (Group 2: 6 mg/kg IV)**	**ENB-0040 (Group 3: 13 mg/kg IV)**	**ENB-0040 (Group 4: 25 mg/kg IV)**	**ENB-0040 (Group 5: 50 mg/kg IV)**
N	5	5	5	5
AUC_{0-24} (mg•h/L)	64.59 (44.7)	245.4 (41.2)	726.8 (14.9)	1964 (19.9)
AUC_{0-t} (mg•h/L)	63.62 (45.5)	253.4 (47.8)	722.6 (15.0)	1954 (21.5)
$AUC_{0-\infty}$ (mg•h/L)	85.77 (NC)[b]	294.6 (33.5)[c]	728.7 (15.3)	1855 (20.2)[c]
C_{max} (mg/L)	36.2 (42.8)	128 (32.9)	370 (14.0)	785 (16.9)
T_{max}[a] (h)	0.50 (0.50, 0.50)	0.50 (0.50, 0.50)	0.50 (0.50, 0.50)	0.50 (0.50, 0.50)
$t_{1/2}$ (h)	5.22 (NC)[b]	4.90 (139.1)[c]	3.51 (79.6)	2.35 (122.3)[c]
CL (L/h/kg)	0.136 (91.8)	0.0641 (57.3)	0.0351 (17.0)	0.0264 (22.7)
V_{ss} (L/kg)	0.176 (NC)[b]	0.0894 (90.1)[c]	0.0511 (36.1)	0.0421 (25.5)[c]

[a] Median (Min, Max), [b] n = 2, [c] n = 4, NC = Not calculated

The TK data for the high dose group were amended to avoid the impact of the sample results associated with optical density (OD) oversaturation. The impact of the exclusion of samples associated with OD oversaturation on TK parameters of ENB-0040 is presented in the applicant's Table below

Text Table 4 Mean TK Parameters of ENB-0040 in Gravid Female Rabbits on Gestation Day 19

TK Parameters	**Mean (CV%)**	
	All samples	**Samples Associated to OD Oversaturation Excluded (NR Sample)**
	ENB-0040 (Group 5: 50 mg/kg IV)	**ENB-0040 (Group 5: 50 mg/kg IV)**
AUC_{0-24} (mg•h/L)	**1964 (19.9)**	**2037 (25.0)**
C_{max} (mg/L)	**785 (16.9)**	**785 (16.9)**
CL (L/h/kg)	**0.0264 (22.7)**	**0.0259 (26.0)**

The analysis showed that exclusion of samples with OD oversaturation had no impact on C_{max} of ENB-0040 and had a minor impact (less than 4% changes) on mean AUC_{0-24} and CL parameters.

Dosing Solution Analysis

One sample for analysis and one sample for retention were collected from each dose formulation on the day of preparation for concentration verification. Samples were analyzed using a validated analytical procedure.

All study samples analyzed were within the acceptance criteria of ±10% (individual values within ±15%) of their nominal concentrations.

Necropsy

Cesarean Section Data (Implantation Sites, Pre- and Post-Implantation Loss, etc.)

Animals were euthanized on Day 29 of gestation and a complete gross pathology examination of the carcass was performed as. All scheduled necropsies included evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues. After necropsy examinations, the reproductive tract was dissected out, the ovaries removed and the corpora lutea counted. The gravid uterus was weighed, the uterine contents (including the placentas) were examined and the number and position of live and dead fetuses and early and late resorptions and/or empty implantation sites were recorded. The uterus of animals judged to be non-pregnant was stained with 10% (v/v) aqueous ammonium sulfide solution and examined for implantation sites.

The pregnancy rates were 80%, 100%, 80%, 100% and 100% for control, 6, 13, 25 and 50 mg/kg dose groups, respectively.
The implantation sites, live and dead fetuses, sex ratio and pre and post implantation losses (%) were unaffected by administration of ENB-0040 when compared to control. There was a significant increase ($p \leq 0.05$) in the number of corpora lutea at 25 mg/kg/day. This was likely due to biological variations and not considered to be test article-related. A slightly higher number of early and late resorptions compared to control animals were noted at 25 and 50 mg/kg/day. However, these values were within the historical control range, and not considered treatment-related. Data are presented in the applicant's Tables below.

Table 7 Summary of Uterine Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 6 mg/kg/day
Group 3 - ENB-0040 13 mg/kg/day
Group 4 - ENB-0040 25 mg/kg/day
Group 5 - ENB-0040 50 mg/kg/day

Group	Summary Information	Total Number of Corpora Lutea	Total Implantation Sites	Male Fetuses	Female Fetuses	Sex Ratio (% Males)
1	Mean	6.5	5.8	3.3	2.5	66.38
	SD	1.9	2.5	1.3	3.1	33.71
	N	4	4	4	4	4
2	Mean	8.6	8.6	3.8	4.8	45.08
	SD	1.3	1.3	1.3	1.9	15.70
	N	5	5	5	5	5
3	Mean	9.3	8.5	4.8	3.8	57.08
	SD	1.5	1.3	0.5	1.5	12.18
	N	4	4	4	4	4
4	Mean	10.6 a	9.2	5.0	3.8	57.94
	SD	0.9	1.6	1.2	1.8	17.04
	N	5	5	5	5	5
5	Mean	9.2	8.2	4.0	3.6	52.50
	SD	1.8	0.8	1.4	1.3	18.39
	N	5	5	5	5	5

Significantly different from control group (group 1) value: a - $P \leq 0.05$ b - $P \leq 0.01$ c - $P \leq 0.001$ (Wilcoxon)

Table 7 Summary of Uterine Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 6 mg/kg/day
Group 3 - ENB-0040 13 mg/kg/day
Group 4 - ENB-0040 25 mg/kg/day
Group 5 - ENB-0040 50 mg/kg/day

Group	Summary Information	Live Fetuses	Dead Fetuses	Early Resorptions	Late Resorptions	Sum of Resorptions
1	Mean	5.8	0.0	0.0	0.0	0.0
	SD	2.5	0.0	0.0	0.0	0.0
	N	4	4	4	4	4
2	Mean	8.6	0.0	0.0	0.0	0.0
	SD	1.3	0.0	0.0	0.0	0.0
	N	5	5	5	5	5
3	Mean	8.5	0.0	0.0	0.0	0.0
	SD	1.3	0.0	0.0	0.0	0.0
	N	4	4	4	4	4
4	Mean	8.8	0.0	0.2	0.2	0.4
	SD	1.8	0.0	0.4	0.4	0.5
	N	5	5	5	5	5
5	Mean	7.6	0.0	0.4	0.2	0.6
	SD	0.5	0.0	0.5	0.4	0.5
	N	5	5	5	5	5

Significantly different from control group (group 1) value: a - $P \leq 0.05$ b - $P \leq 0.01$ c - $P \leq 0.001$ (Wilcoxon)

Table 7 Summary of Uterine Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 6 mg/kg/day
Group 3 - ENB-0040 13 mg/kg/day
Group 4 - ENB-0040 25 mg/kg/day
Group 5 - ENB-0040 50 mg/kg/day

Group	Summary Information	Number of Empty Implantation Sites	Preimplantation Loss (%)	Post Implantation Loss (%)	Gravid Uterus Weight (g)
1	Mean	0.0	13.58	0.00	393.0
	SD	0.0	18.86	0.00	154.8
	N	4	4	4	4
2	Mean	0.0	0.00	0.00	514.4
	SD	0.0	0.00	0.00	83.7
	N	5	5	5	5
3	Mean	0.0	7.50	0.00	467.8
	SD	0.0	9.57	0.00	95.3
	N	4	4	4	4
4	Mean	0.0	12.94	4.50	521.2
	SD	0.0	15.46	6.22	50.9
	N	5	5	5	5
5	Mean	0.0	9.10	6.94	459.2
	SD	0.0	12.87	6.36	74.3
	N	5	5	5	5

Significantly different from control group (group 1) value: a - $P \leq 0.05$ b - $P \leq 0.01$ c - $P \leq 0.001$ (Wilcoxon)

Offspring (Malformations, Variations, etc.)

Each fetus was weighed, examined and their sex determined. All fetuses were examined for abnormalities (major external malformations or minor external anomalies) and signs of ectopic calcification.

The fetal weights (males, females and total) of the treatment groups were slightly lower than controls at 6, 13, 25 and 50 mg/kg/day (88%, 80%, 88% and 91% for total fetal weight, respectively). However, this was considered to be related to the differences in the live litter size for these groups. No major external malformations or minor external anomalies were noted in any fetuses. Data are presented in the applicant's Tables below.

Table 8 Summary of Litter Mean Fetal Weights (g)

Group 1 - Vehicle Control
Group 2 - ENB-0040 6 mg/kg/day
Group 3 - ENB-0040 13 mg/kg/day
Group 4 - ENB-0040 25 mg/kg/day
Group 5 - ENB-0040 50 mg/kg/day

Group	Summary Information	Males	Females	Total
1	Mean	48.84	49.16	48.51
	SD	2.85	4.48	3.00
	N	4	3	4
2	Mean	44.47	40.79	42.56
	SD	1.61	5.52	3.22
	N	5	5	5
3	Mean	38.68	39.16	39.05
	SD	3.74	2.55	2.99
	N	4	4	4
4	Mean	43.52	43.07	42.88
	SD	8.31	5.96	6.90
	N	5	5	5
5	Mean	43.13	45.05	43.91
	SD	4.53	4.32	4.32
	N	5	5	5

Significantly different from control group (group 1) value: A - $P \leq 0.05$ B - $P \leq 0.01$ C - $P \leq 0.001$ (Dunnett)
D - $P \leq 0.05$ E - $P \leq 0.01$ F - $P \leq 0.001$ (Dunn)

Table 9 Summary of Fetal External Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 6 mg/kg/day
Group 3 - ENB-0040 13 mg/kg/day
Group 4 - ENB-0040 25 mg/kg/day
Group 5 - ENB-0040 50 mg/kg/day

	Group 1		2		3		4		5	
	L/E	F/E	L/E	F/E	L/E	F/E	L/E	F/E	L/E	F/E
External	4	23	5	43	4	34	5	44	5	38
	L/A	F/A	L/A	F/A	L/A	F/A	L/A	F/A	L/A	F/A
Major Malformations (Total)	0	0	0	0	0	0	0	0	0	0
Minor Anomalies (Total)	0	0	0	0	0	0	0	0	0	0

L/E = Litters examined L/A = Litters affected
F/E = Fetuses examined F/A = Fetuses affected

Study title: An intravenous Injection Study of Embryo-fetal Development with ENB-0040 in Rabbits.

Study no.:	902237
Study report location:	Electronic submission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	March 19, 2012
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Lot 3259248; purity: 98.9%

Key Study Findings

ENB-0040 was administered once daily by intravenous injections to mated New Zealand White rabbits from Day 7 to day19 of gestation at dose levels of 10, 25 and 50 mg/kg/day. There were no ENB-0040-related effects on the number of corpora lutea, implantation sites, live fetuses, dead fetuses, resorptions and pre and post implantation losses at any dose levels. There was no evidence of fetal toxicity, teratogenicity or embryolethality. The no-observed-effect-level (NOEL) for embryo-fetal development was considered to be 50 mg/kg/day. Tubular mineralization was noted in the kidneys of two pregnant animals at 50 mg/kg/day. Based on this finding, the maternal no-observed-adverse-effect level (NOAEL) was considered to be 25 mg/kg/day.

Methods

Doses:	0, 10, 25 and 50 mg/kg/day
Frequency of dosing:	Once daily
Dose volume:	2 mL/kg
Route of administration:	IV
Formulation/Vehicle:	25 mM sodium phosphate pH 7.4, 150 mM sodium chloride
Species/Strain:	New Zealand White Rabbits
Number/Sex/Group:	20 females /dose group in main study
Satellite groups:	Yes, for TK analysis- 2 females in control group and 3 females/dose group
Study design:	Experimental Design

Group No.	Test Material	Dosage Level (mg/kg)	Concentration (mg/mL)	Dosage Volume (mL/kg)	No. of Animals	
					Main Study	Toxicokinetic
1	Vehicle Control	0	0	2	20	2
2	ENB-0040	10	5	2	20	3
3	ENB-0040	25	12.5	2	20	3
4	ENB-0040	50	25	2	20	3

Deviation from study protocol:	No

Observations and Results

Mortality

Animals were observed twice daily for general health/mortality and moribundity.

There was no mortality in any group.

Clinical Signs

Cage side observations were performed once daily, from Days 7 to 19 of gestation.

There were no ENB-0040-related clinical signs noted.

Body Weight

Body weights were recorded for all animals on Days 0, 5, 7, 10, 13, 16, 20, 23, 25 and 29 of gestation.

Body weight was not affected by ENB-0040 IV administration.

Feed Consumption

Food consumption was measured daily from Day 5 of gestation onward.

Food consumption was not affected by ENB-0040 administration.

Toxicokinetics

For toxicokinetic evaluation, blood samples (0.5 mL) were collected from an auricular artery. Sample collection schedule is described in the applicant's Table below.

TK Sample Collection Schedule

Group No.	No. of Females	Sample Collection Time Points (Time Postdose) on Days 7 and 19 pc						
		0[a] hr	0.5 h	1.5 hr	3 hr	6 hr	12 hr	24 hr
1	2	X	X	-	-	-	-	-
2	3	X	X	X	X	X	X	X
3	3	X	X	X	X	X	X	X
4	3	X	X	X	X	X	X	X

X = Sample collected; - = Not applicable; [a] Sample collected before dosing.

Following intravenous administrations, plasma concentrations of ENB-0040 declined rapidly from the peak levels over the sampling intervals. Mean plasma concentrations were quantifiable up to 24 h post dose except for Animal Nos. 2522, 2523 and 3521 for which measurable concentrations were observed up to 12h. The concentrations on Day 19 pc were associated with more variability. Plasma concentrations were BLQ for all animals in the vehicle control group.

Following a single intravenous administration, on Day 7 pc, the mean CL values of ENB-0040 at 10, 25 and 50 mg/kg/day dose levels were 0.0169, 0.0117 and 0.0113 L/h/kg, respectively. Mean V_{ss} of ENB-0040 at 10, 25 and 50 mg/kg/day dose levels were 0.287, 0.145 and 0.0904 L/kg, respectively. Mean $AUC_{0-\infty}$ values of ENB-0040 at 10, 25 and 50 mg/kg/day dose levels were 614, 2301 and 4600 mg.h/L, respectively, while the mean C_{max} values were 121, 584 and 1135 mg/L, respectively.

Following repeated daily IV administrations, the mean CL values on Day 19 pc were greater than those observed on Day 7 pc values at the 10, 25 and 50 mg/kg/day dose levels (0.0347, 0.0164 and 0.0156 L/h/kg, respectively). Mean $AUC_{0-\infty}$ values on Day 19 pc were 332, 1608 and 2880 mg.h/L, respectively, while mean C_{max} values were 125, 504 and 1021 mg/L, respectively. A summary of TK parameters is presented in the applicant's Table below.

Summary TK Parameters Derived from Concentrations of ENB-0040 in Pregnant Female Rabbits on Days 7 and 19 pc

ENB-0040 TK Parameters	Mean (CV%)					
	Day 7 pc			Day 19 pc		
	Group 2 10 mg/kg/day IV	Group 3 25 mg/kg/day IV	Group 4 50 mg/kg/day IV	Group 2 10 mg/kg/day IV	Group 3 25 mg/kg/day IV	Group 4 50 mg/kg/day IV
	N = 3	N= 3	N = 3	N = 3	N = 3	N = 3
AUC_{0-24} (mg•h/L)	446 (13.5)	1978 (46.0)	4188 (17.6)	322 (43.0)	1573 (20.8)	3435 (31.4)
AUC_{0-t} (mg•h/L)	447 (13.5)	1860 (55.4)	4189 (17.6)	321 (43.5)	1566 (21.5)	3435 (31.4)
$AUC_{0-\infty}$ (mg•h/L)	614 (24.9)	2301 (35.0)	4600 (NC)[b]	332 (47.4)	1608 (24.1)	2880 (NC)[b]
C_{max} (mg/L)	121 (34.6)	584 (84.2)	1135 (13.2)	125 (13.9)	504 (22.4)	1021 (3.2)
t_{max}[a] (h)	0.50 (0.50, 0.50)	0.50 (0.50, 0.52)	0.50 (0.50, 0.50)	0.52 (0.50, 0.53)	0.52 (0.50, 0.52)	0.52 (0.50, 0.52)
$t_{1/2}$ (h)	16.8 (41.2)	10.9 (30.7)	10.4 (NC)[b]	3.64 (78.4)	4.07 (70.7)	2.14 (NC)[b]
CL (L/h/kg)	0.0169 (22.8)	0.0117 (30.0)	0.0113 (NC)[b]	0.0347 (37.2)	0.0164 (22.0)	0.0156 (32.0)
V_{ss} (L/kg)	0.287 (27.3)	0.145 (60.5)	0.0904 (NC)[b]	0.0792 (79.2)	0.0557 (44.4)	0.0435 (NC)[b]

[a] Median (Min, Max); [b] n = 2; NC = Not calculated.

Dosing Solution Analysis

Duplicate samples were collected in containers from each dose formulation on the days of preparation for concentration verification. Samples were analyzed using a validated analytical procedure ((b) (4) Validation Study).

All study samples analyzed had mean sample concentrations within the acceptance criteria of ±10% (individual values within ±15%) of their theoretical concentrations.

Necropsy

Cesarean Section Data (Implantation Sites, Pre- and Post-Implantation Loss, etc.)

Animals in the main study groups were euthanized on Day 29 of gestation. Animals were subjected to a complete necropsy examination, which included evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues and injection sites. Toxicokinetic animals euthanized on Day 20 of gestation had their pregnancy status recorded and the carcasses were discarded without any further examination. For animals in the main study groups, after euthanasia on Day 29, the reproductive tract was dissected out, the ovaries removed and the corpora lutea counted. The gravid uterus was weighed, the uterine contents were examined (including the placentas) and the number and position of live and dead fetuses and early and late resorptions and/or empty implantation sites were recorded. The uterus of any animal judged to be non-pregnant was stained with 10% (v/v) aqueous ammonium sulfide solution and examined for implantation sites.

One doe (No. 2511) littered early with 11 kits on Day 29 of gestation before it was euthanized. Ovarian and uterine examinations were conducted as described above except that the uterus was not weighed.

The pregnancy rate was 100, 95, 100 and 90% for the control, 10, 25 and 50 mg/kg/day groups, respectively. There were no ENB-0040-related effects on the mean number of corpora lutea (10.3, 9.6, 9.7 and 9.1), implantation sites (8.8, 8.8, 8.8 and 8.2), live fetuses (8.5, 8.5, 8.7 and 8.1), dead fetuses (0, 0, 0 and 0), resorptions (0.3, 0.2, 0.2 and 0.2) and pre- (15.6, 8.4, 8.4 and 9.1) and post-implantation (2.8, 3.0, 1.5 and 2.8) losses for the control, 10, 25 and 50 mg/kg/day groups, respectively. Data are presented in the applicant’s Tables below.

Table 7 Summary of Ovarian and Uterine Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 10 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Summary Information	Total Number of Corpora Lutea	Total Number of Implantation Sites	Male Fetuses	Female Fetuses	Sex Ratio (% Males)
1	Mean	10.3	8.8	4.3	4.3	48.69
	SD	2.0	2.5	1.9	1.4	13.12
	N	20	20	20	20	20
2	Mean	9.6	8.8	4.6	3.9	53.77
	SD	1.3	1.4	1.3	1.4	14.75
	N	18	18	18	18	18
3	Mean	9.7	8.8	4.8	3.9	53.99
	SD	1.7	1.6	2.0	1.6	19.24
	N	20	20	20	20	20
4	Mean	9.1	8.2	3.6	4.5	43.46
	SD	1.4	1.4	1.6	1.5	15.39
	N	18	18	18	18	18

Significantly different from control group (Group 1) value: D - $P \leq 0.05$ E - $P \leq 0.01$ F - $P \leq 0.001$ (Dunn)

Table 7 Summary of Ovarian and Uterine Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 10 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Summary Information	Live Fetuses	Dead Fetuses	Early Resorptions	Late Resorptions	Sum of Resorptions
1	Mean	8.5	0.0	0.2	0.1	0.3
	SD	2.5	0.0	0.5	0.2	0.6
	N	20	20	20	20	20
2	Mean	8.5	0.0	0.2	0.1	0.2
	SD	1.3	0.0	0.4	0.2	0.4
	N	18	18	18	18	18
3	Mean	8.7	0.0	0.1	0.1	0.2
	SD	1.5	0.0	0.3	0.2	0.4
	N	20	20	20	20	20
4	Mean	8.1	0.0	0.1	0.1	0.2
	SD	1.6	0.0	0.3	0.2	0.4
	N	18	18	18	18	18

Significantly different from control group (Group 1) value: D - $P \leq 0.05$ E - $P \leq 0.01$ F - $P \leq 0.001$ (Dunn)

Table 7 Summary of Ovarian and Uterine Findings

Group 1 - Vehicle Control
Group 2 - ENB-0040 10 mg/kg/day
Group 3 - ENB-0040 25 mg/kg/day
Group 4 - ENB-0040 50 mg/kg/day

Group	Summary Information	Number of Empty Implantation Sites	Preimplantation Loss (%)	Post Implantation Loss (%)	Gravid Uterus Weight (g)
1	Mean	0.0	15.55	2.83	508.2
	SD	0.0	17.83	6.23	141.7
	N	20	20	20	20
2	Mean	0.0	8.43	2.97	506.3
	SD	0.0	9.09	5.01	64.1
	N	18	18	18	18
3	Mean	0.0	8.42	1.50	512.9
	SD	0.0	9.99	3.71	82.5
	N	20	20	20	20
4	Mean	0.0	9.12	2.78	476.2
	SD	0.0	7.71	6.85	91.8
	N	18	18	18	18

Significantly different from control group (Group 1) value: D - $P \leq 0.05$ E - $P \leq 0.01$ F - $P \leq 0.001$ (Dunn)

Gross lesions were found in 3 maternal animals; animal Nos. 4510 and 4519 in the 50 mg/kg/day dose group showed pale discoloration of the kidneys and animal no 2512 in the 10 mg/kg/day showed mediastinal lymph node enlargement and dark discoloration. Two animals (Nos. 4510 and 4519) in the 50 mg/kg/day dose group had pale discoloration of the kidneys, which correlated with the microscopic finding of moderate tubular mineralization. Other gross findings were considered incidental and unrelated to the test item.

Offspring (Malformations, Variations, etc.)

All fetuses were examined for external abnormalities. Body weight of each live fetus was recorded. The fetuses in each litter were examined for visceral abnormalities. Each fetus was examined for skeletal abnormalities after staining with alizarin red S.

Fetal weights (male, female and total) were not affected by ENB-0040 administration. No minor external and visceral abnormalities were observed in the fetuses. No test item-related minor skeletal anomalies or common skeletal variations were noted. There were no major malformations attributed to ENB-0040. However, two fetuses from two separate litters in the 10 mg/kg/day dose group showed the following malformations; the fetus from animal # 2501 had a small right eye bulge, and another fetus from animal # 2517 showed a small lower jaw/tongue with a cleft lip/palate and a narrowed pharynx, an absent eye bulge (anophthalmia) and some liver protruding into the umbilicus (omphalocele). These findings were not considered treatment-related as no other findings were observed at the higher doses.

9.3 Prenatal and Postnatal Development

Study title: A Pre- and Postnatal Study of ENB-0040 by Intravenous Injection in Rats.

Study no.:	902238
Study report location:	Electronic submission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	February 13, 2012
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Lot # 259248; purity: 98.9%

Key Study Findings

ENB-0040 was administered to female SD rats (25 females per treatment group) by intravenous injection from Day 6 of gestation to Day 21 post-partum at doses of 10, 25 and 50 mg/kg/day. For the F_0 generation dams, transient non-adverse clinical signs (red skin on the fore- and hindpaws, pinnae and muzzle, blue skin on the forepaws and hindpaws, blue skin of the muzzle, swollen and/or soft forepaws, forelimbs and hindpaws and swollen soft muzzle) were noted in all treated groups within 4 hours post-dose throughout the dosing period. A low incidence of cannibalism of their litter was noted for F_0 dams in the treated groups. In the mid dose group, the number of females that cannibalized one or more pups in the litter was above the historical range (0-2 females per group compared to 3 females in the mid dose group). It is not clear whether the cannibalism was treatment-related. The F_1 generation males in the 50 mg/kg/day dose group showed slightly lower body weight and food consumption during the post weaning period. For offspring (F_1 and F_2 generations), there were no effects on survival, physical development, behavior or reproductive performance. Gross pathology examinations did not show any evidence of treatment-related calcification. The No Observed Adverse Effect Level (NOAEL) for the F_0, F_1 and F_2 generations was considered to be 50 mg/kg/day.

Methods

Doses: 0, 10, 25 and 50 mg/kg/day
Frequency of dosing: Once daily
Dose volume: 5 mL/kg
Route of administration: Intravenous
Formulation/Vehicle: 25 mM sodium phosphate pH 7.4, 150 mM sodium chloride
Species/Strain: Sprague Dawley (Crl:CD[SD]) rats
Number/Sex/Group: Main study; 25 females/dose group; TK study: 2 females in vehicle control and 6 females/dose group
Satellite groups: Yes, for TK study.
Study design:

Experimental Design

Group No.	Dose Level (mg/kg/day)	Dose Volume (mL/kg)	Dose Concentration (mg/mL)	Females (F_0)		F_1 Adults[b]	
				Main Study	TK Study[a]	Males	Females
1/ Vehicle Control	0	5	0	25	2	24	24
2/ ENB-0040	10	5	2	25	6	22	22
3/ ENB-0040	25	5	5	25	6	19	19
4/ ENB-0040	50	5	10	25	6	23	23

[a] Toxicokinetic animals were used for toxicokinetic evaluation only.
[b] 1 weanling/sex/litter (when possible) was randomly selected to form the F_1 adult generation. These were the numbers that were obtained.

Deviation from study protocol: No

Observations and Results (Optional Table)

F_0 Dams

Survival: All animals were observed twice daily for mortality.

There was no ENB-0040 related mortality.

On Day 0 of lactation, 6 dams (No. 2516 at 10 mg/kg/day, Nos. 3510, 3514 and 3517 at 25 mg/kg/day and Nos. 4503 and 4512 at 50 mg/kg/day) were euthanized following cannibalization of their litters. One dam (No. 3516) at 25 mg/kg/day was also euthanized on Day 0 of lactation as there were no live pups at birth.

Clinical signs: Cage side observations were performed pre-dose, and at 15 min, 1 and 4 hours following dosing during the first 3 days of the lactation period (lactation Days 1 to 3) and then on a weekly basis (lactation Days 7, 14 and 20). Detailed clinical observation was performed on Days 0 and 3 of gestation, on the first 3 days of treatment (Days 6 to 8 of gestation), prior to dosing and at 15 min, 1h and 4h following dosing, and on a weekly basis thereafter.

ENB-0040-related clinical signs were noted in all treated groups from Day 1 of drug administration and persisting throughout the dosing period. These observations were generally noted between 15 minutes and 4 hours post-dose, and included red skin on the fore and hindpaws and the pinnae and muzzle, blue skin on the forepaws and hindpaws, blue skin of the muzzle, swollen and/or soft forepaws, fore limbs and hindpaws and swollen soft muzzle.
Six animals in the 25 mg/kg/day and 12 animals in the 50 mg/kg/day dose groups showed decreased activity. Decreased muscle tone for 2 females at 25 and 4 females at 50 mg/kg/day and slight to moderate incoordination for 4 animals at 50 mg/kg/day were also noted on Day 6 of gestation. On Day 7 of gestation, decreased activity and decreased muscle tone were noted 15 minutes post-dose in two females at 50 mg/kg/day (Animal Nos. 4504 and 4505, respectively). There were no observations of decreased activity noted in females at any dose level during the lactation period.

Body weight: Animals were individually weighed on Day 0 pc and daily thereafter, starting on Day 3 of gestation. A terminal weight was recorded on the day of necropsy. There were no ENB-0040-related effects on body weights or body weight gains of dams during the gestation or lactation periods.

Feed consumption: Food consumption was measured on Days 3, 7, 10, 12, 15, 18, and 20 pc, and on Days 1, 4, 7, and 14 of lactation.

There were no ENB-0040-related effects on food consumption.

Necropsy observation: All main study F_0 animals surviving to the end of the study were euthanized followed by a complete necropsy examination. Main study dams were euthanized after weaning (on Day 21 of lactation) and the number of implantation site scars was recorded. Pups were subjected to a necropsy examination.

The length of gestation, the duration of parturition, the number of live and malformed pups, the number of implantation scars, sex ratio and the live birth index were unaffected by the administration of ENB-0040.

A statistically significant increase in the numbers of dead pups at birth was noted at all doses and was attributed to four dams with an elevated number of dead pups noted at birth (Animal No. 2516 at 10 mg/kg/day had 6 dead pups, Animal No. 3510 at 25 mg/kg/day had 6 dead pups and, at 50 mg/kg/day Animal Nos. 4503 and 4512 had 5 and 8 dead pups, respectively). As the increases were not statistically significant on a litter basis, and the group mean numbers of dead pups per litter for these groups was within the historical control range of the Test Facility (0.00 to 0.79 dead pups per litter), these differences were considered not to be treatment related and could be related to cannibalism.

A slight decrease in the gestation index (96%) at 25 mg/kg/day when compared to controls (100%) was due to one dam (No. 3516) at this dose level that had no live pups (i.e., 1 dead pup was found at birth).

Toxicokinetics: For toxicokinetic analysis blood samples (0.5 mL) were collected on Days 6 and 19 of gestation. From control animals, samples were collected at 0 and 30 min after dosing. From animals in the ENB-0040 treated groups, samples were collected at 0 and 30 min, and 1.5, 3, 6, 12 and 24 hours after dosing.

Following the first dose of ENB-00400 administration on Day 6 of gestation, systemic clearance (CL) values of ENB-0040 at 10 and 25 mg/kg/day were 0.0294 and 0.0263 L/h/kg, respectively. Mean total volume of distribution (V_{ss}) at 10 and 25 mg/kg/day were 0.552 L/kg and 0.449 L/kg, respectively. The mean AUC_{0-24} values of ENB-0040 at the 10, 25 and 50 mg/kg/day were 243, 703 and 1502 mg.h/L, respectively, while the mean C_{max} values were 65.9, 247 and 612 mg/L, respectively.

Following repeated daily administrations of ENB-0040, mean CL values on Day 19 pc were greater than Day 6 pc values at 10 and 25 mg/kg/day (0.113 and 0.0735 L/h/kg, respectively). Mean AUC_{0-24} values were 88.2, 340 and 1339 mg.h/L, respectively, while mean C_{max} values were 36.9, 134 and 672 mg/L at 10, 25 and 50 mg/kg, respectively. The TK data are presented in the applicant's Table below.

Summary TK Parameters of ENB-0040 in Pregnant Female Rats

	Baseline-corrected Concentrations of ENB-0040					
	Day 6 pc			Day 19 pc		
TK Parameters	ENB-0040 (Group 2: 10 mg/kg/day)	ENB-0040 (Group 3: 25 mg/kg/day)	ENB-0040 (Group 4: 50 mg/kg/day)	ENB-0040 (Group 2: 10 mg/kg/day)	ENB-0040 (Group 3: 25 mg/kg/day)	ENB-0040 (Group 4: 50 mg/kg/day)
AUC_{0-24} (mg•h/L)	243	703	1502	88.2	340	1339
AUC_{0-t} (±SE) (mg•h/L)	243 (8.95)	703 (34.8)	1502 (142)	86.3 (10.6)	340 (53.2)	1339 (117)
$AUC_{0-\infty}$ (mg•h/L)	340*	952*	NC	87.2	NC	1339
C_{max} (±SE) (mg/L)	65.9 (3.85)	247 (8.25)	612 (140)	36.9 (4.87)	134 (14.7)	672 (143)
T_{max} (h)	0.50	0.50	0.50	0.50	0.50	0.50
$t_{1/2}$ (h)	17.8*	18.1*	NC	1.97	NC	2.11
CL (L/h/kg)	0.0294*	0.0263*	NC	0.113	0.0735	0.0373
V_{ss} (L/kg)	0.552	0.449	NC	0.172	NC	0.0748

NC = Not Calculated; pc = postcoitum.
* Extrapolation exceeded 25%.

Dosing Solution Analysis: Duplicate samples were collected from each dose formulation on the days of preparation for concentration verification. The mean sample concentrations for all study samples analyzed were within the acceptance criteria of ±10% (individual values within ±15%) of their nominal concentrations.

Other: On Day 0 post-partum, 6 dams (No. 2516 at 10 mg/kg/day, Nos. 3510, 3514 and 3517 at 25 mg/kg/day and Nos. 4503 and 4512 at 50 mg/kg/day), which had high numbers (up to 8) of dead pups at birth (except for No. 3514), cannibalized the remaining pups in their litters within 5 hours of parturition. The incidence of the dams showing signs of cannibalism on Day 0 post-partum was within the historical control range of the Test Facility (0 to 2 females) at 10 and 50 mg/kg/day but slightly higher (3 females per group) at 25 mg/kg/day. However, this behavior was not dose dependent.

F_1 Generation

Survival: F_1 animals were observed twice daily for mortality and moribundity.

There were no ENB-0040-related effects on litter size, viability, and survival or lactation indices. The viability index was 99.4% in the control group, 92.2% at 10 mg/kg/day, 89.9% at 25 mg/kg/day and 94.6% at 50 mg/kg/day. There was no mortality at any dose levels in F_1 generation adults.

Clinical signs: The animals were observed twice daily for clinical signs.

There were no treatment-related clinical signs in the F1 pups and adults.

Body weight: F_1 males were individually weighed weekly, following weaning. F_1 females were individually weighed weekly from weaning until at least Day 77 post-partum, on Days 0, 7, 10, 14, 17 and 20 of gestation, and Days 0 and 4 of lactation.

The pup body weights (male and female combined) for the treated groups were comparable to controls throughout the pre-weaning period. A slight reduction in body weight gains was noted for males between Days 21 and 70 post-partum attaining statistical significance ($P \leq 0.05$) between Days 21 and 35 post-partum. There was no ENB-0040 related effect on the body weights at any dose level for F_1 females during the premating, gestation and lactation periods.

Feed consumption: The food consumption for the F_1 males was measured once weekly following weaning until Day 77 post-partum, and for females, the food consumption was measured once weekly until Day 77 post-partum, and on Days 0, 7, 10, 14, 17 and 20 of gestation.

There were statistically significant ($P \leq 0.05$, $P \leq 0.01$) reductions in food consumption for males at 50 mg/kg/day during the premating period after the end of dosing (i.e., between Days 35 to 56 post-partum), including the overall food consumption (i.e., between Days 35 to 77 post-partum). These differences correlated with the body weight changes during the same period and were not considered adverse due to minimal changes in food consumption.

Physical development: The physical development parameters of F_1 pups were assessed as described below: Pinna unfolding was assessed from Day 1 post-partum until all pups in the litter have a positive response. If the assessment continued past Day 4 post-partum, it was assessed before and after culling on Day 4 post-partum. Tooth eruption was assessed from Day 7 post-partum and eye opening from Day 12 post-partum until each pup has a positive response.

The mean day of development for the pinna unfolding, tooth eruption and eye opening were unaffected.

Neurological assessment: Pupillary closure and visual placing of F_1 pups was assessed on Day 21 post-partum. The startle habituation was measured on Day 55 (±2) post-partum. Locomotor activity was assessed on Day 60 (±2) post-partum for 1 hour. Animals were tested for Water Maze between Days 63 and 73 post-partum.

There were no ENB-0040-related effects on pupillary closure and visual placing. Motor activity for the males and females was unaffected by the administration of ENB-0040. There were no ENB-0040-related effects on the auditory startle habituation. There were no test item-related differences noted for the performance times to complete the Water Maze.

Reproduction: Vaginal opening was assessed beginning on Day 26 post-partum and continuing until opening was complete for all females. Preputial separation was assessed from Day 35 post-partum until separation was noted for all males. On Days 80 to 84 post-partum, 1 female was placed with 1 male (sibling mattings were avoided) in the same dosage group for up to 14 days. The females were examined for mating by examination of the vaginal lavage for spermatozoa. The day of positive identification of spermatozoa was termed as Day 0 of gestation.

The mean day to development of preputial separation in males and vaginal opening in females was unaffected by ENB-0040 administration.

The conception rate and the mean day to mating for the F_1 animals were unaffected by treatment of F_0 animals by ENB-0040.

The gestation index was 100% in each group. The length of gestation, the duration of parturition, the number of live and dead pups, the number of implant scars, live birth and the sex ratio for the treated groups were comparable to control values. There were no malformed pups in the ENB-0040-treated groups.

There were no macroscopic findings in F_1 animals attributed to the administration of ENB-0040.

F_2 Generation

Survival: On Day 0 post-partum, the F_2 pups were examined for malformations, sexed, and the numbers of live and dead pups recorded.

The viability index on Day 4 post-partum was comparable to controls in all treated groups.

Body weight: In addition to the assessment of body weight at birth, the pups were weighed individually on Day 4 post-partum.

The pup body weights (male and female combined) for the treated groups were comparable to controls on Days 0 and 4 post-partum.

External evaluation: The general condition of the F_2 pups was evaluated each day during the lactation period. Any pups found dead or euthanized between Days 0 and 6 post-partum was stored in Bouin's fluid for subsequent examination.

There were no findings related to the administration of ENB-0040.

Male/Female ratio: The litter size and male and female ratio for F_2 pups were determined on Days 0 and 4 post-partum.

There was no difference in litter size and male and female ratio.

10 Special Toxicology Studies

No special toxicology studies were submitted.

11 Integrated Summary and Safety Evaluation

In the current BLA, the applicant is seeking approval of STRENSIQ™ (asfotase alfa) SC injection for (b) (4) in patients with infantile- and juvenile-onset hypophosphatasia (HPP). The proposed dosing regimen is 2 mg/kg of body weight, administered three times per week, or a dosage regimen of 1 mg/kg administered six times per week.

Hypophosphatasia (HPP) is an autosomal recessive disease caused by a deficiency of one of the isoenzymes of alkaline phosphatase (ALP). Three isoenzymes of ALP are tissue specific (intestine, placenta, and germ cell) and the fourth is called tissue non-specific ALP (TNSALP) found in the liver, bone, and kidney. Deficiency of the TNSALP leads to problems with bone mineralization and can affect the central nervous system .

Hypophosphatasia has an estimated frequency of 1/100,000. TNSALP has a critical role in bone mineralization. First, it cleaves inorganic phosphate (PPi). When PPi accumulates, it inhibits bone mineralization by inhibiting the formation of hydroxyapatite crystals. TNSALP deficiency leads to accumulation of not only PPi but phosphoethanolamine (PEA) and pyridoxal 5'-phosphate (PLP; a cofactor form of vitamin B6), believed to be the natural substrate of TNSALP. ENB-0040 (asfotase alfa) is a fusion protein that consists of the catalytic domain of human tissue nonspecific alkaline phosphatase, human IgG1 Fc domain and a decamer aspartate peptide used as a bone targeting domain. *In vitro* study showed that asfotase alfa binds with high affinity (up to 97%) to hydroxyapatite, most common mineral component of the bone. A mouse model ($Akp2^{-/-}$) that mimics HPP, was generated by genetic inactivation of TNSALP. These mice are growth impaired, and while normal at birth, develop abnormal bone mineralization and dentition as well as seizures and apnea. When $Akp2^{-/-}$ mice were treated with ENB-0040 (asfotase alfa) intravenously and subcutaneously, they showed significant improvement in bone mineralization and survival rate.

This BLA is supported by primary pharmacology, safety pharmacology, pharmacokinetic and single- and repeated-dose toxicology studies in rats and monkeys and reproductive toxicology studies in rats and rabbits. Treatment with asfotase alfa completely prevented the increase in plasma PPi in $Akp2^{-/-}$ mice. Asfotase alfa had dose-dependent depressant effects on the respiratory function in rats. CNS adverse effects of asfotase alfa included reduced motor activity and abnormal gait and reduced mobility. Asfotase alfa did not show any adverse cardiovascular effects in monkeys at the highest dose tested (10 mg/kg/day for 6 months).

Four-week to 6 month repeated dose toxicity studies were conducted in rats and monkeys following IV or SC administration of asfotase alfa. The NOAEL doses in the 4-week IV toxicity studies in rats and monkeys, with 4 weeks recovery periods, were 30 and 45 mg/kg, respectively. The NOAEL doses in the 6-month IV toxicology study in rats and the 6-month SC toxicology study in monkeys were 13 mg/kg/day and 10 mg/kg/day, respectively. No clinical and pathological findings were noted in the monkey studies. However, rats showed some transient clinical signs including partly closed eyes, decreased muscle tone, lying on the side, hunched posture, cold to touch, uncoordinated movements, decreased activity, abnormal gait and/or blue, red and/or firm swollen hindpaws and/or forepaws and swollen muzzle. A dose related increase in alkaline phosphatase was observed in all repeated dose toxicology studies in rats and monkeys, and this increase was principally due to the presence of the drug in the bloodstream of the animals after each dose. Reproductive and developmental toxicology studies showed that asfotase alfa was not fetotoxic, embryo lethal or teratogenic; had no effect on fertility, and had no adverse effects on pre- and postnatal development in rats.

In conclusion, in support of the nonclinical safety, the applicant has submitted adequate nonclinical studies (pharmacology, pharmacokinetic, toxicology and reproductive and developmental toxicology) of asfotase alfa. The NOAEL doses in the six-month IV toxicity study in rats and SC toxicity study in juvenile monkeys were 13 and 10 mg/kg/day, respectively. The proposed dose for (b) (4)

in patients with infantile- and juvenile-onset hypophosphatasia (HPP) is 2 mg/kg of body weight administered subcutaneously three times per week, or a dosage regimen of 1 mg/kg of body weight administered six times per week. In the 6-month toxicology studies, the AUC in rats at 13 mg/kg/day was 406 mg.h/L and the AUC in monkeys at 10 mg/kg/day was 303 mg.h/L, which are approximately 6 and 5 times the human AUC of 65486 ng.h/mL at the 2 mg/kg/day dose administered three times weekly, respectively. There are no pharm-tox related safety issues for the approval of STRENSIQ™ (asfotase alfa).

12 Appendix/Attachments: None

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/s/

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08/27/2015

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