CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER:

208114Orig1s000

PHARMACOLOGY REVIEW(S)

MEMORANDUM

Date: January 12, 2016
To: File for NDA 208,114
From: Haleh Saber, PhD
Deputy Director, Division of Hematology Oncology Toxicology
Office of Hematology and Oncology Products
Product: DEFITELIO (defibrotide)
Indication: Treatment of adult and pediatric patients with hepatic veno-occlusive disease (VOD), also known as sinusoidal obstruction syndrome (SOS), with (b) (4) dysfunction following hematopoietic stem-cell transplantation (HSCT)

I have examined pharmacology/toxicology review conducted by Drs. Gehrke and Gudi, and the labeling and team leader memorandum provided by Dr. Sheth. Fertility and pre- and post-natal toxicology studies were conducted in the rat; however, the studies were not fully reviewed for the following reasons: non-GLP studies conducted in 1970's with a route of administration (i.m.) different from the proposed clinical route (i.v.), no clear drug-related toxicities observed in the studies and no toxicokinetic data included to ascertain systemic exposure; no rationale provided in the study reports for doses selected; incomplete assessment of reproductive parameters ; lack of product/process-related information to ensure the similarity of the material used in animal studies to the to-be-marketed product.

I agree with Dr. Sheth that DEFITELIO may be approved for the proposed indication.

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/s/

HALEH SABER
01/12/2016

MEMORANDUM

Date: January 4, 2016
From: Christopher Sheth, PhD
Division of Hematology Oncology Toxicology (DHOT)
Office of Hematology and Oncology Products (OHOP)
Re: Approvability for Pharmacology and Toxicology
NDA: 208114
Drug: Defitelio (defibrotide)
Indication: Treatment of adult and pediatric patients with hepatic veno-occlusive disease (VOD), also known as sinusoidal obstruction syndrome (SOS), with (b) (4) dysfunction following hematopoietic stem-cell transplantation (HSCT).
Applicant: Gentium S.p.A. (a Jazz Pharmaceuticals Company)

Defibrotide is a sodium salt of a complex of mainly single-stranded polydeoxyribonucleotides derived from porcine intestinal tissue, being developed as a treatment for hepatic VOD with (b) (4) dysfunction following HSCT. The drug will be formulated into a sterile aqueous solution for intravenous administration. Defitelio is to be administered at a dose of 6.25 mg/kg by a 2-hour intravenous infusion every 6 hours (total daily dose of 25 mg/kg/day) for a minimum of 21 days.

The pharmacology and toxicology studies reviewed included those that assessed the pharmacodynamics, pharmacokinetics, genotoxicity, safety pharmacology, repeat dose toxicology (continuous and intermittent infusion) in rats and dogs, effects on embryo-fetal development in rats and rabbits and pre- and post-natal development in rats, juvenile animal toxicology in rats, and oral carcinogenicity of defibrotide in mice and rats.

Hepatic VOD following HSCT involves chemotherapy-induced sinusoidal endothelial cell damage (leading to apoptosis) followed by thrombosis, which may lead to organ dysfunction. The Applicant's studies demonstrated defibrotide ameliorates chemotherapy-induced stress responses of endothelial cells in addition to reversing deleterious effects on fibrinolysis. There remains an incomplete understanding of the mechanism of action of defibrotide; (b) (4)

Target organs of toxicity were identified in a 13-week rat toxicology study, which included adverse findings in the kidneys, liver and lymphoid tissues. Defibrotide also prolonged prothrombin time (PT) in rats, in addition to activated partial thromboplastin time (APTT) in rats and dogs. Defibrotide may have direct effects on coagulation based on the dose-dependent effects of the drug on PT and APTT.

The Applicant's proposal for Section 8.1 of the label is consistent with the Pregnancy and Lactation Labeling Rule. Defitelio may cause fetal harm based on findings in animals. Exposure to defibrotide was associated with embryo-fetal toxicity (decreased number of implantations and viable fetuses compared to controls) in pregnant rabbits at doses approximately equivalent to the recommended clinical dose on a mg/m^2 basis. Studies of

fertility and pre- and postnatal toxicology were not conducted with defibrotide administered by the intravenous route. Defibrotide, given intramuscularly, was tested in non-GLP studies for effects on fertility and pre- and postnatal development; however these studies were not fully reviewed. The repeat dose general toxicology studies included evaluation of reproductive tissues, and there were no effects due to treatment with intravenously administered defibrotide on male or female reproductive organs in studies up to 13-weeks in duration in rats or dogs. The juvenile rat toxicology study revealed a delayed mean age to preputial separation in defibrotide treated males.

No carcinogenicity studies have been conducted with defibrotide administered by the intravenous route and the oral studies were inadequate to conduct an assessment of the potential for carcinogenicity. Defibrotide was not mutagenic in the in vitro bacterial reverse mutation assay (Ames assay) nor was it clastogenic in an in vivo bone marrow micronucleus assay in rats. Defibrotide was devoid of clastogenic activity in the in vitro chromosomal aberration assay in Chinese hamster ovary cells in the presence or absence of an exogenous metabolic activation system.

The nonclinical studies needed to support product labeling were reviewed by Drs. Brenda Gehrke and Ramadevi Gudi. The nonclinical findings are summarized in the "Executive Summary" of the NDA review and reflected in the product label.

Recommendation: I concur with the pharmacology/toxicology reviewers that from a nonclinical perspective, Defitelio may be approved and that no additional nonclinical studies are needed to support approval of Defitelio in patients with hepatic VOD with (b) (4) dysfunction following HSCT.

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/s/

CHRISTOPHER M SHETH
01/04/2016

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

PHARMACOLOGY/TOXICOLOGY NDA REVIEW AND EVALUATION

Application number:	208114
Supporting document/s:	1 and 3
Applicant's letter date:	July 30, 2015
CDER stamp date:	July 31, 2015
Product:	Defibrotide (Defitelio)
Indication:	Treatment of hepatic veno-occlusive disease with [redacted] (b) (4) dysfunction following hematopoietic stem-cell transplantation
Applicant:	Gentium S.p.A. (a Jazz Pharmaceuticals company)
Review Division:	Division of Hematology Oncology Toxicology (for Division of Hematology Products)
Reviewers:	Brenda J Gehrke, PhD Ramadevi Gudi, PhD
Supervisor/Team Leader:	Christopher M Sheth, PhD
Division Director:	John Leighton, PhD, DABT Ann Farrell, MD (DHP)
Project Manager:	Beatrice A Kallungal, BS

Disclaimer

Except as specifically identified, all data and information discussed below and necessary for approval of NDA 208114 are owned by Gentium S.p.A. or are data for which Gentium S.p.A. has obtained a written right of reference. Any information or data necessary for approval of NDA 208114 that Gentium S.p.A. does not own or have a written right to reference constitutes one of the following: (1) published literature, or (2) a prior FDA finding of safety or effectiveness for a listed drug, as reflected in the drug's approved labeling. Any data or information described or referenced below from reviews or publicly available summaries of a previously approved application is for descriptive purposes only and is not relied upon for approval of NDA 208114.

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1 Executive Summary

1.1 Introduction

Veno-occlusive disease (also known as sinusoidal obstruction syndrome) is a life-threatening complication following hematopoietic stem cell transplant. Sinusoidal endothelial cells and hepatocytes are damaged by toxic metabolites generated by the myeloablative conditioning therapy administered prior to transplant. Defibrotide is a mixture of polydisperse oligonucleotides (b) (4) from porcine intestinal mucosa. Defibrotide primarily targets the endothelium and appears to modulate endothelial cell injury. NDA 208114 has been submitted for defibrotide for the proposed indication of treatment of patients with hepatic veno-occlusive disease with (b) (4) dysfunction following hematopoietic stem cell transplantation. Defibrotide is to be administered at a dose of 6.25 mg/kg by a 2-hour intravenous infusion every 6 hours (total daily dose of 25 mg/kg/day) for a minimum of 21 days. Nonclinical pharmacology, pharmacokinetics, and toxicology studies have been submitted and reviewed to support the approval of the proposed indication.

1.2 Brief Discussion of Nonclinical Findings

The pharmacological assessment of defibrotide included an investigation of the effects of defibrotide on endothelial cell damage induced by various types of stress. Defibrotide protected cells from density stress and endothelial cell damage induced by serum free medium. Treatment with defibrotide significantly decreased caspase-3 gene expression in endothelial cells, suggesting that one way defibrotide may protect endothelial cells is through interfering with the apoptotic signaling pathway involving active caspase-3. The effects of defibrotide on fludarabine-induced apoptosis and cellular changes in endothelial cells were also investigated using a variety of assays. Defibrotide decreased fludarabine-induced apoptosis, protected cells from the fludarabine-induced increases in caspase-3 that lead to cell death, downregulated several genes induced by fludarabine associated with apoptosis, angiogenesis/migration, and inflammatory activation/innate immunity, and decreased fludarabine-induced gene expression of heparanase and IL-8. Collectively, these data suggest that defibrotide protects endothelial cells from fludarabine-induced cytotoxicity through inhibition of factors of apoptosis, inflammation, and cell-adhesion processes, and that these inhibitory activities may be involved in the mechanism of the activity of defibrotide in reducing or preventing chemotherapeutic-related veno-occlusive disease.

Degradation serum products of defibrotide were tested for their ability to decrease fludarabine-induced apoptosis in endothelial cells. Results indicate that the degradation products of defibrotide did not decrease fludarabine-induced apoptosis. This suggests that the loss of the defibrotide polymer structure results in the loss of the pharmacologic activity.

One study investigated the effects of defibrotide on the thrombotic effects of thalidomide in endothelial cells. Results from this study indicate that defibrotide reduces the pro-

thrombotic effects of thalidomide in endothelial cells by producing changes in the levels and gene expression of the fibrinolytic proteins tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) and increasing the ability of endothelial cells to degrade clots.

While the mechanism of action for defibrotide in the treatment of veno-occlusive disease is not fully understood, nonclinical pharmacology studies published in the literature or submitted in the NDA suggest that defibrotide protects endothelial cells by protecting cells from further damage including apoptosis and by producing fibrinolytic effects. Defibrotide has been shown to have effects on various factors and chemicals believed to be involved the pathophysiology of veno-occlusive disease. Effects include increases in t-PA and thrombomodulin expression and decreases in the expression of von Willebrand factor, tissue factor, PAI-1, intracellular adhesion molecule 1 (ICAM-1), heparanase, and cytokines. Defibrotide has also been shown to protect endothelial cells from damage caused by tumor necrosis factor-alpha (TNFα). Although some of the pharmacological activities involved in the mechanism of action of defibrotide have been identified, it is not known at this time how defibrotide is interacting with the endothelial cells to produce these effects. Based on the incomplete understanding of the mechanism of action, no pharmacological classification will be assigned at this time.

General toxicology studies included 13-week repeat-dose studies of intravenous administration of defibrotide in rats and dogs. In the rat study, defibrotide (240, 1200, or 4800 mg/kg/day; 1440, 7200, or 28,800 mg/m^2/day) or vehicle was administered as a continuous intravenous infusion for a planned duration of 13 consecutive weeks. Due to extensive mortality and dosing complications associated with the intravenous catheters (loss of catheter patency and function), the study was terminated early following 9 weeks of treatment with defibrotide. In the dog study, defibrotide (60, 300, or 1600 mg/kg/day; 1200, 6000, or 32,000 mg/m^2/day) or vehicle was administered as a 2-hour intravenous infusion four times daily for 13 consecutive weeks. This administration schedule is the same used clinically in humans. Unlike the rat study, there was no defibrotide-related mortality observed in the dog study.

Due to the high mortality rate in the rat study and the complexity of the macroscopic and microscopic findings, it is difficult to interpret which findings were defibrotide-related. Dose-dependent, defibrotide-related findings identified in the rat included basophilic granules in tubular cells, tubular degeneration/regeneration, glomerulonephropathy, and lymphohistiocytic infiltration in the kidneys, basophilic granules and hypertrophy/hyperplasia in kupffer cells in the liver, and vacuolated macrophages in the mandibular and mesenteric lymph nodes and spleen. The liver was the main organ of toxicity in the dog as indicated by increased liver weights and microscopic findings of basophilic granules and hypertrophy in kupffer cells, acute inflammation, and necrosis of individual hepatocytes.

The other defibrotide-related toxicity observed in both rats and dogs was decreased coagulation. Increases in activated partial thromboplastin time (APTT) were observed at 1200 and 4800 mg/kg/day (7200 and 28,800 mg/m^2/day) in rats and 300 and 1600

mg/kg/day (6000 and 32,000 mg/m^2/day) in dogs. Prothrombin time (PT) was also increased at 4800 mg/kg/day (28,000 mg/m^2/day) in rats. These findings were observed at doses much higher than the recommended clinical dose of 25 mg/kg/day (925 mg/m^2/day in adults and 625 mg/m^2/day in children). Decreased coagulation is an expected toxicity of defibrotide based on its pharmacologic activity and is consistent with bleeding including various types of hemorrhage observed in patients treated with defibrotide at a dose higher than the recommended dose.

The potential for defibrotide to induce genotoxicity was assessed in an in vitro bacterial reverse mutation (Ames) assay, an in vitro chromosome aberrations assay in Chinese hamster ovary cells, and in an in vivo micronucleus test in the bone marrow of male rats. Defibrotide was negative in all three tests under the conditions tested.

To assess carcinogenicity, 2-year dietary carcinogenicity studies of defibrotide administered orally as an admixture with the diet once daily were conducted in mice and rats. (b) (4) Carcinogenicity studies are not required for the proposed duration of administration of defibrotide.

The embryo-fetal development effects of defibrotide were studied in the rat and rabbit. Preliminary studies were conducted in pregnant females with intravenous administration of defibrotide as a continuous infusion in rats or as a 2-hour infusion 4 times daily in rats or rabbits. In both the rat and rabbit studies, intravenous infusion of defibrotide resulted in severe maternal toxicity including imminent abortion and mortality in the majority of the defibrotide-treated females. In the rabbit study, total resorption was observed in 60% of pregnant females treated with defibrotide. Increased resorption was also observed in the rat study. Due to the limited number of defibrotide-treated females with live fetuses at the end of the studies, meaningful comparisons could not be made for parameters regarding body weight, the cesarean section, or offspring.

Based on the mortality observed in the preliminary study in rabbits, a modified design was used for the GLP embryo-fetal development study in rabbits in order to increase the probability of evaluable litters at the end of the study. Defibrotide or vehicle was administered to pregnant females by a 2-hour infusion 4 times daily during three different brief critical windows of organogenesis instead of a consecutive 13-day dosing period. The dose of 80 mg/kg/day (960 mg/m^2/day) was comparable to the recommended clinical dose of 25 mg/kg/day (925 mg/m^2/day in adults). There was a low pregnancy rate in this study and only 11 females in the control group and 11 females in the defibrotide group had live fetuses at the cesarean section. A statistically significant lower gravid uterus weight in females treated with defibrotide compared to controls corresponded to an increased incidence of unilateral implantation noted in the defibrotide group. Two females treated with defibrotide had only one live fetus present and with these females excluded from the analysis, the mean number of viable fetuses was still significantly decreased in the defibrotide group compared to the control group. Additionally, the mean number of implantations was lower and post-implantation loss

was increased in the defibrotide group compared to the control group. No defibrotide-related teratogenicity was observed in this study.

To evaluate the effects of defibrotide on the development of rats, a 28-day juvenile toxicology study with a 28-day recovery period was conducted in Sprague-Dawley rats. Defibrotide was administered by intravenous injection once daily for 28 days starting on Day 21 post-partum to at least Day 48 post-partum. The toxicities observed in this study were similar to the toxicities observed in the general toxicology studies. Toxicities included hematology changes of increased reticulocytes and lymphocytes and decreased partial thromboplastin time. Organs of toxicity were the liver and the spleen. In general, these toxicities were resolved or were resolving following the 28-day recovery period. Evaluations of sexual maturation showed that preputial separation was significantly delayed by approximately 3 days in males treated with defibrotide at all doses compared to controls, suggesting a delay in the onset of male puberty. In neurobehavioral tests, defibrotide had no effects on general motor activity, motor coordination, or learning ability in the juvenile rats.

1.3 Recommendations

1.3.1 Approvability

Recommended for approval. The nonclinical studies submitted to this NDA provide sufficient information to support the use of defibrotide for treatment of patients with hepatic veno-occlusive disease with (b) (4) dysfunction following hematopoietic stem cell transplantation.

1.3.2 Additional Non Clinical Recommendations

None

1.3.3 Labeling

Changes have been made to the proposed labeling including changes consistent with the Pregnancy and Lactation Labeling Rule. Any dose comparisons between animals and humans are made on the basis of dose in mg/m^2 due to limited pharmacokinetic data in patients. (b) (4)

2 Drug Information

2.1 Drug

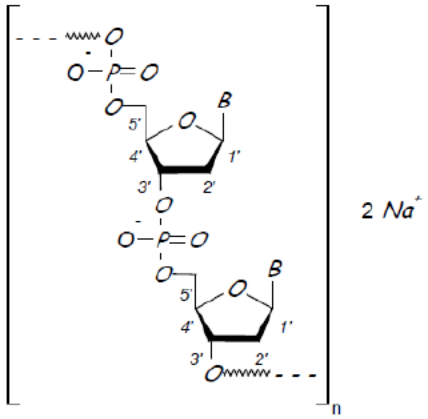
CAS Registry Number: 83712-60-1

Generic Name: Defibrotide

Molecular Formula/Molecular Weight: Mean molecular weight of 13-20 kDa

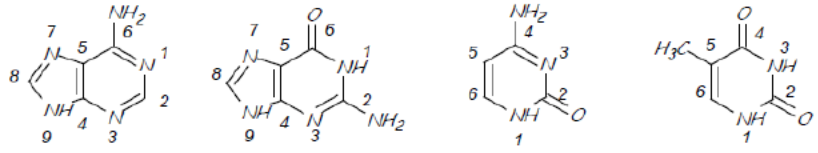
Structure or Biochemical Description:

Defibrotide is the sodium salt of a polydisperse mixture of predominately single-stranded polydeoxyribonucleotides [redacted]. The primary structure of defibrotide is shown below (excerpted from Applicant's submission):



n = from about 2 to 50

B =



Adenine Guanine Cytosine Thymine

The general composition of defibrotide [redacted] is the following:

- Nucleic phosphorus: [redacted]%
- Nucleic bases
  - Adenine: [redacted]%
  - Guanine: [redacted]%
  - Thymine: [redacted]%
  - Cytosine: [redacted]%
- Purine/pyrimidine ratio: ≥ [redacted]
- Phosphorus/bases ratio: [redacted]

Pharmacologic Class: None assigned

2.2 Relevant INDs, NDAs, BLAs and DMFs

IND 62118

2.3 Drug Formulation

The defibrotide drug product is a sterile aqueous solution for intravenous administration supplied in 2.5 mL clear glass vials containing 200 mg defibrotide (80 mg/mL concentration). The composition of defibrotide product is provided in the table below.

Table 1: Quantitative composition of defibrotide drug product
(excerpted from Applicant's submission)

Component	Reference to Standard Quality	Function	80 mg/mL for Infusion	Concentration per mL
Defibrotide	In-house standard	Drug Substance	200.0 mg	80.0 mg
Sodium Citrate (b) (4)	USP – Ph. Eur.	(b) (4)		
Water-for-injection	USP – Ph. Eur.			
Sodium hydroxide (b) (4) hydrochloric acid	NF – Ph. Eur. NF – Ph. Eur.	pH adjustment	As required for pH 6.8–7.8	As required for pH 6.8–7.8

[a] In order to assure the correct extractable volume of drug product, the filling volume for each vial is (b) (4)%, based on a theoretical density of (b) (4) g/mL.

2.4 Comments on Novel Excipients

None

2.5 Comments on Impurities/Degradants of Concern

None

2.6 Proposed Clinical Population and Dosing Regimen

The proposed clinical population is patients with hepatic veno-occlusive disease with (b) (4) dysfunction following hematopoietic stem cell transplantation. Defibrotide is to be administered at a dose of 6.25 mg/kg by a 2-hour intravenous infusion every 6 hours (total daily dose of 25 mg/kg/day) for a minimum of 21 days. If signs and symptoms of veno-occlusive disease have not resolved after 21 days of treatment, treatment should continue until resolution.

2.7 Regulatory Background

Defibrotide is being developed by Gentium S.p.A., a Jazz Pharmaceuticals company, for the treatment of hepatic veno-occlusive disease in patients with a hematopoietic stem cell transplant under IND 62118. Defibrotide was granted orphan drug designation by the FDA in 2003 for the treatment of hepatic veno-occlusive disease, and was granted Fast Track designation for the treatment of severe hepatic veno-occlusive disease in hematopoietic stem cell transplantation recipients in May 2005. (b) (4) A CMC only pre-NDA meeting and a separate pre-NDA meeting to discuss clinical and nonclinical questions were held in August 2014. NDA 208114 was submitted for

defibrotide as a rolling submission with Module 4 submitted in December 2014, Module 3 submitted in March 2015, and the final submission including Modules 1, 2, and 5 in July 2015.

Defibrotide was approved in Italy in 1986 for the prophylaxis of deep vein thrombosis and treatment of thrombophlebitis, and was marketed as Prociclide®, Noravid®, and Dinelasi®. Following the request of the marketing authorization holder, the Italian marketing authorizations for these indications were withdrawn in 2009. Defibrotide was granted marketing authorization under the trade name Defitelio® by the European Commission for the treatment of severe hepatic veno-occlusive disease in adults and children undergoing hematopoietic stem cell transplantation in October 2013. In May 2015, Gentium S.p.A. was granted license to market Defitelio in Israel for the same indication.

3 Studies Submitted

3.1 Studies Reviewed

Study Number	Study Title	eCTD Location
RB2008010	Effects of defibrotide on endothelium protection	4.2.1.1.
RB2008020	Defibrotide protects endothelial cell without influence the immunosuppressive and antitumor activity of chemotherapeutics	4.2.1.1.
RB2010010	Defibrotide modulates the expression of genes in endothelial cells induced by fludarabine: A microarray analysis	4.2.1.1.
RB2009010	Defibrotide blunts the pro-thrombotic effect of thalidomide on endothelial cells	4.2.1.1.
0040-2010	Effect on hERG tail current recorded from stably transfected HEK 293 cells	4.2.1.3.
150210	Absorption, distribution, and excretion of [^{125}I]-defibrotide following oral or intravenous administration in rats	4.2.2.2.
1529-001	A 13-week intravenous toxicity study of defibrotide in rats	4.2.3.2.
1529-002	A 13-week intravenous toxicity study of defibrotide in dogs	4.2.3.2.
0245-2010	Defibrotide: Bacterial reverse mutation assay	4.2.3.3.1.
A0076	Evaluation of in vitro chromosome aberrations in Chinese hamster ovary cells with defibrotide	4.2.3.3.1.
A0077	In vivo micronucleus test in rats with intravenous administration of defibrotide (multiple dosing)	4.2.3.3.2.
253-094-024	Defibrotide: Dietary oncogenicity study in mice	4.2.3.4.3.
237-094-021	Defibrotide: Dietary oncogenicity study in rats	4.2.3.4.3.
79820EXT	Defibrotide preliminary intravenous infusion embryo-fetal development study in rats	4.2.3.5.2.
79840EXT	Defibrotide preliminary intravenous infusion embryo-fetal development study in rabbits	4.2.3.5.2.
95800	Defibrotide embryo-fetal development study in rabbits by intravenous administration	4.2.3.5.2.
82640	Defibrotide 28 days intravenous repeated toxicity study in juvenile rats followed by a 28 days recovery period	4.2.3.5.4.

3.2 Studies Not Reviewed

Study Number	Study Title	eCTD Location
RQCVL049R011	Defibrotide biological assay	4.2.1.1.
RQCVL050R011	Defibrotide drug substance biological plasmin assay	4.2.1.1.
12-650	Transfer, adaptation and partial validation of analytical methods for the determination of defibrotide in minipig plasma and urine	4.2.2.1.
13-891	Validation of analytical method for the qualification of defibrotide in rabbit plasma	4.2.2.1.
13-919	Bioanalysis analysis of samples obtained from Study # 95800	4.2.2.1.
150184	Studies into methods for the detection and quantitation of free ^{125}I and [^{125}I]-defibrotide in biological samples in vitro and ex vivo	4.2.2.1.
1529-007	Full validation of an HPLC-UV assay for defibrotide in dog sodium citrate plasma	4.2.2.1.
1529-008	Partial validation of an HPLC-UV assay for defibrotide in rat sodium citrate plasma	4.2.2.1.
79790	Defibrotide in rabbit plasma and urine validation of the HPLC-UV analytical method	4.2.2.1.
79800	Defibrotide in rat plasma and urine partial validation of the HPLC-UV analytical method	4.2.2.1.
82070	Defibrotide in rabbit plasma validation of the fluorimetric spectrometer analytical method	4.2.2.1.
0251-2010	Defibrotide: Evaluation of the in vitro metabolism with human hepatocytes from young pediatric and adult donors	4.2.2.2.
150205	Studies in whole blood kinetics of radioactivity with ^{125}-I defibrotide in mice	4.2.2.2.
80870	Defibrotide pharmacokinetic study in rabbits following intravenous infusion	4.2.2.2.
8313003	In vitro plasma protein binding and blood-to-plasma partitioning of defibrotide in mouse, rat, rabbit, dog, and human	4.2.2.2.
94830	Defibrotide pharmacokinetic study in juvenile and adult male rats	4.2.2.2.
A124079	Pharmacokinetics of defibrotide after IV infusion to young and adult Gottingen minipigs	4.2.2.2.
A134330	Pharmacokinetics of defibrotide after intravenous infusion to young and adult Gottingen minipigs	4.2.2.2.
0184-2010	Defibrotide: Determination of the potential inhibition of CYP450 activity in human liver microsomes	4.2.2.6.
8305353	Evaluation of cytochrome P450 and UGT1A1 induction following exposure of defibrotide to primary cultures of human hepatocytes	4.2.2.6.
8305354	Evaluation of defibrotide as a substrate and inhibitor of a panel of human drug transporters	4.2.2.6.
8309087	Inhibitory potential of defibrotide towards human hepatic microsomal UDP-glucuronosyl transferase (UGT)1A1 and UGT2B7	4.2.2.6.
15/75712	Compound P intravenous toxicity study in Beagle dogs	4.2.3.2.

Study Number	Study Title	eCTD Location
	(repeated dosage for 13 weeks)	
619	26 weeks intravenous toxicity study in dogs	4.2.3.2.
1529-003	A 7-day (four times daily for 2 hours) intravenous infusion tolerability study in dogs	4.2.3.2.
151-0-094-012	Defibrotide: 17 day intravenous perfusion study in rabbits	4.2.3.2.
1529-004	A 7-day continuous intravenous infusion tolerability study in rats	4.2.3.2.
654	4 weeks intravenous toxicity in rats	4.2.3.2.
094007-M-03784	Micronucleus test- test substance: Defibrotide	4.2.3.3.2.
16/76299	Effect of compound P on fertility and general reproductive performance of the rat	4.2.3.5.1.
19/75687	Effect of compound P on pregnancy of the New Zealand white rabbit 2, intravenous administration	4.2.3.5.2.
21/75822	Effect of compound P on pregnancy of the rat 2, intravenous administration	4.2.3.5.2.
17/75802	Effect of compound P on peri- and post-natal development of the rat	4.2.3.5.3.
85800FR	Defibrotide 28 day intravenous repeated toxicity study in juvenile rats followed by a 6 day recovery period	4.2.3.5.4.
111035	Studies of the allergenic potential of 3 samples of fraction P	4.2.3.7.1.
RB2010020	Extended administration of defibrotide in rats and dogs for immunogenic analysis	4.2.3.7.1.
10189	Experimental study of possible immunological effects of defibrotide	4.2.3.7.2.
10200	Immunological effects of defibrotide	4.2.3.7.2.
10194b	Toxicological investigation on a polydeoxyribonucleotidic substance of mammalian origin (Code: “Fraction P”)	4.2.3.7.7.
10627b	Pharmacological investigation on a polydeoxyribonucleotidic substance of mammalian origin (Code “Fraction P”)	4.2.3.7.7.

3.3 Previous Reviews Referenced

IND 62118 Nonclinical Review by David B. Joseph, PhD, 2005

4 Pharmacology

4.1 Primary Pharmacology

Veno-occlusive disease, also known as sinusoidal obstruction syndrome, is a life-threatening complication that can occur following hematopoietic stem cell transplant. Although the pathophysiology of veno-occlusive disease is complex and still being investigated, it has been described in published literature reviews[1] [2]. The high-dose

[1] Ho, VT, C Revta, and PG Richardson, 2008, Hepatic veno-occlusive disease after hematopoietic stem cell transplantation: Update on defibrotide and other current investigational therapies, Bone Marrow Transplantation, 41:229-237.

alkylating chemotherapy agents administered as myeloablative conditioning therapy prior to transplant result in endothelial cell damage and hepatocellular injury. Sinusoidal endothelial cells and hepatocytes are damaged by toxic metabolites generated by the conditioning therapies. Locally released cytokines induce activation of cell adhesion molecules on endothelial cells, resulting in cell damage and detachment, and activation of the coagulation pathway. Subsequent activation of the fibrinolytic pathway then leads to fibrosis of sinusoids, perivascular necrosis, and the venular blockage observed in veno-occlusive disease[2]. A number of markers of endothelial cell injury and adhesion molecules are upregulated in patients with veno-occlusive disease including plasma thrombomodulin, P- and E-selectins, tissue factor pathway inhibitor, plasminogen activator inhibitor (PAI-1)[1], and intracellular adhesion molecule 1 (ICAM-1) [2].

Defibrotide is a mixture of polydisperse oligonucleotides (b) (4) from porcine intestinal mucosa that primarily targets the endothelium and appears to modulate endothelial cell injury. The various pharmacological effects of defibrotide including pro-fibrinolytic and anti-thrombotic activities have been investigated and presented in the extensive literature on defibrotide. Thrombomodulin is an endothelial cell membrane glycoprotein that is involved in the regulation of the anticoagulant system. Defibrotide has been shown to increase the expression of thrombomodulin in endothelial cells in a concentration-dependent manner[3]. In a study using sera from patients undergoing autologous hematopoietic stem cell transplant, treatment with defibrotide prevented the increased expression of von Willebrand factor and tissue factor on the extracellular matrix of endothelial cells induced by the sera[4].

Defibrotide has been shown to protect cells against damage produced by various types of stress, cell injury, and drugs. Data in the pharmacology studies submitted with the NDA demonstrate that defibrotide protects endothelial cells from fludarabine- and thalidomide-induced damage. Defibrotide has also been shown to protect endothelial cells from tumor necrosis factor-alpha (TNFα)-mediated cytotoxicity.[5] In a rat model of liver ischemia-reperfusion injury, results suggested that defibrotide protected the liver, as measured by lower malondialdehyde levels in liver tissue compared to controls, through increasing antioxidant (superoxide dismutase and glutathione peroxidase)

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[2] Dalle, J and SA Giralt, 2015, Hepatic veno-occlusive disease after hematopoietic stem cell transplantation: Risk factors and stratification, prophylaxis, and treatment, Biology of Blood and Marrow Transplantation, Epub ahead of print: 1-10.
[3] Zhou, Q, X Chu, and C Ruan, 1994, Defibrotide stimulates expression of thrombomodulin in human endothelial cells, Thrombosis and Haemostasis, 71(4):507-510.
[4] Palomo, M, M Diaz-Ricart, M Rovira, et al., 2011, Defibrotide prevents the activation of macrovascular and microvascular endothelia caused by soluble factors released to blood by autologous hematopoietic stem cell transplantation, Biology of Blood and Marrow Transplantation, 17:497-506.
[5] Schröder, H, 1995, Defibrotide protects endothelial cells, but not L929 tumour cells, from tumour necrosis factor-alpha-mediated cytotoxicity, The Journal of Pharmacy and Pharmacology,47(3):250-252.

enzyme levels[6]. In a study investigating the mechanism of the anti-ischemic effects of the drug, defibrotide inhibited adhesion of neutrophils to endothelial cells through the ICAM-1/LFA-1 adhesion system[7]. ICAM-1 is an immunoglobulin located on endothelial cells and lymphocytes and is the ligand for lymphocyte function-associated molecule-1 (LFA-1), an integrin located on polymorphonucleates, monocytes, and lymphocytes. Defibrotide has also been shown to reduce the increased expression of ICAM-1 on endothelial cells induced by sera from patients undergoing autologous hematopoietic stem cell translplant.[4]

While the mechanism of action for defibrotide in the treatment of veno-occlusive disease is not fully understood, nonclinical pharmacology studies published in the literature or submitted in the NDA suggest that defibrotide protects endothelial cells by protecting cells from further damage including apoptosis and by producing fibrinolytic effects. The Applicant and the literature refers to the fibrinolytic activities of defibrotide as the (b) (4) The proposed mechanism of action of defibrotide has been described in the pharmacology written summary submitted in the NDA and the literature[8]; the figure below from the literature shows the proposed mechanism. In the proposed mechanism, defibrotide protects sinusoidal endothelial cells from TNF-α toxicity, decreases the expression of adhesion molecules, and inhibits heparanase activation, which then prevents or reduces the influx of inflammatory mediators. Defibrotide also restores the thrombotic-fibrinolytic balance by increasing the function of t-PA, decreasing the activity of PAI-1, decreasing synthesis of tissue factor (TF) and von Willebrand factor (vWF), and increasing expression of thrombomodulin.

[6] Aydemir, EO, A Var, BS Uyanik, et al., 2003, The protective mechanisms of defibrotide on liver ischaemia-reperfusion injury, Cell Biochemistry and Function, 21(4):307-310.
[7] Pellegatta, F, Y Lu, A Radaelli, et al., 1996, Drug-induced in vitro inhibition of neutrophil-endothelial cell adhesion, British Journal of Pharmacology, 118:471-476.
[8] Richardson, PG, S Corbacioglu, VT Ho, et al., 2013, Drug safety evaluation of defibrotide, Expert Opinion on Drug Safety, 12(1):123-136.

Studies submitted to the NDA to support the mechanism of action and pharmacology of defibrotide are reviewed below.

Study title: Effects of defibrotide on endothelium protection

Study No.:	RB2008010
Report Date:	November 6, 2008
Study report location:	eCTD 4.2.1.1.
Conducting Laboratory:	(b) (4)
GLP:	No

This study investigated a possible protective effect of defibrotide against stress-induced damage of endothelial cells using various types of stress. To identify how defibrotide protects endothelial cells, the ability of defibrotide to modulate gene expression of caspase-3 was also investigated.

A thiazolyl blue tetrazolium bromide assay (MTT) was conducted to determine the proliferation and viability of the endothelial cells. This method is based on the cleavage of tetrazolium salts by mitochondrial dehydrogenase in viable cells leading to the

production of formazan dye. After incubation with MTT solution that contains the tetrazolium compound, the formation of formazan product was measured by spectrophotometry. The level of formazan salt is proportional to the number of living/proliferating cells.

To determine the expression of caspase-3, quantitative PCR was conducted using mRNA extracts from cells from the human microvascular endothelial cell line CDC/EU.HMEC-1 (HMEC) treated with different defibrotide batches at 150 µg/mL for 24 hours. An expression index was calculated by comparing the levels of specific mRNA transcripts with the housekeeping gene β-actin. Additionally, cytometric bead arrays were used to verify whether the extrinsic apoptosis pathway is related with the protective effect of defibrotide from fludarabine (F-Ara)-induced cell death.

Stress via cellular density
To assess stress via cellular density, HMECs were cultured under both normal and high density conditions. Cells were incubated in 96-well plates with $10x10^4$ cell/well for 24 hours for normal cell culture or with $20x10^4$ cell/well for 3 days for high density cell culture. Subsequently, cells were treated for 24 hours in the presence or absence of different batches of defibrotide at increasing concentrations (12.5, 50, and 150 µg/mL).

Stress via serum starvation
To assess the protective effect of defibrotide against stress induced by serum free medium, HMECs were incubated in 96-well plates with different medium conditions with different batches of defibrotide at concentrations of 50, 150, and 400 µg/mL tested for each experimental condition. The MTT assay was conducted for these experiments.

- **Defibrotide for treatment:** $10x10^4$ cells/well of HMEC and human hepatic sinusoidal cells (HHSEC) were seeded in serum free medium for 24 hours and treated for another 24 hours with and without defibrotide
- **Defibrotide for prevention:** $10x10^4$ cells/well were seeded in growth medium with and without defibrotide. After 24 hours normal medium was removed and serum free medium was added for 24 hours.
- **Defibrotide for prevention and treatment:**
    - $10x10^4$ cells/well were cultured for 24 hours in growth medium supplemented with defibrotide. Following incubation, the medium was removed and a new serum free medium supplemented with defibrotide was added for 24 hours.
    - $10x10^4$ cells/well were plated in culture medium containing defibrotide for 24 hours. After aspiration of the medium, cells were incubated firstly in serum free medium for 24 hours and then treated with defibrotide in serum free medium for another day.

Oxidative stress

To evaluate the oxidative stress on HMECs, cells were treated with different concentrations of H_2O_2. In an initial experiment, cells ($10x10^4$ cells/well) were stressed for 1 hour with three different concentrations of H_2O_2 (25, 50, and 100 µM). The MTT

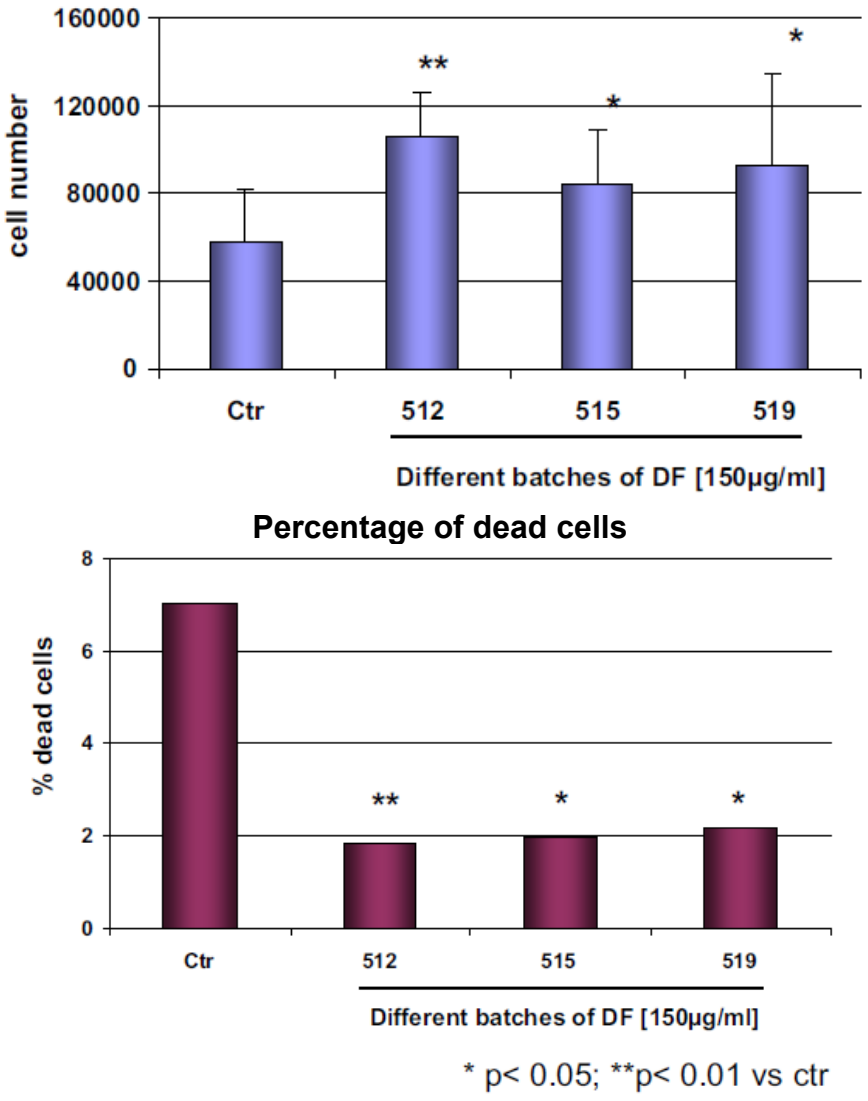
assay was performed after 24 hours of culture in growth medium. In a follow-up experiment, cells were seeded for 24 hours in growth medium and then stressed with H_2O_2 at 50 or 100 µM for 1 hour. Following the removal of H_2O_2 solution, HMECs were treated for 24 hours with or without defibrotide at concentrations of 50, 150, and 400 Gudiµg/mL.

Results

Protective effect of defibrotide on HMEC cells from density stress

- Defibrotide (DF) at a concentration of 150 µg/mL protected HMECs from density stress induced cell death as demonstrated by significantly increasing the number of live cells and decreasing the percentage of dead cells compared to untreated control cells.

Figure 2: Effects of defibrotide on density stress induced cell death
(excerpted from study report for RB2008010)

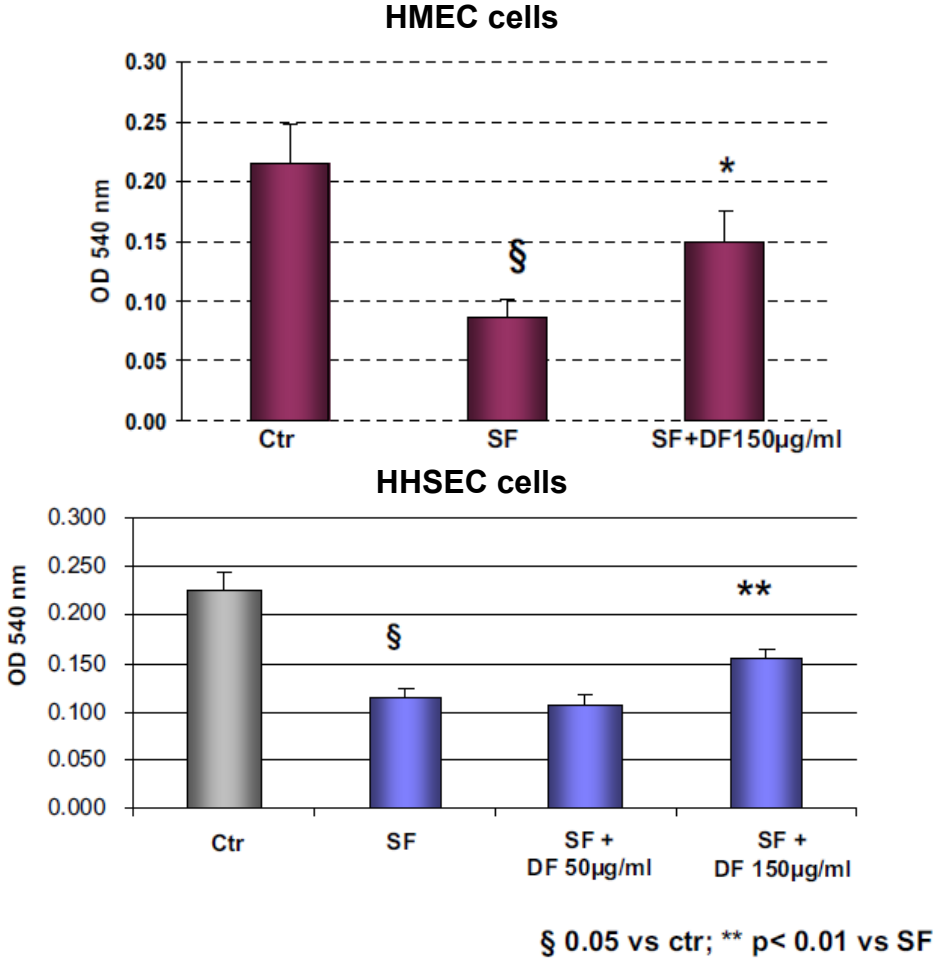


Effects of defibrotide against stress induced by serum free medium

- Treatment with defibrotide significantly reversed endothelial cell damage induced by serum starvation in HMEC and HHSEC cells by enhancing survival of the cells under serum-free conditions compared to untreated controls in MTT experiments.
- Defibrotide also prevented damage induced by serum starvation in HMECs in MTT experiments.

Figure 3: Effects of treatment with defibrotide on damage induced by serum free (SF) medium

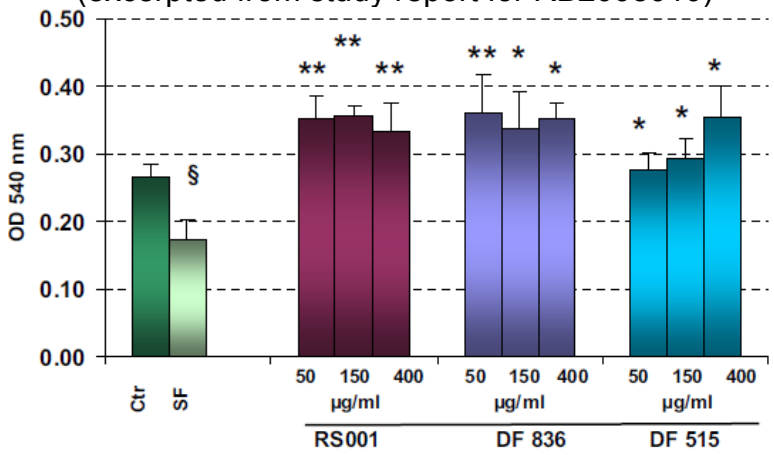
(excerpted from study report for RB2008010)



§ 0.05 vs ctr; ** p< 0.01 vs SF

Figure 4: Effect of defibrotide in the prevention of damage induced by serum free (SF) medium in HMEC

(excerpted from study report for RB2008010)



§ <0.05 vs Ctr *p<0.05 vs SF **p<0.01 vs SF

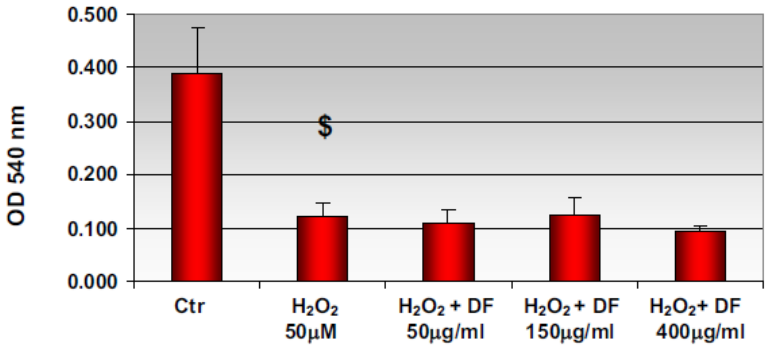
Effects of defibrotide against oxidative stress

- The MTT assay showed that defibrotide was not able to protect endothelial cells from oxidative stress at either H_2O_2 concentration tested.

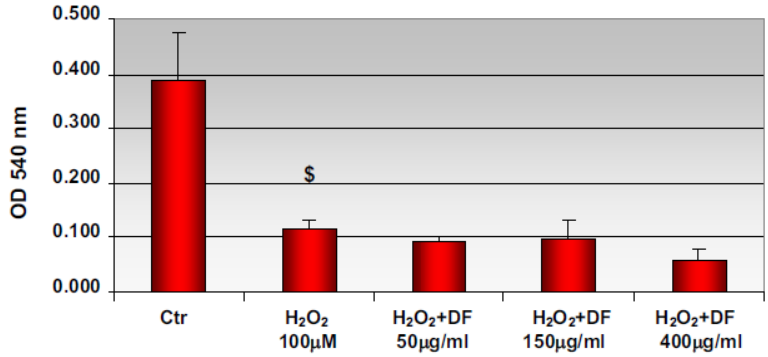
Figure 5: Results of oxidative stress experiments with defibrotide

(excerpted from study report for RB2008010)

50 µM H_2O_2



100 µM H_2O_2

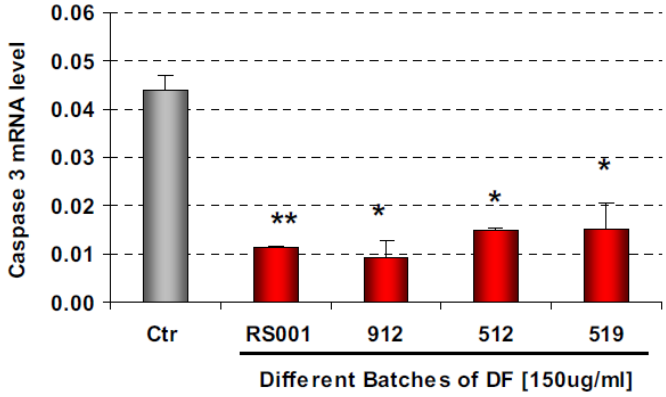


$< 0.05 vs ctr

Effects of defibrotide on caspase-3 expression in endothelial cells

- Treatment with defibrotide significantly decreased caspase-3 gene expression, suggesting that one way defibrotide may protect endothelial cells is through interfering with the apoptotic signaling pathway involving active caspase-3.

Figure 6: Effect of defibrotide on caspase-3 expression
(excerpted from study report for RB2008010)

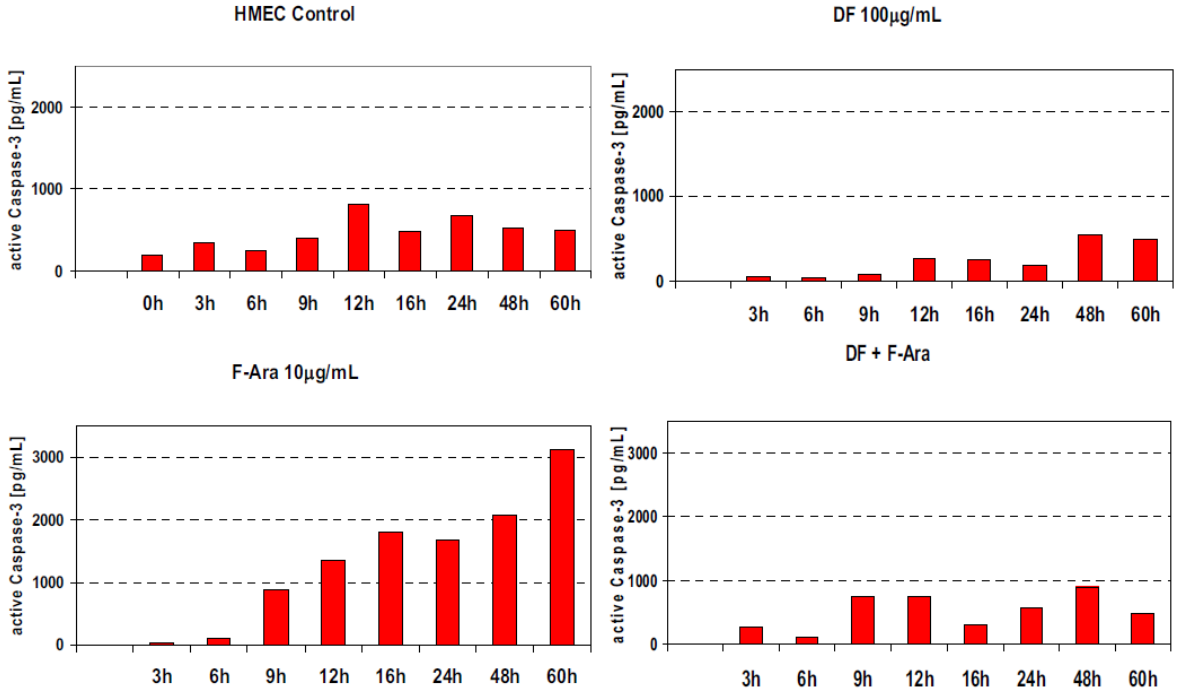


* p< 0.05; ** p< 0.01 vs ctr

Protective effects of defibrotide from fludarabine-induced increases in caspase-3

- Results of the cytometric bead arrays demonstrated that levels of active caspase-3 were increased with treatment of fludarabine (F-Ara) alone, but were not increased with treatment of both fludarabine and defibrotide. This finding suggests that defibrotide protects endothelial cells from the fludarabine-induced increases in caspase-3 that lead to cell death. Levels of Bcl-2 were not modulated by defibrotide (data not shown).

Figure 7: Effect of defibrotide on fludarabine-induced increases in caspase-3
(excerpted from study report for RB2008010)



Study title: Defibrotide protects endothelial cell without influence the immunosuppressive and anti-tumor activity of chemotherapeutics

Study No.: RB2008020
Report Date: November 13, 2008
Study report location: eCTD 4.2.1.1.
Conducting Laboratory: (b) (4)
GLP: No

Previous studies published in the literature have shown that defibrotide protects endothelial cells from fludarabine-induced damage. The current study further investigated the effects of defibrotide in the protection of endothelial cells from fludarabine-induced apoptosis.

Apoptosis assay
An established method for detecting apoptosis in human endothelial cells was conducted using flow cytometry. Human endothelial cells (HMEC), primary acute myeloid leukemia (AML) cells, peripheral blood monoclonal cells (PBMC), and human colon carcinoma cell line (CX+/CX-) were left untreated or were incubated with fludarabine (F-Ara) and 5-fluoro-uracil (5-FU) in descending concentrations (10 g/mL to 0.1 µg/mL) in the presence or absence of defibrotide for 48 hours. Cells were then washed in phosphate-buffered saline (PBS)-10% FCS and were stained with the necrosis-detecting dye propidium iodide. Apoptotic cells were identified by propidium

iodide-negative staining and by a characteristic side scatter image distinct from that of non-apoptotic cells.

Microscopic counting of stained cells
An alternative method for the detection of apoptosis involved microscopic analysis of DNA fluorescence-labeled cells. Endothelial cells ($1x10^5$/plate) seeded in 35-mm Petri dishes were treated as described above and subsequently fixed with methanol-acetone 1:1 for 2 minutes, washed once with PBS, stained with 4,6-diamidino-2-phenylindole (DAPI; 0.5 µg/mL), and dissolved in 20% glycerin-PBS. Samples were mounted and subjected to microscopic analysis. Nuclear condensation revealed by the DAPI staining in the absence of trypan blue uptake is considered characteristic of apoptosis instead of necrosis. Quantitative analysis was conducted with computer-assisted counting of the number of apoptotic cells relative to all identifiable cells from at least 10 microscopic fields, with an average of 70 cells/field.

Flow cytometric proliferation assay
Cellular proliferation was induced by the addition of CD3/CD28 Dynabeads for T-cells, or a cytokine cocktail containing stem cell factor (SCF; 50 ng/mL), granulocyte-macrophage colony stimulating factor (GM-CSF; 100 ng/mL), interleukin 3 (IL-3; 25 ng/mL), G-CSF (100 ng/mL), erythropoietin (EPO; 2 U/mL), transferring (0.47 g/L), and 2-mercaptoethanol (50 µM) for the myeloid AML blasts. Proliferation was assayed using flow cytometric analysis of carboxyfluorescein diacetate succinylester (CFSE)-labeled cells, cultured in the presence or absence of defibrotide. CFSE-labeled cells were assayed for loss of dye as a parameter for proliferation at Day 5 and 7 of incubation by flow cytometry after calibration with TO-PRO3 beads for the detection of viable cells.

Results

Protection of endothelial cells from fludarabine-induced apoptosis

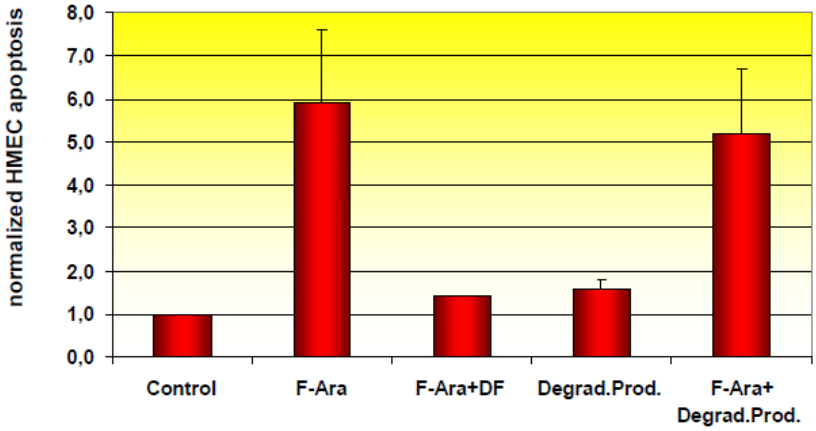
- Microscopic counting of DAPI-stained cells shows that apoptosis induced by fludarabine (10 µg/mL) is decreased when cells are treated with both defibrotide (DF; 100 µg/mL) and fludarabine.
- In experiments with the apoptosis assay using flow cytometry, images of cellular granularity with the characteristic side scatter also demonstrate that defibrotide decreases apoptosis induced by fludarabine (10 µg/mL) in a concentration-dependent manner.
- Additional experiments were conducted on the effects of defibrotide and oligotide, a defibrotide derivative manufactured by prolong controlled chemical denaturation and depolymerization, on apoptosis produced by 5-FU. Results showed that both defibrotide and oligotide decreased the apoptosis induced by 5-FU (10 µM) in HMEC cells when cells were treated with 5-FU and either defibrotide or oligotide (10 µg/mL).

Effects of defibrotide degradation products

- Degradation serum products of defibrotide, as determined by HPLC-SEC, were tested for their ability to decrease fludarabine-induced apoptosis in endothelial cells. It is unclear from the report what the degradation products compound tested contained. Results indicate that the degradation products of defibrotide did not decrease fludarabine-induced apoptosis. This suggests that the loss of the defibrotide polymer structure results in the loss of the pharmacologic activity.

Figure 8: Effects of defibrotide degradation products on fludarabine-induced apoptosis

(excerpted from study report for RB2008020)



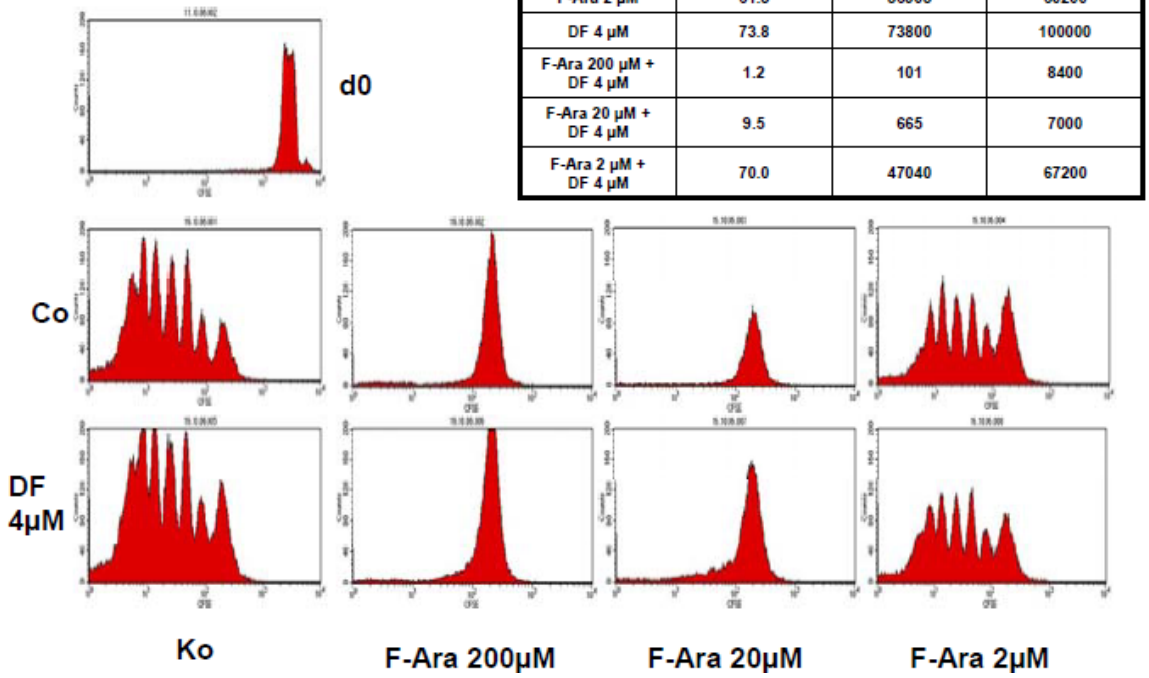
Effects of defibrotide on the treatment-related effects of fludarabine and 5-FU

Experiments were conducted to determine if defibrotide interferes with the therapeutic effects of chemotherapy including reduction of tumor load (anti-leukemic effects) and immunosuppression (anti-hematopoietic effects) activities.

- Results indicated that defibrotide did not decrease the anti-leukemic effect of fludarabine measured by the percent vitality using trypan blue exclusion analysis in primary AML cells or the immunosuppressive effects of fludarabine measured in the side scatter apoptosis assay in PBMC cells from a healthy donor.
- The flow cytometric proliferation assay described above was used to investigate the effect of defibrotide on fludarabine-mediated inhibition of cell proliferation in TCR (CD3/CD28)-stimulated T-cells. The results are shown in the figure below and are reported as the number of proliferating cells, total number of viable cells, and the % CFSE decline as a measure of proliferation. While defibrotide did not completely inhibit the fludarabine-mediated inhibition of cell proliferation or cell death of TCR (CD3/CD28)-stimulated T-cells, defibrotide did appear to lessen the sensitivity of the TCR (CD3/CD28)-stimulated T-cells to the effects of fludarabine.

Figure 9: Effects of defibrotide on fludarabine-mediated inhibition of cell proliferation in TCR (CD3/CD28)-stimulated T-cells
(excerpted from study report for RB2008020)

Stimulation	% CFSE decline	Total no. of prolif. cells	Total no. of viable cells
Control (CO)	71.2	68115	95000
F-Ara 200 µM	0.5	35	7000
F-Ara 20 µM	1.4	118	8400
F-Ara 2 µM	61.3	36903	60200
DF 4 µM	73.8	73800	100000
F-Ara 200 µM + DF 4 µM	1.2	101	8400
F-Ara 20 µM + DF 4 µM	9.5	665	7000
F-Ara 2 µM + DF 4 µM	70.0	47040	67200



- Flow cytometric proliferation assays were also conducted with blasts from AML patients. The results are shown in the table below and are reported as the number of proliferating cells and the % CFSE decline as a measure of proliferation. Once again, while defibrotide did not completely inhibit the fludarabine-mediated inhibition of cell proliferation in AML cells, defibrotide did appear to lessen the sensitivity of the cells to the effects of fludarabine.

Table 2: Effects of defibrotide on fludarabine-mediated inhibition of cell proliferation in AML cells
(excerpted from study report for RB2008020)

Stimulation	% CFSE decline		Total no. of prolif. Cells	
	Day 5	Day 7	Day 5	Day 7
Control	92.4	91.9	224070	268808
F-Ara 50 µM*	4.2	3.5	2415	1050
F-Ara 5	5.5	5.7	3300	1853
F-Ara 0.5	6.1	11.9	19520	19338
F-Ara 50+DF 50	3.5	6.0	1925	1950
F-Ara 50+DF 5	4.6	4.8	2415	1560
F-Ara 5+DF 0.5	4.1	4.1	1538	1333
F-Ara 5+DF 50	7.3	10.0	3103	3500
F-Ara 5+DF 5	6.9	8.1	4140	2835
F-Ara 0.5+DF 0.5	5.2	6.3	1950	1733
F-Ara 0.5+DF 50	5.7	14.9	7410	31290
F-Ara 0.5+DF 5	5.4	9.4	9450	17625
F-Ara 0.5+DF 0.5	5.2	10.4	10270	21840

Study title: Defibrotide modulates the expression of genes in endothelial cells induced by fludarabine: A microarray analysis

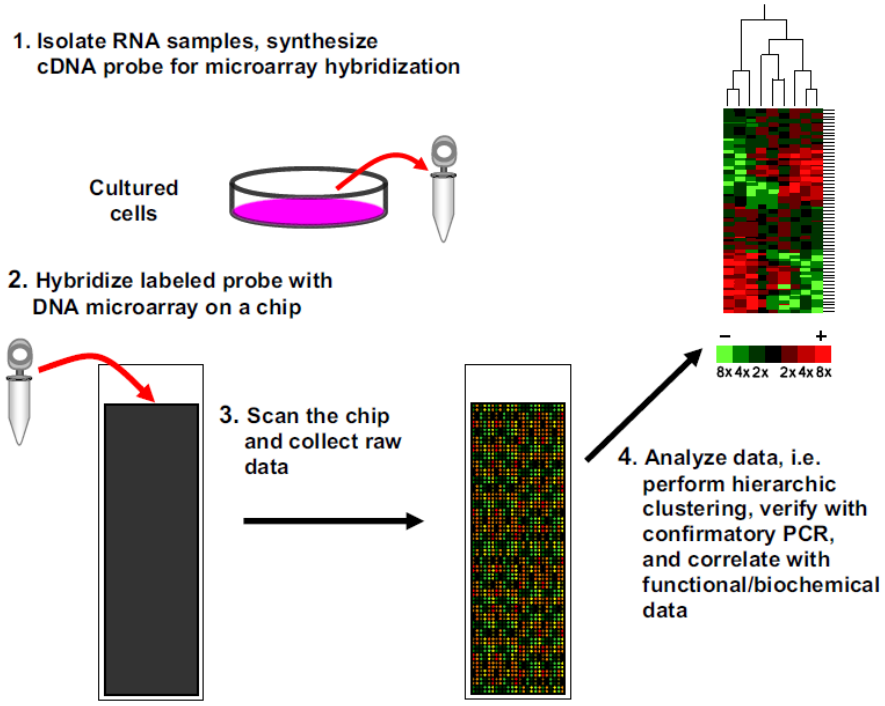
Study No.: RB2010010
Report Date: July 7, 2010
Study report location: eCTD 4.2.1.1.
Conducting Laboratory: (b) (4)
GLP: No

Defibrotide has been shown to protect endothelial cells from fludarabine-induced apoptosis without much effect on the therapeutic effects of fludarabine in previous studies. This study investigated whether the protective effect of defibrotide occurs at the transcriptional level and can be related with the modulation of other fludarabine-activated endothelial cell pathways.

Microarray expression profile

HMECs were incubated with fludarabine (10 µg/mL) in the presence or absence of defibrotide (100 µg/mL) for 6 and 24 hours. Total cellular RNA was then isolated by using TRIZOL reagent according to the manufacture's protocol. The Affymetrix analysis was conducted at [redacted] (b) (4) using the standard affymetrix genechip protocol. The microarray procedure is shown in the figure below.

Figure 10: Schematic description of the microarray procedure
(excerpted from study report for RB2010010)



Real time PCR

HMECs (30,000/well) were cultured in 48 well plates for 3 days and were then incubated with fludarabine with and without defibrotide for 24 hours. Each experimental condition was performed in triplicates. RNA was isolated by RNeasy Mini Kit according to the manufacturer's instructions. The 1% agarose gel electrophoresis, stained with ethidium bromide, was performed on all samples to check the purity of the RNA. The mRNA levels of heparanase and interleukin-8 (IL-8) were measured through were measured through Syber-green real-time PCR of cDNA prepared by iScript™ cDNA synthesis kit. The expression of the genes was quantified by normalizing to β-actin expression.

Immunoenzymatic assay interleukin-8 (ELISA for IL-8)

The immunoenzymatic assay was conducted to verify the impact of fludarabine in both the presence and absence of defibrotide on the release of IL-8 by HMEC cells. The cells were incubated at three different times (24, 48, and 72 hours). IL-8 was determined in culture supernatants using a sandwich ELISA assay; the minimum detectable dose of the IL-8 ELISA was 3.5 pg/mL. Culture supernatants were all tested in triplicate. In all tests, a standard cytokine preparation (recombinant cytokine at

defined concentration) was used as an internal control. Results are expressed as pg/number of viable cells.

Cytometric bead array
The effect of defibrotide to modulate the level of caspase-3 protein in fludarabine-activated endothelial cells was examined with a commercially available cytometric bead array kit. The bead population with fluorescence intensity had been captured with the antibody against active caspase-3. HMECs were treated with fludarabine in the presence and absence of defibrotide, and the cell lysates were incubated with the beads and the protein levels assessed using cytometric bead array analysis software.

Surface marker immunostaining for flow cytometric analysis
HMEC cells were surface stained using a mouse anti-human antibody to detect the antimajor histocompatibility complex (MHC) Class II and with mouse anti-human CD146/ melanoma cell adhesion molecule (MCAM) antibody. Cell-surface labeling was conducted by a direct fluorescence technique with cells incubated with each primary antibody in a total volume of 100 µL for 1 hour on ice. HMECs were then washed with PBS/1% BSA and incubated in the dark with fluorescein-conjugated secondary antibody IgG for 30 minutes on ice. Cells were washed and analyzed for the expression of MHC Class II and CD146 by FACS Calibur.

Results

Microarray gene expression analysis

- Defibrotide did not significantly induce or reduce transcriptional activity of control HMECs after 6 or 24 hours.
- When cells were activated by fludarabine after 24 hours, the presence of defibrotide downregulated several genes induced by fludarabine, particularly those associated with apoptosis, angiogenesis/migration, adhesion, and inflammatory activation.

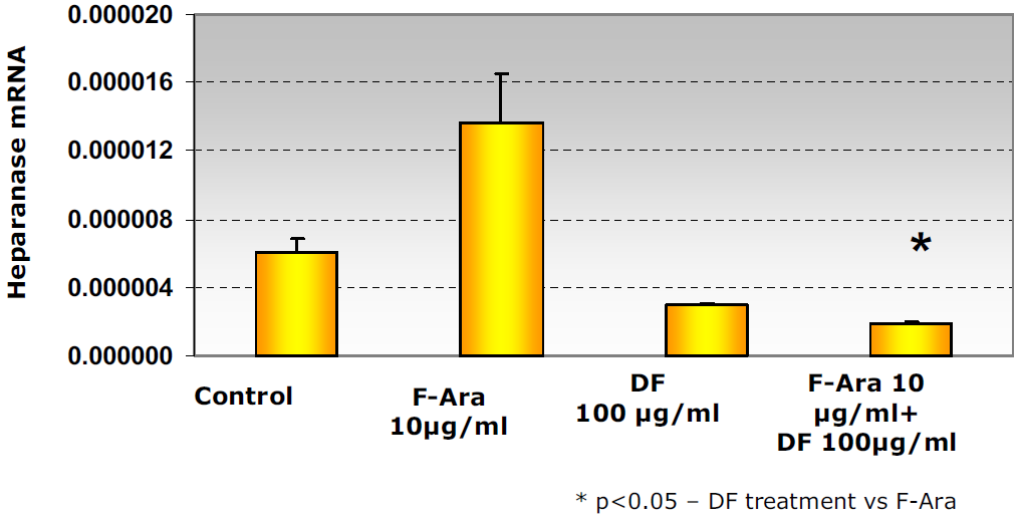
Table 3: Effects of 24 hours of defibrotide and fludarabine with and without defibrotide on the expression of selected genes
(excerpted from study report for RB2010010)

APOPTOSIS	DF vs Ctr	F-ara vs Ctr	F-Ara+DF vs F-Ara
PDCD6	-	↑	↓
FAS	-	↑	↓
APAF-1	-	↑	↓
MOAP-1	-	↑	↓
Caspase 3	-	↑	↓
Caspase 9	-	↑	↓
BAD	-	↑	↓
INFLAMMATION/ INNATE IMMUNUTY	**DF vs Ctr**	**F-ara vs Ctr**	**F-Ara+DF vs F-Ara**
IL-1β	-	↑	↓
IL-32	-	↑	↓
IL-11	-	↑	↓
TRL1	-	↑	↓
TLR3	-	↑	↓
ICAM1	-	↑	↓
MHC Class II	-	↑	↓
ANGIOGENESIS/ MIGRATION	**DF vs Ctr**	**F-ara vs Ctr**	**F-Ara+DF vs F-Ara**
Heparanase	-	↑	↓
FGFR3	-	↑	↓
CCL5	-	↑	↓
MCAM (CD146)	-	↑	↓
PLAU	-	↑	↓
VEGF	-	↑	↓
IL8	-	↑	↓
THBS1	-	↓	↑

Effects of defibrotide on heparanase and IL-8 expression in activated endothelial cells

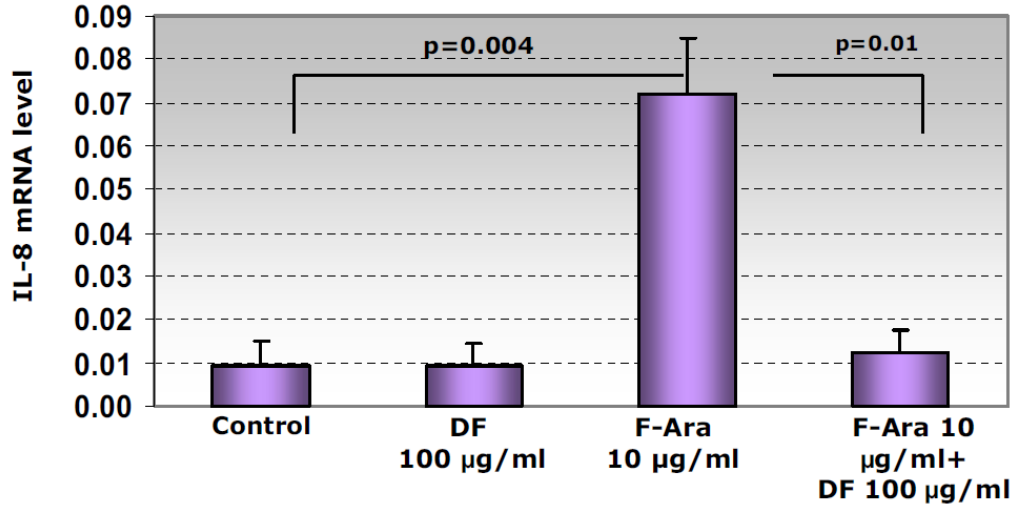
- Real time PCR experiments showed that fludarabine alone increased the gene expression of heparanase and IL-8 in HMEC cells. Treatment of defibrotide with fludarabine decreased the gene expression of both heparanase and IL-8 compared to fludarabine alone.

Figure 11: Effect of defibrotide on heparanase gene expression
(excerpted from study report for RB2010010)



* p<0.05 – DF treatment vs F-Ara

Figure 12: Effect of defibrotide on IL-8 gene expression
(excerpted from study report for RB2010010)

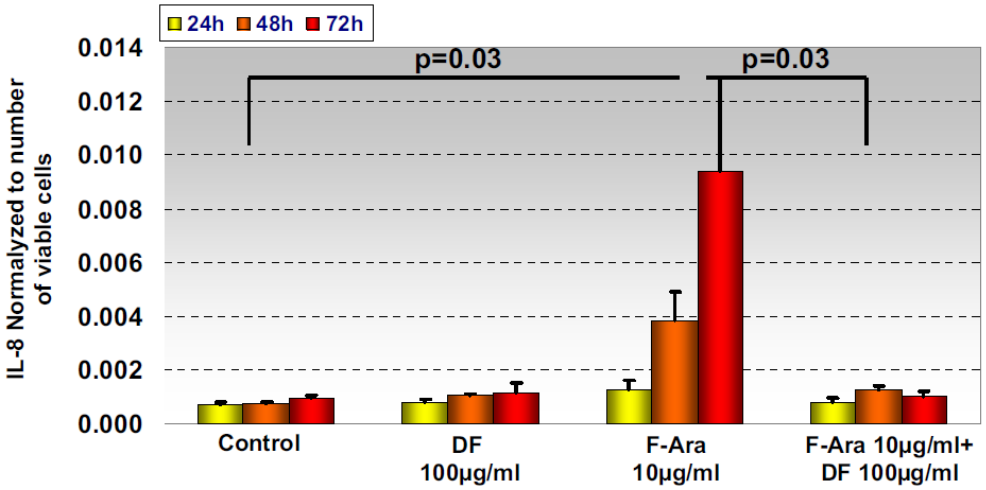


* p< 0.05; ** p< 0.01
DF treatment vs F-Ara

Effect of defibrotide on the release of IL-8 by HMECs treated with fludarabine

- In an ELISA assay, fludarabine increased the release of IL-8 in a time-dependent manner in HMEC cells and treatment of defibrotide with fludarabine significantly decreased the release of IL-8 by fludarabine.

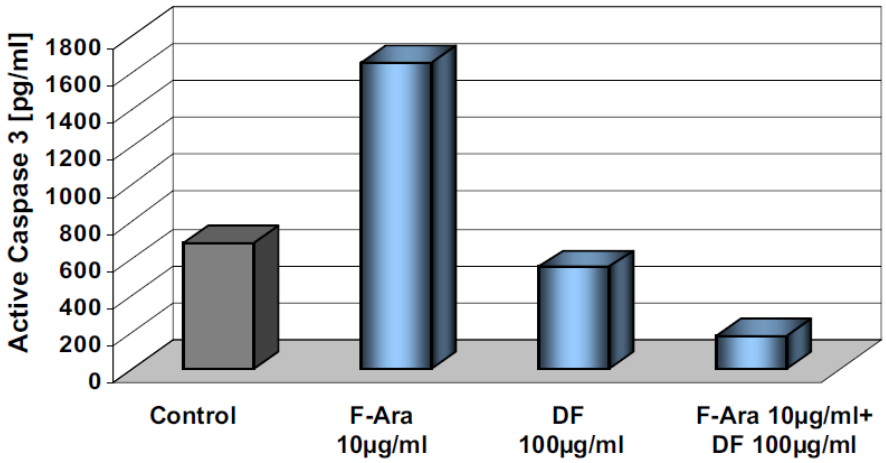
Figure 13: Effect of defibrotide on IL-8 secretion induced by fludarabine
(excerpted from study report for RB2010010)



Effect of defibrotide on fludarabine-induced increases in active caspase-3

- Experiments with the cytometric bead array showed that treatment of defibrotide with fludarabine protected endothelial cells from increases in active caspase-3 induced by fludarabine.

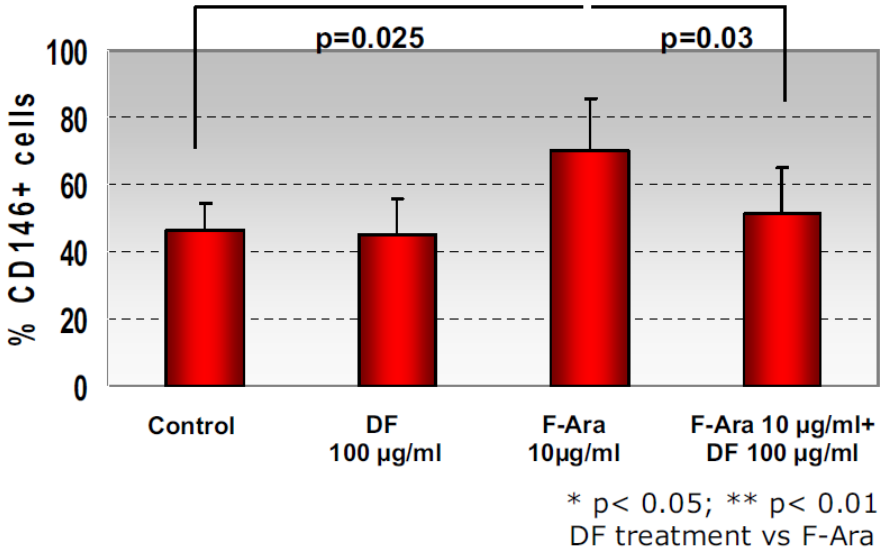
Figure 14: Effect of defibrotide on fludarabine-induced increases in active caspase-3
(excerpted from study report for RB2010010)



Flow cytometric analysis of MCAM/CD146 and MHC Class II on HMECs

- Treatment of defibrotide with fludarabine significantly reduced the rates of CD146-positive HMECs increased with fludarabine alone.

Figure 15: Flow cytometric analysis of CD146 expression
(excerpted from study report for RB2010010)



- MHC Class II molecules were expressed on 36% of HMEC cells stimulated by fludarabine compared to 7.5% of control HMEC cells. Treatment of defibrotide with fludarabine reduced the number of cells expressing MHC Class II molecules to 10.8%.

Study title: Defibrotide blunts the pro-thrombotic effect of thalidomide on endothelial cells

Study No.:	RB2009010
Report Date:	November 2009
Study report location:	eCTD 4.2.1.1.
Conducting Laboratory:	(b) (4)
GLP:	No

Previous nonclinical studies have demonstrated that defibrotide has pro-fibrinolytic and anti-thrombotic activities. This study was conducted to investigate the effect of defibrotide on the thrombotic effect of thalidomide on endothelial cells.

In addition to the use of HMEC endothelial cells in these experiments, a co-culture system was used in order to verify if multiple myeloma cells by themselves can modify the antithrombotic phenotype of endothelial cells and the effect of thalidomide and thalidomide plus defibrotide on this system. For co-culture, $3x10^5$ cells/well of HMEC were seeded in a 6 well plate for 24 hours, then $3 x10^6$ cells/well of RPMI 8226 multiple myeloma cells were added. After 24 hours, the cells were stimulated with thalidomide (100 µg/mL) and thalidomide plus defibrotide (100-400 µg/mL) for 4-24 hours. The cells

were used in ELISA assays, mRNA extraction and gene expression, and the MTT assay.

Enzyme linked immune-sorbent assay (ELISA)
To assess both the impact of thalidomide on endothelial cells and the ability of defibrotide to modulate thalidomide-induced changes in fibrinolysis, HMEC cells ($6x10^4$) were incubated with thalidomide (50 and 100 µg/mL) in the presence and absence of 150 µg/mL of defibrotide for 24 hours. In parallel, the co-culture of endothelial and multiple myeloma cells was applied. The release of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1), in these cells' supernatant was quantified by an ELISA assay performed according to the manufacturer's instructions.

Thiazolyl blue tetrazolium bromide assay (MTT)
Proliferation and viability of endothelium and multiple myeloma cells, treated with thalidomide (10, 25, 50, 75, or 100 µg/mL) with and without defibrotide (150 µg/mL), were determined by the MTT assay. As mentioned above, this method is based on the cleavage of tetrazolium salts by mitochondrial dehydrogenase in viable cells leading to the production of formazan dye. After incubation with MTT solution that contains the tetrazolium compound, the formation of formazan product was measured by spectrophotometry. The level of formazan salt is proportional to the number of living/proliferating cells. A blank well containing only media and drug was also run as a control in all experiments.

Real time PCR
RNA were isolated by RNeasy Mini kit according to the manufacturer's instructions. The 1% agarose gel electrophoresis, stained with ethidium bromide, was performed on all samples to check the purity of the RNA. mRNA levels of tPA and PAI-1 were measured through Syber-green real-time PCR of cDNA prepared by iScript™ cDNA synthesis kit. The expression of the genes was quantified by normalizing to β-actin expression.

Fibrin clot plate assay
The fibrin clot was formed in the wells of a microtitre plate with the purpose of determining the fibrinolytic activity of cell supernatant to degrade the fibrin clots. In this method, the fibrin clot was formed by mixing fibrinogen, plasminogen and thrombin. When the clot was completed, the supernatant of cells treated with thalidomide in the presence and absence of defibrotide and a chromogenic substrate (S-2251 were added to the clot. The plasmin generated by the t-PA present on cell supernatant was able to digest fibrin and also hydrolyzes the substrate S-2251. The generation of *p*-nitroaniline was monitored by following the increase in OD at 405 nm in 2.5 minute steps for a period of 10 to 25 minutes. Plasmin activity was determined from a slope of plots absorbance versus time.

Apoptosis Assays
An established method for detecting apoptosis in human endothelial cells was conducted using flow cytometry. Multiple myeloma and endothelial cells were left

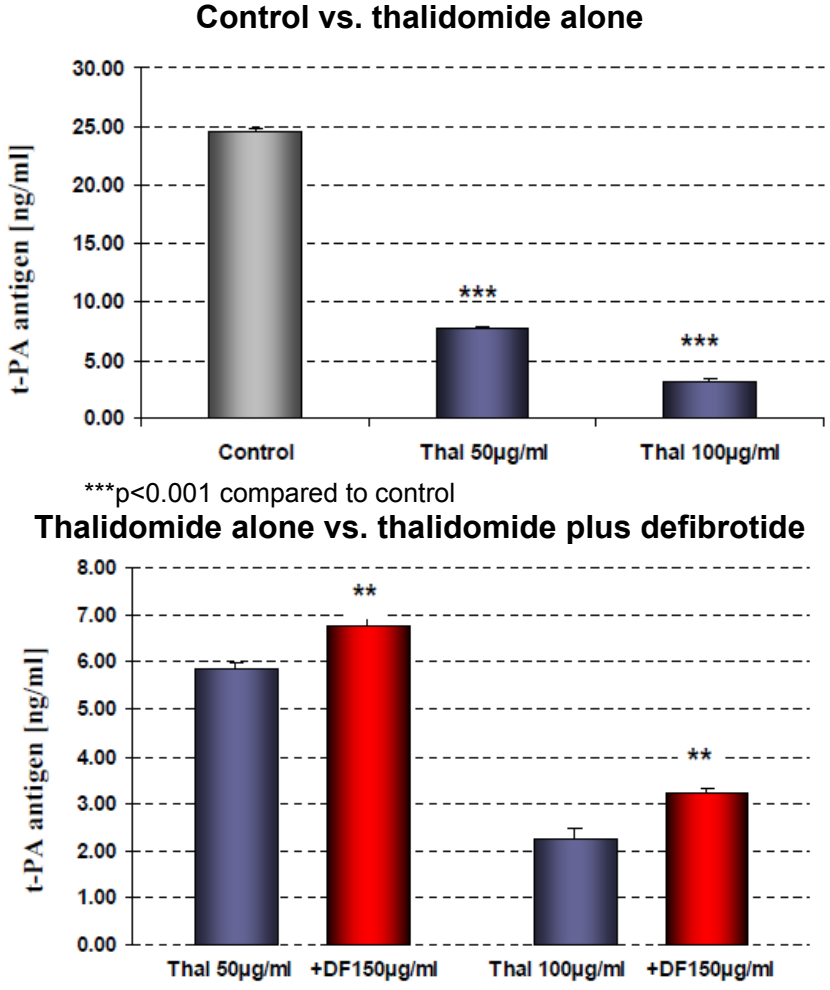
unstimulated or stimulated with thalidomide (100 µg/mL) in the presence or absence of defibrotide (150 µg/mL) for 24 hours. Cells were then washed in PBS and were stained with the necrosis-detecting dye propidium iodide. Apoptotic cells were identified by propidium iodide-negative staining and by a characteristic side scatter image distinct from that of non-apoptotic cells.

Results

Defibrotide increases the release of t-PA in HMEC cells treated with thalidomide

- Results of ELISA assays showed that after 24 hours of incubation, both concentrations of thalidomide significantly reduced the levels of t-PA antigen in HMEC cells supernatant. Treatment of defibrotide (150 µg/mL) with the thalidomide significantly increased the levels of t-PA antigen compared to thalidomide alone. It is noted that the levels of t-PA with defibrotide treatment were still below the levels of control.

Figure 16: Levels of t-PA following treatment with thalidomide and thalidomide plus defibrotide
(excerpted from study report for RB2009010)



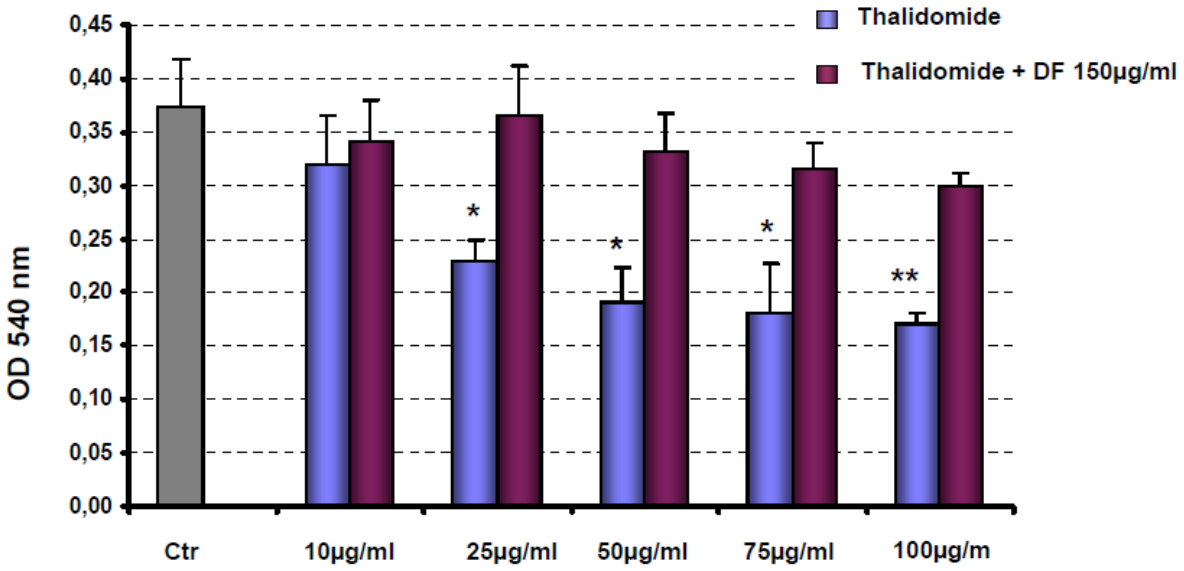
**p<0.01 compared to thalidomide alone

- In the co-cultured system with HMEC and multiple myeloma cells, multiple myeloma cells by themselves changed the pro-fibrinolytic potential of HMEC cells by decreasing the release of t-PA and increasing the expression of PAI-1.
- In this system, the decrease in t-PA levels and increase in PAI-1 were larger with treatment of thalidomide. Treatment of defibrotide with the thalidomide significantly increased the levels of t-PA and decreased the levels of PAI-1compared to thalidomide alone.

Protective effects of defibrotide on the cytotoxic effects of thalidomide

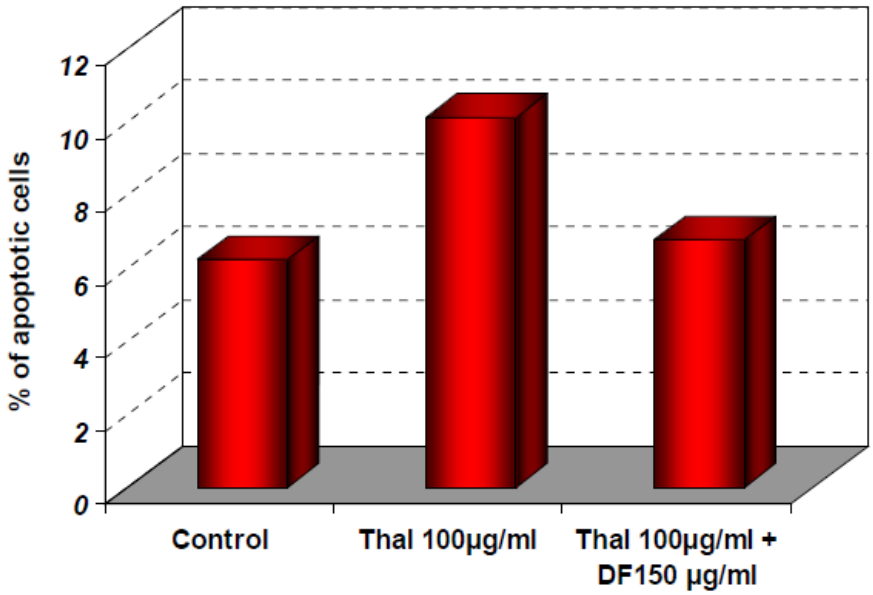
- In MTT experiments, defibrotide demonstrated a protective effect against the cytotoxic effect of thalidomide.

Figure 17: Effect of defibrotide on thalidomide-induced cytotoxicity in MTT assay
(excerpted from study report for RB2009010)



- In apoptosis assays, thalidomide (100 µg/mL) alone produced apoptosis in up to 60% of HMEC cells, and treatment of defibrotide (150 µg/mL) with thalidomide decreased the apoptosis in HMEC cells.

Figure 18: Effect of defibrotide on thalidomide-induced apoptosis
(excerpted from study report for RB2009010)

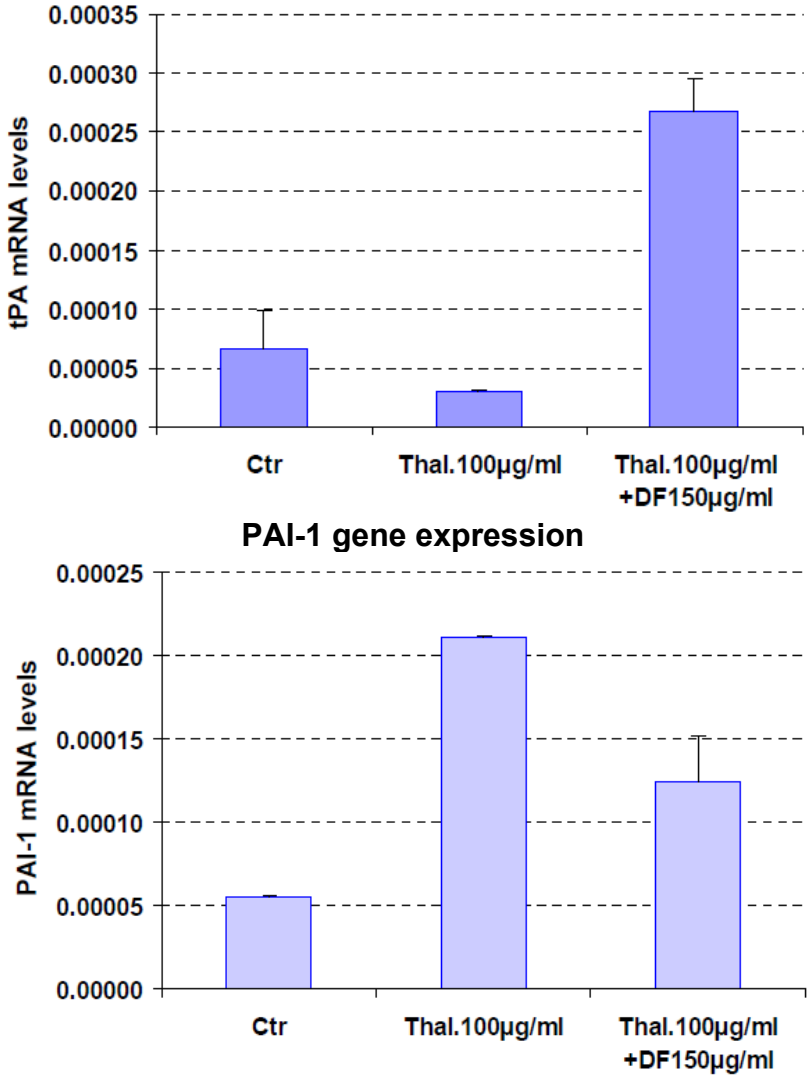


- In experiments in multiple myeloma cells, defibrotide did not protect multiple myeloma cells from the cytotoxic effects of thalidomide. These results indicate that defibrotide can protect endothelial cells but does not interfere with the therapeutic effects of thalidomide.

Effects of defibrotide on t-PA and PAI-1 gene expression in HMEC cells treated with thalidomide

- In experiments with real time PCR, thalidomide reduced the t-PA gene expression by 2.2 fold and increased the PAI-1 expression by 4 fold compared to control HMEC cells.
- Treatment of defibrotide with thalidomide reversed these effects by up-regulating the expression of t-PA and down-regulating the gene expression of PAI-1 compared to thalidomide alone. The amount of t-PA gene expression of the thalidomide plus defibrotide treated cells was actually greater than the expression in control cells.

Figure 19: Effect of defibrotide on thalidomide-induced changes in t-PA and PAI-1 gene expression
(excerpted from study report for RB2009010)

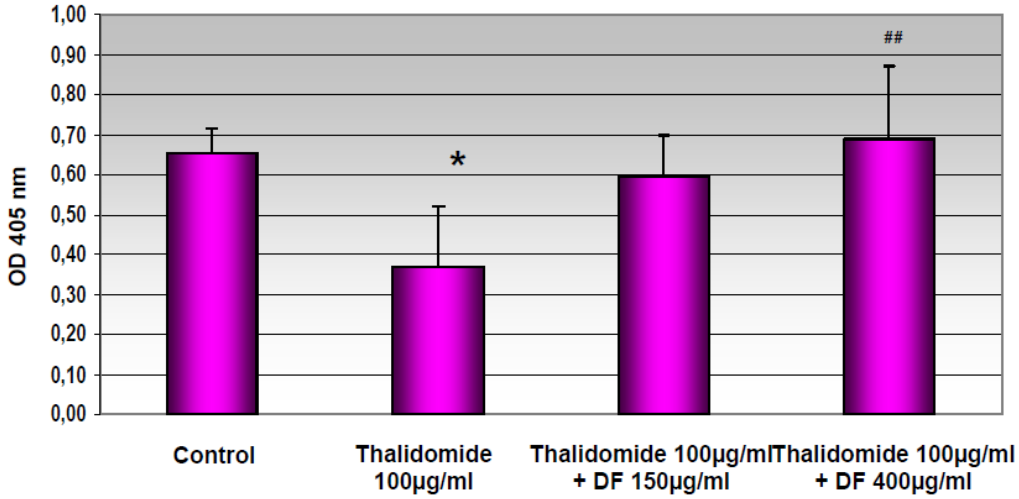


Effects of defibrotide on thalidomide-induced decreases in fibrinolytic activity

- In the fibrin clot plate assay, thalidomide reduced the fibrinolytic activity of HMEC cells, and treatment of defibrotide with thalidomide significantly increased in a concentration-dependent manner the fibrinolytic ability of HMEC to degrade the fibrin clot compared to thalidomide alone.

Figure 20: Effect of defibrotide on thalidomide-induced decreases in fibrinolytic activity

(excerpted from study report for RB2009010)



* p<0.05 thalidomide vs. control
p<0.01 defibrotide + thalidomide vs. thalidomide alone

4.3 Safety Pharmacology

Study title: Defibrotide: Effect on hERG tail current recorded from stably transfected HEK 293 cells

Study No.:	0040-2010
Study report location:	eCTD 4.2.1.3.
Conducting laboratory and location:	(b) (4)
Experimental start date:	February 10, 2010
GLP compliance:	No
QA statement:	No
Drug, lot #, and % purity:	Defibrotide, Lot # 1070010073, Purity: 95.4%

To assess the effect of defibrotide on hERG tail current, HEK 293 cells stably transfected with hERG cDNA were used for patch clamp experiments in voltage clamp configuration. Defibrotide was tested at concentrations of 50, 150, and 500 µg/mL. Groups of cells were treated with vehicle solution, with a single concentration of defibrotide, and with the positive control E4031 (300 nM).

Cells were held at -80 mV. Onset and steady state blockade of the hERG channel current (I_{Kr}) due to defibrotide were monitored using a pulse pattern with fixed amplitudes (first depolarization: -50 mV for 500 ms; second depolarization: +20 mV for 2 seconds; hyperpolarization: -50 mV for 7 seconds). The current amplitude at the onset of the second step to -50 mV, referred to as the peak tail current, was monitored until a steady state was obtained in the vehicle solution and then defibrotide was applied to the bath solution at 37°C. The reference item (E4031) was applied at the end to block

100% of the current for determination of the current "leak" response to the stimulation protocol. The effect of defibrotide was determined by calculating the residual current as a percentage of control (% control) and all data were corrected for "leak" current by subtracting the response in the presence of 300 nM of the positive control E4031.

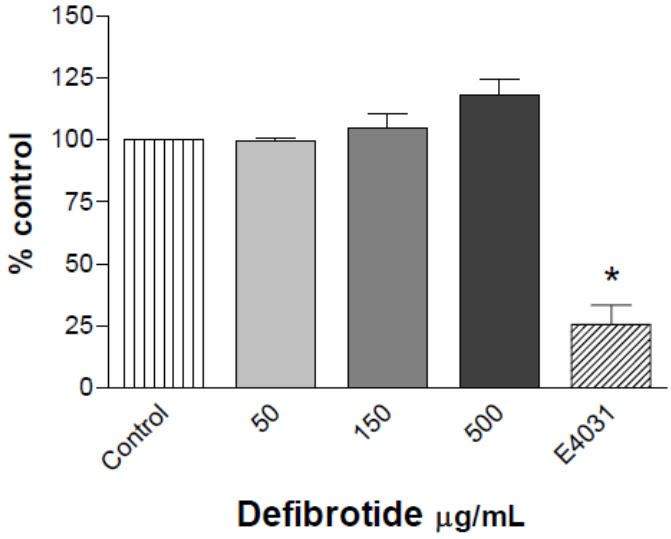
Results

Exposure to defibrotide did not reduce the hERG residual tail current at any of the concentrations tested.

Table 4: Effect of defibrotide on hERG tail current

Treatment	n	Tail current (% control)	
		Mean	Sem
50 µg/mL	3	99.73	1.26
150 µg/mL	3	104.87	5.68
500 µg/mL	3	117.89	6.50

Figure 21: Effect of defibrotide on hERG tail current
(excerpted from study report for 0040-2010)



5 Pharmacokinetics/ADME/Toxicokinetics

5.1 PK/ADME

Study title: Absorption, distribution, and excretion of [^{125}I]-defibrotide following oral or intravenous administration in rats

Study No.:	150210 (8284)
Report Date:	February 1992
Study report location:	eCTD 4.2.2.2.
Conducting Laboratory:	(b) (4)
GLP:	No

This study was reviewed under IND 62118 by David B. Joseph (PhD) in the Division of Gastrointestinal and Coagulation Drug Products (DARRTS date: September 2, 2005). The review is presented below and has been modified slightly for this review.

Plasma Kinetics of Radioactivity in Rats Using Oral and Intravenous Administration of [^{125}I]Defibrotide

Methods: Male Sprague Dawley rats (166-270 g) were treated orally with 12.5 or 25 mg/kg [^{125}I]defibrotide (6 rats/group), or intravenously with 6.25 or 12.5 mg/kg [^{125}I]defibrotide (8 rats/group). Blood samples were collected at 0.25-48 hr post-dose in the orally-treated groups, and at 1 min-48 hr post-dose in the intravenously-treated groups. Plasma radioactivity levels were measured. Additional groups were treated orally with 12.5 or 25 mg/kg/day [^{125}I]defibrotide for seven days (6 rats/group). Blood was collected at 0.25-48 hr after the final dose.

Results: A previous study (reviewed above) demonstrated that a substantial proportion of plasma radioactivity is associated with free ^{125}I following administration of [^{125}I]defibrotide in rats, due to release of ^{125}I after dosing. Since the present study did not include a qualitative analysis of the plasma radioactivity, the results are extremely difficult to interpret. Plasma kinetic parameters for total radioactivity are shown in the following table.

	Plasma Kinetic Parameters for Total Radioactivity					
Dose (mg/kg)	**Route**	**Number of Doses**	**C_{max} (μg eq/ml)**	**t_{max}**	**$t_{1/2}$ (hr)**	**$AUC_{0-48\ hr}$ (μg eq•hr/ml)**
6.25	IV	1	106.1	1 min	< 1	62.5
12.5	IV	1	227.8	1 min	< 1	138.8
12.5	PO	1	5.2	8 hr	nc	91.4
25	PO	1	11.8	8 hr	nc	198.8
12.5*	PO	7	6.2	8 hr	nc	105.4
25*	PO	7	12.9	8 hr	nc	227.7

Values are the mean of 6-8 rats/group/time-point
* mg/kg/day
nc: not calculated

Conclusions: The results are difficult to interpret, given that a substantial proportion of the plasma radioactivity was probably associated with free ^{125}I.

Distribution Study in Rats Using Oral and Intravenous Administration

Methods: Male Sprague Dawley rats (166-270 g) were treated orally with 12.5 or 25 mg/kg [^{125}I]defibrotide, or intravenously with 6.25 or 12.5 mg/kg [^{125}I]defibrotide. Each animal was given a single oral administration of 4 mg/kg potassium iodide at 16 hr prior to dosing with [^{125}I]defibrotide, presumably to minimize the accumulation of ^{125}I in thyroid. The rats were sacrificed at 2, 8, 12, 24, 36, 48, and 168 hr after dosing (1 rat/group/time-point). Total radioactivity was measured in each tissue sample collected. In addition, the proportion of radioactivity associated with free ^{125}I in plasma was measured.

Results: The results are shown in the following tables and are expressed as μg equiv.g or mL.

Table 5: Levels of total radioactivity in selected tissues, organs, and body fluids following single oral administration of [^{125}I]-defibrotide at 12.5 mg/kg
(excerpted from study report for 150210)

Tissue	Animal Number						
	41	42	43	44	45	46	47
	Time of Sacrifice Post Dose (h)						
	2	8	12	24	36	48	168
Whole Blood	4.142	3.879	2.750	0.614	0.313	0.266	0.027
Plasma	4.477	4.024	2.042	0.658	0.429	0.368	0.012
Aorta	3.891	3.469	2.321	0.638	0.281	0.158	0.055
Adrenals	2.669	3.646	1.848	0.328	0.156	0.088	0.000
Brain	0.190	0.171	0.160	0.112	0.012	0.012	0.001
Bone Marrow	3.091	2.611	2.057	0.515	0.282	0.146	0.045
Eyes	1.375	1.103	0.913	3.384	0.142	0.094	0.009
Fat	1.354	1.697	1.095	0.190	0.115	0.116	0.011
Spleen	1.770	1.743	1.148	0.290	0.164	0.122	0.000
Testes	1.453	1.772	1.336	0.327	0.181	0.093	0.000
Thyroid	286.268	848.503	1643.153	4068.853	3019.148	1605.124	5.872
Heart	1.448	1.406	1.228	0.220	0.119	0.129	0.024
Large Intestine + Contents	21.762	105.358	38.032	3.973	0.742	6.062	0.027
Small Intestine + Contents	114.474	28.475	16.016	2.220	1.774	11.851	0.038
Stomach + Contents	162.068	30.440	37.782	3.662	1.837	7.407	0.046
Kidneys	3.740	3.178	2.321	0.484	0.436	0.249	0.029
Liver	2.131	1.918	1.422	0.378	0.315	0.281	0.030
Lungs	2.271	2.435	1.732	0.400	0.250	0.201	0.026
Muscle	0.900	0.938	0.653	0.682	0.061	0.057	0.008
Skin	4.068	5.064	3.482	0.836	0.616	0.672	0.088
Bone	1.879	1.590	0.959	0.211	0.096	0.199	0.008
Blood Cells	3.940	3.553	2.458	0.539	0.346	0.252	0.033
Carcass	2.291	3.937	3.253	0.728	0.621	0.563	0.248

Tissue	Animal Number						
	41	42	43	44	45	46	47
	Time of Sacrifice Post Dose (h)						
	2	8	12	24	36	48	168
Free ^{125}I	2.977	2.531	1.699	0.371	0.185	0.184	0.000
% Free ^{125}I	66.5	62.9	83.2	56.4	43.1	49.9	0.0
% Defibrotide and Related Components	33.5	37.1	16.8	43.6	56.1	50.1	100.0

Table 6: Levels of total radioactivity in selected tissues, organs, and body fluids following single oral administration of [^{125}I]-defibrotide at 25 mg/kg
(excerpted from study report for 150210)

Tissue	Animal Number						
	7A	8	9	10	11	12	13
	Time of Sacrifice Post Dose (h)						
	2	8	12	24	36	48	168
Whole Blood	6.50	6.22	4.22	1.49	0.65	0.41	0.06
Plasma	6.97	6.77	4.30	1.55	0.71	0.53	0.07
Aorta	4.93	7.35	1.87	1.36	0.51	0.44	0.23
Adrenals	3.73	3.54	1.86	0.79	0.47	0.18	0.48
Brain	0.32	0.17	0.13	0.02	0.13	0.03	0.01
Bone Marrow	3.32	9.92	3.94	2.85	0.66	0.39	2.29
Eyes	1.89	1.79	1.21	0.45	1.61	1.10	0.37
Fat	2.43	2.29	1.28	0.47	0.16	0.12	0.02
Spleen	2.58	2.93	1.60	0.65	0.27	0.17	0.00
Testes	2.16	2.82	1.81	0.41	0.30	0.16	0.02
Thyroid	812.24	1234.44	1630.19	95.24+	4668.61	5588.04	2103.53
Heart	2.63	1.92	1.50	0.41	0.23	0.16	0.03
Large Intestine + Contents	209.69	171.54	60.14	4.79	1.59	0.76	0.09
Small Intestine + Contents	173.09	43.69	20.05	5.19	1.23	0.83	0.06
Stomach + Contents	140.92	73.06	18.19	7.75	2.11	0.95	0.03
Kidneys	6.40	5.72	3.38	1.24	0.54	0.31	0.07
Liver	3.25	3.51	1.69	0.68	0.40	0.40	0.07
Lungs	3.57	3.66	1.92	0.57	0.42	0.20	0.02
Muscle	1.45	1.67	0.84	0.34	0.05	0.00	0.01
Skin	6.16	5.00	3.93	1.58	1.16	0.38	0.06
Bone	2.71	2.70	1.82	0.59	0.19	0.09	0.00
Blood Cells	6.14	6.58	3.13	1.30	0.81	0.55	0.03
Carcass	3.40	3.56	4.12	1.84	1.40	0.79	0.35

+ = Atypically low value

Tissue	Animal Number						
	7A	8	9	10	11	12	13
	Time of Sacrifice Post Dose (h)						
	2	8	12	24	36	48	168
Free ^{125}I	4.48	4.13	2.70	0.95	0.38	0.21	0.003
% Free ^{125}I	64.3	61.0	62.9	61.1	53.3	38.7	4.1
% Defibrotide and Related Components	35.7	39.0	37.1	38.9	46.7	61.3	95.9

+ = Atypically low value

Table 7: Levels of total radioactivity in selected tissues, organs, and body fluids following single intravenous administration of [^{125}I]-defibrotide at 6.25 mg/kg
(excerpted from study report for 150210)

Tissue	Animal Number						
	54	55	56	57	58	59	60
	Time of Sacrifice Post Dose (h)						
	2	8	12	24	36	48	168
Whole Blood	2.012	2.702	1.176	0.351	0.150	0.093	0.014
Plasma	2.269	3.121	1.277	0.448	0.209	0.140	0.035
Aorta	1.817	2.755	1.598	0.415	0.201	0.199	0.104
Adrenals	3.956	2.562	2.351	1.275	0.996	0.758	0.332
Brain	0.094	0.116	0.039	0.031	0.004	0.001	0.001
Bone Marrow	5.242	5.609	4.517	2.609	3.005	2.464	1.687
Eyes	0.518	0.762	0.313	0.184	0.059	0.045	0.131
Fat	1.021	0.996	0.552	0.222	0.089	0.097	0.030
Spleen	6.377	5.245	4.434	3.204	2.565	1.639	1.033
Testes	0.854	1.316	0.629	0.223	0.93	0.057	0.031
Thyroid	96.858	506.755	455.346	945.530	871.651	514.548	335.566
Heart	0.831	0.804	0.439	0.192	0.116	0.089	0.034
Large Intestine + Contents	1.262	30.452	5.811	1.030	0.726	0.320	0.092
Small Intestine + Contents	29.261	13.433	6.903	1.782	0.446	0.313	0.082
Stomach + Contents	7.084	15.827	4.749	2.632	0.430	0.584	0.118
Kidneys	15.633	12.175	9.546	7.525	7.037	5.402	2.697
Liver	9.158	9.276	7.108	4.649	4.764	3.430	1.495
Lungs	1.346	1.607	0.780	0.321	0.186	0.134	0.050
Muscle	0.511	0.612	0.291	0.096	0.053	0.032	0.015
Skin	2.092	1.949	1.307	0.610	0.238	0.159	0.039
Bone	2.727	1.610	2.339	0.675	0.654	1.200	0.370
Blood Cells	1.897	2.459	1.007	0.318	0.086	0.079	0.016
Carcass	1.208	1.638	0.934	0.305	0.258	0.196	0.082

Tissue	Animal Number						
	54	55	56	57	58	59	60
	Time of Sacrifice Post Dose (h)						
	2	8	12	24	36	48	168
Free ^{125}I	0.879	1.930	0.671	0.176	0.011	0.0001	0.000
% Free ^{125}I	38.7	61.8	52.5	39.3	5.03	0.7	0.0
% Defibrotide and Related Components	61.3	38.2	47.5	60.7	95.0	99.3	100.0

Table 8: Levels of total radioactivity in selected tissues, organs, and body fluids following single intravenous administration of [^{125}I]-defibrotide at 12.5 mg/kg
(excerpted from study report for 150210)

Tissue	Animal Number						
	24	25	26	27	28	29	30
	Time of Sacrifice Post Dose (h)						
	2	8	12	24	36	48	168
Whole Blood	3.753	2.421	1.823	0.498	0.393	0.157	0.047
Plasma	4.317	2.710	1.984	0.642	0.440	0.297	0.054
Aorta	4.427	2.666	1.976	0.583	0.467	0.306	0.354
Adrenals	8.773	3.769	2.619	3.436	0.759	0.823	0.344
Brain	0.163	0.070	0.064	0.009	0.009	0.006	0.006
Bone Marrow	14.869	9.430	7.701	4.363	4.293	5.591	2.416
Eyes	1.032	0.716	0.541	0.165	0.117	0.103	0.032
Fat	1.421	0.885	0.736	0.269	0.245	0.146	0.047
Spleen	8.652	6.298	5.066	4.040	2.987	3.226	1.395
Testes	1.657	1.286	1.076	0.314	0.221	0.241	0.054
Thyroid	306.636	1408.088	564.609	3257.061	1064.353	1234.713	714.082
Heart	1.824	1.011	0.941	0.319	0.297	0.312	0.072
Large Intestine + Contents	2.454	3.205	16.219	73.656	0.763	0.793	0.406
Small Intestine + Contents	61.344	10.513	7.010	2.056	1.069	0.796	0.162
Stomach + Contents	3.690	16.817	8.225	2.494	1.050	0.664	0.129
Kidneys	31.068	22.271	18.346	15.370	13.350	10.724	4.457
Liver	13.063	10.809	10.423	7.251	5.818	4.272	1.935
Lungs	2.727	1.678	1.178	0.490	0.356	0.293	0.092
Muscle	1.512	0.630	0.423	0.189	0.131	0.069	0.025
Skin	4.135	2.675	1.842	0.942	0.671	0.383	0.147
Bone	4.623	3.556	1.967	3.056	1.232	1.093	0.480
Blood Cells	3.299	1.881	1.636	0.326	0.203	0.195	0.057
Carcass	2.713	1.644	1.232	0.463	0.583	0.436	0.263

Tissue	Animal Number						
	24	25	26	27	28	29	30
	Time of Sacrifice Post Dose (h)						
	2	8	12	24	36	48	168
Free ^{125}I	2.021	1.396	1.154	0.211	0.156	0.084	0.004
% Free ^{125}I	46.8	51.5	58.1	32.9	35.8	28.2	7.4
% Defibrotide and Related Components	53.2	48.5	41.9	67.1	64.2	71.8	92.6

The highest level of radioactivity was present in thyroid following oral or intravenous administration. It is likely that the radioactivity in thyroid was mostly associated with free ^{125}I, given that a large proportion of plasma radioactivity was present in this form, especially with oral administration. Following oral administration of 12.5 or 25 mg/kg [^{125}I]defibrotide, the highest radioactivity levels (excluding thyroid) were present in the gastrointestinal tract with contents. Radioactivity was broadly distributed in many other organs/tissues that were examined, albeit at much lower levels. Trace levels of radioactivity remained at 168 hr after oral dosing. Following intravenous administration of 6.25 or 12.5 mg/kg [^{125}I]defibrotide, the highest radioactivity levels (excluding thyroid) were present in gastrointestinal tract with contents, kidneys, bone marrow, liver, spleen, and adrenals. Radioactivity levels in these organs exceeded plasma levels. Lower concentrations of radioactivity were detected in many other organs/tissues. At 168 hr post-dose, relatively high levels were still present in kidneys, bone marrow, liver, and spleen. Given that a high proportion of plasma radioactivity was associated with free ^{125}I (up to 83% and 62% following oral and intravenous administration, respectively), it is possible that a significant proportion of radioactivity in organs and tissues was actually free ^{125}I. Therefore, the results are extremely difficult to interpret.

Conclusions: This study was deficient in several aspects, including the absence of time-points before 2 hr post-dose and the small number of animals used (1 rat/time-point). However, the most significant problem was related to the use of [^{125}I]defibrotide. Extensive release of ^{125}I from [^{125}I]defibrotide following administration in rats was previously demonstrated in an absorption study (reviewed above). The incidence of ^{125}I release was also demonstrated in the present study, as indicated by the high proportion of plasma radioactivity associated with free ^{125}I. It is possible that a significant proportion of radioactivity in organs and tissues was actually free ^{125}I. Therefore, the results are extremely difficult to interpret.

Distribution Study in Pigmented Rats

Methods: Male Harlan Olac PVG rats (182-214 g) were treated orally with 25 mg/kg [^{125}I]defibrotide or intravenously with 12.5 mg/kg [^{125}I]defibrotide. Each animal was given a single oral administration of 4 mg/kg potassium iodide at 16 hr prior to dosing with [^{125}I]defibrotide, presumably to minimize the accumulation of ^{125}I in thyroid. The rats were sacrificed at 8, 24, 48, and 168 hr after dosing (1 rat/group/time-point). Total radioactivity was measured in blood, plasma, blood cells, eyes, liver, and skin (pigmented and non-pigmented). In addition, the proportion of plasma radioactivity associated with free ^{125}I was measured.

Results: The results are shown in the following tables and are expressed as µg equiv.g or mL.

Table 9: Levels of total radioactivity in selected tissues, organs, and body fluids following single intravenous administration of [^{125}I]-defibrotide at 12.5 mg/kg in pigmented rats

(excerpted from study report for 150210)

Tissue	Animal Number			
	31	32	33	34
	Time of Sacrifice Post Dose (h)			
	8	24	48	168
Whole Blood	3.52	0.41	0.07	0.20
Plasma	4.07	0.62	0.33	0.10
Liver	13.31	8.26	5.64	2.75
Eyes	1.21	0.22	0.13	0.10
Pigmented Skin	5.58	0.90	0.48	0.18
Non Pigmented Skin	3.99	0.72	0.23	0.19
Blood Cells	2.79	0.29	0.15	0.05
Free ^{125}I	2.15	0.17	0.04	0.01
% Free ^{125}I	52.8	27.5	12.1	9.3
% Defibrotide and Related Components	47.2	72.5	87.9	90.7

Table 10: Levels of total radioactivity in selected tissues, organs, and body fluids following single intravenous administration of [^{125}I]-defibrotide at 25 mg/kg in pigmented rats

Tissue	Animal Number			
	14	15	16	17
	Time of Sacrifice Post Dose (h)			
	8	24	48	168
Whole Blood	5.58	0.59	0.31	0.02
Plasma	6.55	0.80	0.53	0.08
Liver	3.58	0.86	0.51	0.07
Eyes	1.63	0.27	0.11	0.07
Pigmented Skin	5.53	3.42	0.37	0.12
Non Pigmented Skin	2.69	0.40	0.24	0.04
Blood Cells	5.05	0.45	0.26	0.00
Free ^{125}I	4.13	0.15	0.02	0.00
% Free ^{125}I	63.0	19.2	4.2	0.0
% Defibrotide and Related Components	37.0	80.8	95.8	100.0

Given that a high proportion of plasma radioactivity was associated with free ^{125}I (up to 63% at 8 hr), it is possible that a significant proportion of radioactivity in tissues was actually free ^{125}I.

Conclusions: The results are extremely difficult to interpret, due to the extensive release of ^{125}I from [^{125}I]defibrotide in plasma.

Excretion Study in Rats Using Oral and Intravenous Administration of [^{125}I]Defibrotide

Methods: Male Sprague Dawley rats (166-270 g) were treated orally with 12.5 or 25 mg/kg [^{125}I]defibrotide or intravenously with 6.25 or 12.5 mg/kg [^{125}I]defibrotide (6 rats/group). Urine, feces, and cage wash were collected at scheduled intervals up to 120 hr post-dose.

Results: No substantial differences in excretion rate occurred at the two dose levels tested for either the oral and intravenous route of administration. The results from rats treated orally or intravenously with 12.5 mg/kg [^{125}I]defibrotide are shown in the following table.

	Cumulative Excretion (%Radioactive Dose)						
	6 hr	**12 hr**	**24 hr**	**48 hr**	**72 hr**	**96 hr**	**120 hr**
	12.5 mg/kg po						
Urine	6.8	16.0	32.0	36.1	37.0	37.5	37.7
Feces	-	-	46.5	48.1	48.3	48.5	48.6
Cage wash	-	-	0.9	1.7	1.8	1.8	1.8
Total	-	-	79.4	85.9	87.1	87.7	88.1
Carcass							3.3
	12.5 mg/kg iv						
Urine	42.0	48.9	60.1	65.2	66.8	67.6	68.1
Feces	-	-	20.5	23.9	24.8	25.3	25.5
Cage wash	-	-	1.0	1.1	1.1	1.1	1.2
Total	-	-	81.6	90.3	92.8	94.0	94.8
Carcass							4.8

Values are the mean of 6 rats.

A pharmacokinetic study (reviewed above) has demonstrated that a large proportion of plasma radioactivity in rats was associated with free ^{125}I following oral or intravenous administration of [^{125}I]defibrotide, due to the release of ^{125}I from the radiolabeled drug. Therefore, the results of the present study are extremely difficult to interpret, since no qualitative analysis of the excreted radioactivity was performed. The extent to which excretion of radioactivity is indicative of drug excretion is uncertain in the absence of qualitative information about the excreted radioactivity.

Conclusions: The results are inconclusive due to the use of [^{125}I]defibrotide in this study, and the lack of qualitative analysis of the excreted radioactivity. A significant proportion of radioactivity excretion was probably due to the release of free ^{125}I after dosing.

6 General Toxicology

6.1 Single-Dose Toxicity

No single-dose toxicology studies were submitted to the NDA.

6.2 Repeat-Dose Toxicity

Study title: A 13-week intravenous toxicity study of defibrotide in rats

Study no.:	1529-001
Study report location:	eCTD 4.2.3.2.
Conducting laboratory and location:	(b) (4)
Date of study initiation:	February 26, 2008
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Defibrotide, lot # 2070010005

Key Study Findings

- Extensive mortality was observed with 89 rats either died or were euthanized *in extremis* during the study. While the number of mortalities observed is not dose-dependent, proportionally there was more mortality in each of the defibrotide-treated groups than in the control group, suggesting that the higher rates of mortality observed were related to the administration of defibrotide.
- Due to the high mortality rate and the complexity of the macroscopic and microscopic findings, many of which were related to complications such as septicemia, urinary tract infections, vegetative valvular endocarditis, and disseminated intravascular coagulation, it is difficult to interpret which findings were defibrotide-related and which were not.
- Defibrotide-related toxicities were observed in the kidney, liver, mandibular and mesenteric lymph nodes, and spleen.
- Clinical chemistry changes were supportive of other toxicities indicating an inflammatory response, anemia, renal failure, and hepatic injury.
- Decreased coagulation was observed with increases activated partial thromboplastin time and prothrombin time.

Methods

Doses:	0, 240, 1200, or 4800 mg/kg/day
Frequency of dosing:	Planned: Continuous infusion for 13 consecutive weeks (planned) Actual: Continuous infusion for 9 consecutive weeks and 1 day
Route of administration:	Intravenous infusion (continuous) via a femoral catheter
Dose volume:	Infusion rate of 2.5 mL/kg/hour; 60 mL/kg/day
Formulation/Vehicle:	0.9% sodium chloride for injection
Species/Strain:	Sprague-Dawley rats (Crl:CD®(SD))
Number/Sex/Group:	15/sex/group
Age:	9.5 weeks at arrival
Weight:	Males: 269-311 g at randomization Females: 162-236 g at randomization
Satellite groups:	Toxicokinetics: 4/sex for control group and 12/sex/group for defibrotide-treated groups

Continuous infusion of vehicle or defibrotide (240, 1200, or 4800 mg/kg/day) was not tolerated well in rats. Eighty nine rats either died or were euthanized *in extremis* during the study. Due to the number of mortalities and the dosing complications with the catheters, the study was terminated approximately 3 weeks early with all surviving animals necropsied between study Days 64 and 68. Only 111 out of a total of 200 animals in the study (33, 21, 29, and 28 animals in the 0, 240, 1200, and 4800 mg/kg/day groups, respectively) survived to this scheduled necropsy.
During Week 4, compromised catheter patency and function was suspected in several animals. These animals were examined under fluoroscopy using a radio-opaque contrast solution injected into the infusion catheters and inferior vena cava. Imaging revealed vascular blockage, suspected intraperitoneal dose administration, and/or retrograde flushing back through the vein. Surgical repair and reposition of the catheter was conducted in these animals in an attempt to re-establish patency and continue the intravenous administration. Subsequently, all animals exhibiting signs of swelling at the inguinal, femoral, and/or abdominal region as well those with multiple catheter complications underwent further fluoroscopic evaluation and surgery as needed.

Observations and times:

Mortality:	Twice daily
Clinical signs:	Cage side observations: Twice daily Detailed examinations: Weekly beginning Day -1 Catheter exit site was inspected daily from Day -4 until Day 68
Body weights:	Weekly beginning on Day -1
Food consumption:	Daily beginning on Day -1
Functional observation battery:	Prior to treatment for first 10 main study animals/sex/group, and Day 2 and prior to the necropsy for all surviving animals
Ophthalmoscopy:	Once prior to treatment
Hematology:	During Week 5 and prior to the necropsy
Clinical chemistry:	During Week 5 and prior to the necropsy
Coagulation:	During Week 5 and prior to the necropsy
Urinalysis:	During Week 5 and prior to the necropsy
Gross pathology:	At necropsy
Organ weights:	At necropsy
Histopathology:	At necropsy
Immunogenicity:	Days 64 or 65, from main study and toxicokinetic animals
Toxicokinetics:	Day 1: From toxicokinetic animals • Predose for the control group • Predose and 0.5, 1, 2, 3, 12, and 24 hours after dosing for defibrotide groups Days 64 or 65: Additional samples were collected immediately prior to the end of infusion in main study or toxicokinetics animals

The first day of dosing was designated as Day 1 and the day prior to dosing was designated as Day -1.

Results

Mortality

- Extensive mortality was observed in this study, with 89 rats either found dead or euthanized *in extremis*. The number of mortalities for each group is listed in the table below.
- The exact cause of death or reason for euthanasia was not apparent and deemed undetermined in some cases. The most common cause of death was heart failure (usually associated with vegetative valvular endocarditis), septicemia, and kidney inflammation/necrosis. Other causes of death included disseminated intravascular coagulation, heart inflammation/necrosis, pulmonary thrombosis, and pulmonary hemorrhage. Some mortality was related to surgical procedures to repair and reposition of the catheter (see surgery-related mortality section below).

- While the number of mortalities observed is not dose-dependent, proportionally there was more mortality in each of the defibrotide-treated groups than in the control group. The number of mortalities in the defibrotide-treated groups along with the information provided on the clinical signs, clinical pathology, and pathology findings suggests that the higher rates of mortality observed were related to the administration of defibrotide.

Table 11: Mortality observed in 13-week rat study
(excerpted from study report for 1529-001)

Study Mortality									
Dose Group	Number of Animals		Dose		DOS/Euthanized *in extremis*		Total Mortality by Group	Total Survivors by Group*	
	M	F	Test/Control Article	Dose Level (mg/kg/day)	M	F		M	F
Main Study									
1	15	15	Saline	0	2	2	4	13	13
2	15	15	Defibrotide	240	13	14	27	2	1
3	15	15	Defibrotide	1200	10	5	15	5	10
4	15	15	Defibrotide	4800	9	5	14	6	10
TK									
5	4	4	Saline	0	1	0	1	3	4
6	12	12	Defibrotide	240	2	4	6	10	8
7	12	12	Defibrotide	1200	4	6	10	8	6
8	12	12	Defibrotide	4800	5	7	12	7	5
	100	100		Total by gender	46	43	89 Total Animals	54	57

M = Male; F = Female; DOS = Died on study – found dead; TK = Toxicokinetic
* 111 out of a total of 200 study animals survived to scheduled necropsy (33, 21, 29, and 28 animals at 0, 4, 20, and 80 mg/mL, respectively).

Clinical signs associated with mortality

- Clinical signs observed in animals prior to death or euthanasia *in extremis* included the following: Decreased activity, hunched posture, prostration, soft, mucoid, or watery feces, impaired limb function, righting reflex impaired, red/brown/black material around the nose or eyes, eyes discolored (cloudy/dark/pale), swelling of the abdominal/inguinal/thoracic/cervical region or foot/limb/face, swelling along catheter line, swelling at incision sites, pale/black/blue skin discoloration of ear, limbs, or entire body, red discoloration of the hair, red/black/white skin discoloration of the tail, penis extended, yellow/white/red discharge from penis, white/yellow/red/clear discharge from incision site or limbs, red discharge from lip/mouth, clear discharge from eyes, skin cold or warm to the touch, red/brown discolored urine, excessive drinking, rapid breathing, thin, red material in pan/bedding, and unkempt appearance.
    - In general, these findings increased in occurrence with increased dose level.
    - These observations were a progression in a deteriorating health condition that ultimately lead to death or early euthanization.
- Control animals that died during the study showed findings of swelling along the catheter line/incision site, scabbed areas at the axillary site, abrasions of the axillary/cervical region, and red discharge at the cervical region. Although some of these findings were similar to those observed in animals treated with

defibrotide, they were of a lesser incidence and severity and most were associated with the infusion, tether equipment, or were secondary to a repair surgery.

Clinical pathology findings associated with mortality

- Changes in hematology and clinical chemistry were generally dose-dependent.
- Hematology changes included increases in leukocytes, neutrophils, lymphocytes, and monocytes, and decreased red blood cell parameters and platelets.
- Clinical chemistry changes included increases in total bilirubin, alkaline phosphatase (ALP), γ-glutamyltransferase (GGT), AST, ALT, urea nitrogen, creatinine, globulins, amylase, and lipase and decreases in albumin. These changes are compatible with renal failure and hepatic injury.
- Activated partial thromboplastin time (APTT) and prothrombin time (PT) were also increased in these animals.

Microscopic and microscopic findings associated with mortality

- Due to the high mortality rate and the complexity of the macroscopic and microscopic findings, many of which were related to complications (septicemia, urinary tract infections, vegetative valvular endocarditis, and disseminated intravascular coagulation), it is difficult to interpret which findings were defibrotide-related and which were not.
- Many macroscopic and microscopic findings were observed in these animals including findings related to the infusion sites and complications associated with the continuous presence of indwelling catheters.
- Liver toxicities were observed. While some findings may be secondary to systemic infections, the finding of kupffer cell hyperplasia was associated with the presence of basophilic granules was considered a defibrotide-related effect.

Surgery-related mortality

- Surgery was conducted on animals with catheter complications to repair and reposition of the catheter an attempt to re-establish patency and continue the intravenous administration. The table below summarizes the number of animals that underwent repair surgery and the mortality that resulted from complications associated with or following the surgical procedure.

Table 12: Surgery-related mortality in 13-week rat study
(excerpted from study report for 1529-001)

Surgery Related Mortality									
Dose Group	Number of Animals		Dose		Animals Surgically Repaired		Total Repaired by Group	Mortality following Surgery*	
	M	F	Test/Control Article	Dose Level (mg/kg/day)	M	F		M	F
Main Study									
1	15	15	Saline	0	8	8	16	1	1
2	15	15	Defibrotide	240	8	11	19	4	6
3	15	15	Defibrotide	1200	5	6	11	4	0
4	15	15	Defibrotide	4800	0	1	1	0	1
TK									
5	4	4	Saline	0	1	2	3	0	0
6	12	12	Defibrotide	240	9	4	13	1	1
7	12	12	Defibrotide	1200	4	6	10	0	0
8	12	12	Defibrotide	4800	3	3	6	1	1
	100	100		Total by gender	38	41	79 Total Animals	11	10

M = Male; F = Female; TK = Toxicokinetic
* Animals died during or following surgical repair or were determined un-repairable during the procedure and were euthanized soon thereafter.

Clinical Signs

- The clinical signs observed in animals found dead or euthanized early are discussed in the mortality section above.
- The prominent clinical signs and incidences observed in all the main study animals including those found dead or euthanized early are presented by group in the table below.

Table 13: Clinical signs in 13-week rat study

Clinical signs on Days -1 to 65	Number of animals affected/ number of times observed							
	Males				Females			
Dose (mg/kg/day)	0 Control	240	1200	4800	0 Control	240	1200	4800
Number of animals examined	15	15	15	15	15	15	15	15
Activity decreased	1/3	9/30	11/46	7/33	1/1	1/3	3/7	6/31
Prostration	-	-	3/3	1/4	-	1/1	-	-
Feces soft	1/1	-	2/5	3/17	-	-	-	2/2
Red material in pan/bedding	-	-	-	3/11	-	1/1	-	-
Red discharge	3/4	-	1/2	7/14	-	1/2	-	4/5
Limb function impaired	1/2	6/26	6/21	5/26	-	3/3	6/18	3/25
Red material around eyes	-	2/3	3/4	4/20	4/14	5/7	3/6	2/4
Red material around mouth	1/1	-	2/2	3/9	-	1/3	-	1/2
Red material around nose	3/10	7/8	10/27	7/30	3/13	4/9	3/12	5/14
Hunched posture	-	4/11	3/14	6/19	-	-	2/7	3/14
Swelling	10/60	11/67	12/66	12/69	7/53	12/54	10/72	9/87
Thin	-	1/1	2/2	2/8	-	1/2	1/2	2/10
Eye discolored, cloudy	1/4	-	-	1/9	-	-	1/5	1/3
Eye discolored, pale	-	-	-	2/13	-	-	-	-

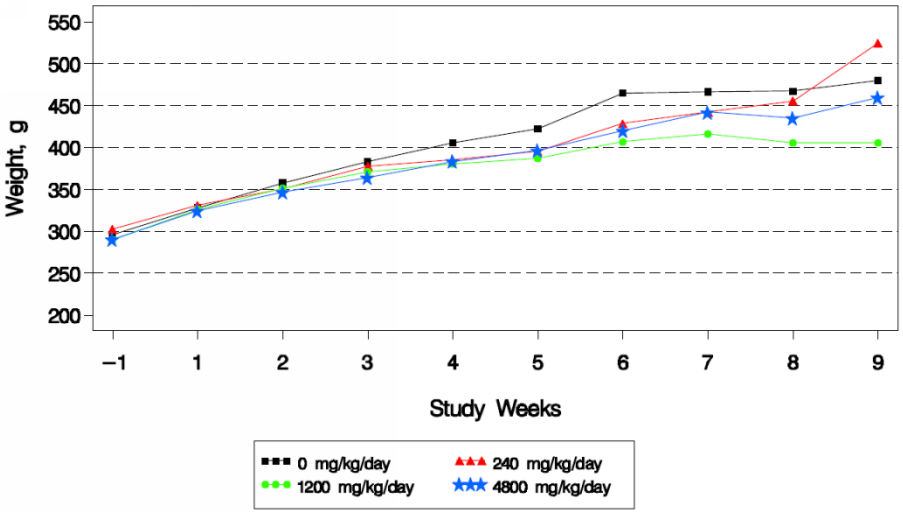
Clinical signs on Days -1 to 65	Number of animals affected/ number of times observed							
	Males				Females			
Dose (mg/kg/day)	0 Control	240	1200	4800	0 Control	240	1200	4800
Abrasions	12/48	11/33	9/40	12/90	9/31	7/22	6/17	5/22
Hair sparse	3/64	3/46	2/8	1/8	3/57	3/63	5/88	6/89
Skin cold to touch	-	-	3/7	2/6	-	1/1	1/1	2/3
Skin discolored, black	-	-	-	3/18	1/1	-	2/6	-
Skin discolored, pale	-	-	3/11	3/19	-	-	-	2/33
Unkempt appearance	1/1	8/28	10/46	6/33	-	-	-	1/9
Breathing rapid	-	1/2	3/11	-	-	-	-	-

- = Clinical sign not observed in this group

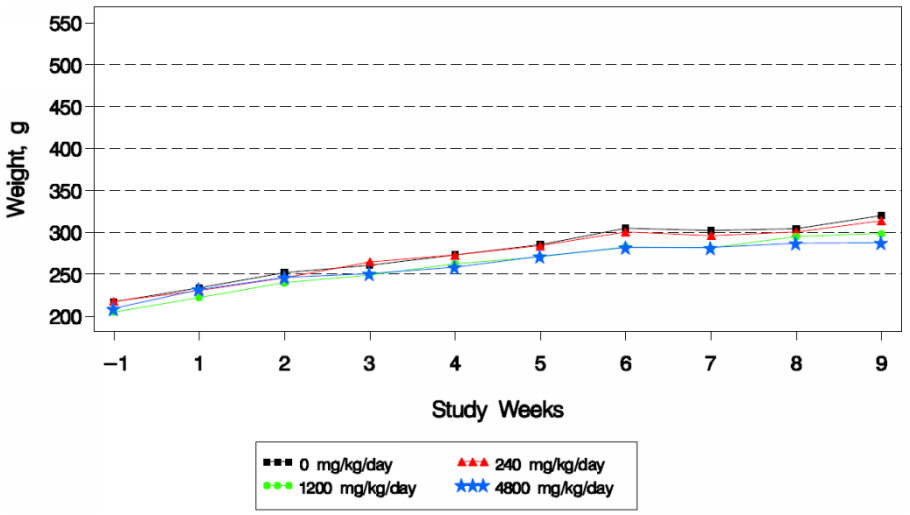
Body Weights

- Mean body weights were significantly lower (12-16%) on Weeks 6 to 9 in males treated with 1200 mg/kg/day compared to controls, which resulted in a 37% decrease in mean body weight gain compared to controls.
- In females treated with 4800 mg/kg/day, mean body weights were significantly lower compared to controls on Week 6 (8%) and Week 9 (10%), which resulted in a 24% decrease in mean body weight gain compared to controls.
- Mean body weights were also lower in females treated with 1200 mg/kg/day with a 9% decrease in body weight gain compared to controls and in males treated with 4800 mg/kg/day with an 8% decrease in body weight gain compared to controls.

Figure 22: Mean body weights in 13-week rat study
(excerpted from study report for 1529-001)
Males



Females

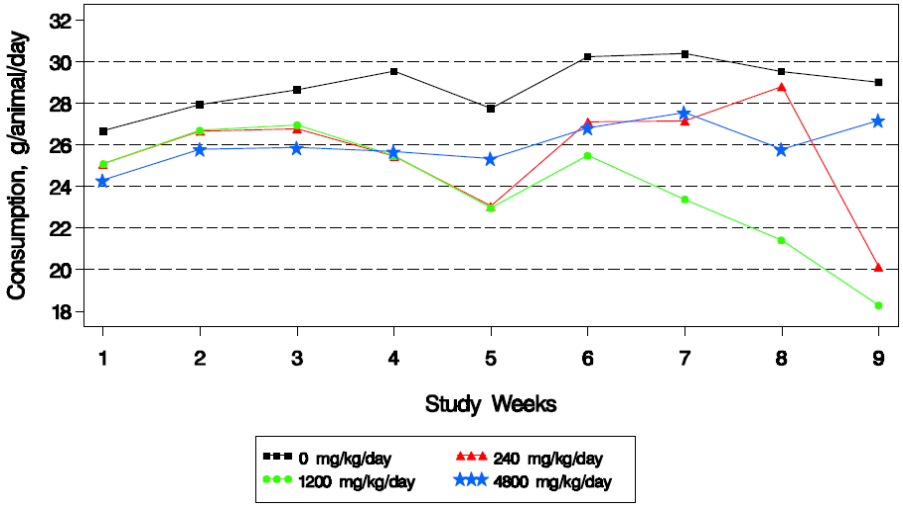


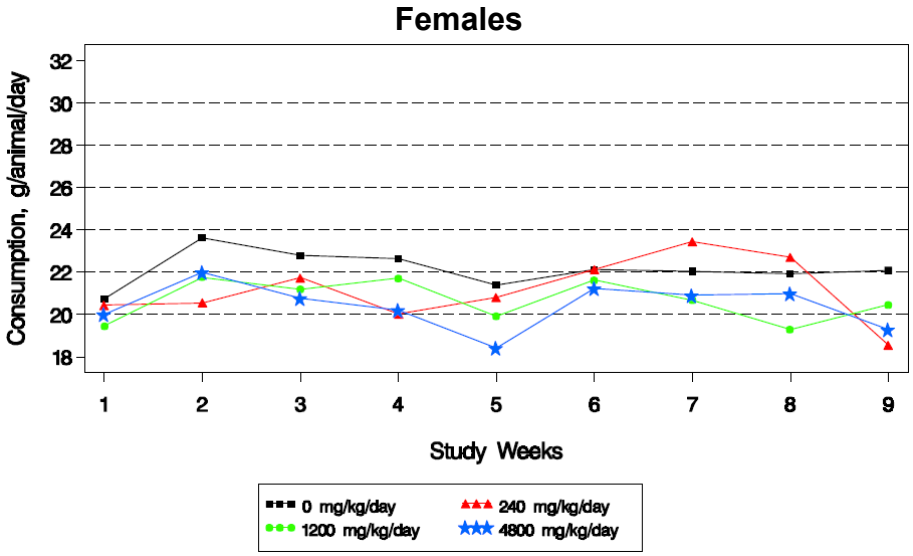
Food Consumption

- Mean food consumption was significantly decreased (14-37%) on Weeks 4 to 9 in males treated with 1200 mg/kg/day compared to controls. This finding corresponds to the decreased body weight also observed in this group.
- Food consumption was also decreased in males treated with 240 and 4800 mg/kg/day compared to controls, with statistical significance on particular weeks. At 240 mg/kg/day, food consumption was significantly decreased by 31% on Week 9.
- In females, mean food consumption was also decreased on various weeks compared to controls. On Week 9, decreases were 16%, 7%, and 13% at 240, 1200, and 4800 mg/kg/day, respectively.

Figure 23: Mean food consumption in 13-week rat study
(excerpted from study report for 1529-001)

Males





Ophthalmoscopy

- Due to the early termination of the study, ophthalmoscopic examinations were only conducted prior to treatment.

Hematology and coagulation

- Changes observed in samples collected prior to necropsy in animals euthanized *in extremis* are described in the mortality section above.
- Changes observed at the scheduled collection times of 5 weeks and prior to the necropsy (terminal) are presented in the table below.
    - An inflammatory response was observed with increases in neutrophils, lymphocytes, and monocytes at all levels.
    - Anemia was also observed variably at 240 and 1200 mg/kg/day and consistently at 4800 mg/kg/day.
    - Activated partial thromboplastin time (APTT) was increased at 1200 and 4800 mg/kg/day and prothrombin time (PT) was also increased at 4800 mg/kg/day.

Table 14: Changes in hematology and coagulation in 13-week rat study
(excerpted from study report for 1529-001)

Summary of Effects on Hematology and Coagulation Parameters at Scheduled Intervals[a]							
		240 mg/kg/day		1200 mg/kg/day		4800 mg/kg/day	
	interval	M	F	M	F	M	F
erythrocytes	W5		↓24%			↓20%	↓19%
	term			↓25%	↓25%	↓17%	↓28%
hemoglobin	W5		↓24%			↓20%	↓18%
	term			↓27%	↓26%	↓17%	↓28%
hematocrit	W5		↓21%	↓16%	↓14%	↓23%	↓21%
	term			↓22%	↓23%	↓14%	↓28%
reticulocytes	W5		↑1.72				
	term			↑2.13	↑2.15		
total leukocytes	W5	↑1.56	↑1.75	↑1.56	↑1.48	↑2.82	↑3.46
	term	↑1.22 NS	↑1.69 NS	↑1.44 NS	↑1.53	↑1.72	↑1.73
neutrophils	W5		↑2.85	↑1.83 NS	↑ 1.50 NS	↑2.95	↑2.99
	term		↑2.65 NS	↑1.70	↑1.97	↑1.26 NS	↑1.61
lymphocytes	W5	↑1.43		↑1.40	↑1.44	↑2.67	↑3.53
	term	↑1.19 NS	↑1.33 NS	↑1.26 NS	↑1.35	↑1.96	↑1.75
monocytes	W5	↑3.18	↑3.95	↑3.16	↑2.99	↑5.25	↑7.79
	term		↑2.29 NS	↑1.80 NS	↑1.97 NS	↑1.80 NS	↑2.23
APTT	W5			↑1.83	↑1.79	↑3.35	↑3.99
	term					↑1.64	↑1.14 NS
PT	W5					↑1.17	↑1.18
	term				↑1.05		
fibrinogen	W5						
	term			↑1.29	↑1.40		↑1.33

[a]: change relative to controls; ↓ = percent decrease; ↑ = fold increase; M = male; F = female
NS: not statistically significant; term = termination; W5 = Week 5
APTT: activated partial thromboplastin time; PT: prothrombin time

Clinical Chemistry

- Changes observed in samples collected prior to necropsy in animals euthanized *in extremis* are described in the mortality section above.
- Changes observed at the scheduled collection times of 5 weeks and prior to the necropsy (terminal) are presented in the table below.

Table 15: Changes in clinical chemistry in 13-week rat study
(excerpted from study report for 1529-001)

Summary of Effects on Clinical Chemistry Analytes at Scheduled Intervals[a]							
		240 mg/kg/day		1200 mg/kg/day		4800 mg/kg/day	
	interval	M	F	M	F	M	F
total protein	W5			↓8%	↓10%	↓17%	↓19%
	term				↓8%	↓13%	↓22%
albumin	W5	↓16%	↓25%	↓15%	↓13%	↓17%	↓23%
	term		↓15%	↓15%	↓21%	↓9%[NS]	↓22%
globulins	W5	↑1.12	↑1.16			↓17%	↓16%
	term	↑1.08[NS]				↓16%	↓21%
A/G	W5	↓24%	↓36%				
	term	↓12%[NS]		↓16%	↓24%		
urea nitrogen	W5	↑1.41		↑1.10[NS]		↑1.19[NS]	↑1.22[NS]
	term	↑1.25[NS]		↑2.45	↑2.27[NS]	↑1.29[NS]	↑1.46[NS]
amylase	term					↑2.08	↑1.46
lipase	W5		↑2.17				↑2.44
potassium	W5					↓12%	↓14%
	term				↓16%		↓23%
chloride	W5			↓3%	↓3%	↓4%	↓6%
calcium	W5					↑1.08	↑1.11
ALT	W5				↑1.32[NS]		↑1.65[NS]
SDH	W5				↑1.46[NS]		↑1.31[NS]
	term						↑1.57[NS]
total bilirubin	W5						↑1.41

[a]: change relative to controls; ↓ = percent decrease; ↑ = fold increase; M = male; F = female [NS]: not statistically significant; term = termination; W5 = Week 5
APTT: activated partial thromboplastin time; PT: prothrombin time
ALT: alanine aminotransferase; SDH: sorbitol dehydrogenase

Urinalysis

For urinalysis only data from the sampling prior to the terminal necropsy were presented.

- Urine specific gravity was significantly increased in both males and females at 4800 mg/kg/day compared to controls.

- Protein levels in the urine were increased in both males and females treated with 4800 mg/kg/day compared to controls. At 4800 mg/kg/day, levels of 300 mg/dL were observed in 2/6 males and 3/10 females and levels of ≥1000 mg/dL were observed in 3/6 males and 6/10 females compared with protein levels of negative, trace, 30, 100, or 300 (males only) mg/dL in controls.

Gross Pathology

- Due to the high mortality rate and the complexity of the macroscopic findings, many of which were related to complications such as septicemia, urinary tract infections, vegetative valvular endocarditis, and disseminated intravascular coagulation, it is difficult to interpret which findings were defibrotide-related and which were not.
- Macroscopic findings associated with the infusion and indwelling catheters were observed in both vehicle and defibrotide-treated groups. Findings included yellow discoloration, swollen/thickened, edema, adhesion, and abscess at the infusion sites, and abscess, adhesions, and fluid accumulation in the abdominal cavity and subcutis.
- Prominent macroscopic findings observed in the defibrotide-treated groups are presented in the table below.

Table 16: Macroscopic findings in 13-week rat study

Treatment-Related Macroscopic Findings			No. of animals affected							
			Males				**Females**			
Dose (mg/kg/day)			0	240	1200	4800	0	240	1200	4800
Number of animals examined			2*/13	13*/2	10*/5	9*/6	2*/13	14*/1	5*/10	5*/10
Organ	Finding									
Adrenal glands	Enlarged	Total	-	4*/1	5*/4	1*/2	1*/0	2*/1	2*/5	5*/9
		Minimal	-	1*/1	-	-	-	-	-	-
		Mild	-	3*/0	2*/3	0/1	1*/0	1*/0	1*/3	4*/8
		Moderate	-	-	2*/1	1*/1	-	1*/1	1*/2	1*/1
		Severe	-	-	1*/0	-	-	-	-	-
Kidney	Enlarged	Total	0/1	-	1*/0	1*/2	-	-	-	-
		Mild	0/1	-	1*/0	1*/1	-	-	-	-
		Severe	-	-	-	0/1	-	-	-	-
	Tan focus/foci	Total	-	1*/0	2*/0	3*/0	-	-	1*/1	-
		Mild	-	-	1*/0	1*/0	-	-	0/1	-
		Moderate	-	-	1*/0	2*/0	-	-	1*/0	-
		Severe	-	1*/0	-	-	-	-	-	-
	White focus/foci	Total	-	-	1*/2	-	-	-	0/1	-
		Mild	-	-	-	-	-	-	0/1	-
		Moderate	-	-	1*/1	-	-	-	-	-
		Severe	-	-	0/1	-	-	-	-	-
	Irregular surface	Total	-	-	2*/2	-	-	-	-	-
		Mild	-	-	1*/0	-	-	-	-	-
		Moderate	-	-	1*/1	-	-	-	-	-
		Severe	-	-	0/1	-	-	-	-	-
Liver	Enlarged	Total	-	3*/1	2*/0	4*/1	-	1*/0	1*/1	-
		Minimal	-	0/1	-	-	-	-	-	-
		Mild	-	2*/0	2*/0	1*/0	-	1*/0	1*/0	-
		Moderate	-	1*/0	-	2*/1	-	-	0/1	-
		Severe	-	-	-	1*/0	-	-	-	-
Lymph node, mandibular	Enlarged	Total	1*/1	1*/1	1*/0	0/3	-	-	0/2	-

Treatment-Related Macroscopic Findings			No. of animals affected							
			Males				Females			
Dose (mg/kg/day)			0	240	1200	4800	0	240	1200	4800
Number of animals examined			2*/13	13*/2	10*/5	9*/6	2*/13	14*/1	5*/10	5*/10
Organ	Finding									
		Minimal	-	-	1*/0	-	-	-	-	-
		Mild	0/1	1*/0	-	-	-	-	0/1	-
		Moderate	1*/0	0/1	-	0/2	-	-	0/1	-
		Severe	-	-	-	0/1	-	-	-	-
Spleen	Enlarged	Total	0/1	9*/2	9*/4	6*/4	-	6*/0	3*/5	3*/5
		Minimal	-	0/1	-	-	-	-	-	-
		Mild	-	5*/1	4*/1	3*/0	-	2*/0	2*/0	2*/1
		Moderate	0/1	4*/0	2*/3	2*/2	-	4*/0	1*/3	0/2
		Severe	-	-	3*/0	1*/2	-	-	0/2	1*/2

Number of animals examined and affected: Early deaths*/Terminal necropsy
- = no test-article related changes

Organ Weights

Table 17: Organ weights in 13-week rat study

Group and Dose		Mean		Percentage deviation from Control					
		Control 0 mg/kg/day		240 mg/kg/day		1200 mg/kg/day		4800 mg/kg/day	
Sex		Males	Females	Males	Females	Males	Females	Males	Females
Number of animals examined		13	13	2	1	5	10	6	10
Adrenal gland	Absolute (g)	0.082	0.083	↑54	↑83	↑112*	↑86**	↑73	↑146**
	Relative (BW, %)	0.0191	0.0304	↑75	↑86	↑168	↑106**	↑91	↑177**
Kidney	Absolute (g)	3.709	2.216	↑44	↑40	↑55*	↑35*	↑63	↑72**
	Relative (BW, %)	0.8587	0.8055	↑36	↑44	↑91**	↑48*	↑78	↑96**
Liver	Absolute (g)	13.932	9.438	↑43	↑38	↑73*	↑51**	↑59*	↑57**
	Relative (BW, %)	3.2206	3.4203	↑33	↑41	↑116*	↑69*	↑73*	↑78**
Spleen	Absolute (g)	1.302	0.795	↑52	↑78	↑203*	↑178*	↑117	↑139**
	Relative (BW, %)	0.3050	0.2892	↑41	↑82	↑269*	↑210*	↑132	↑171**

BW= Body weight
↑= increase - = no test-article related changes
* P≤0.05 ** P≤0.01

Histopathology

Microscopic examination was conducted on all specified tissues from all animals found dead or euthanized early and all animals in the control and 4800 mg/kg/day groups from the terminal necropsy. The spleen, liver, kidney, mesenteric and mandibular lymph nodes, and infusion sites were determined to be the target organs and were examined from all animals in the 240 and 1200 mg/kg/day from the terminal necropsy.

Adequate Battery: Yes

Peer Review: No

Histological Findings

- Due to the high mortality rate and the complexity of the microscopic findings, many of which were related to complications such as septicemia, urinary tract infections, vegetative valvular endocarditis, and disseminated intravascular coagulation, it is difficult to interpret which findings were defibrotide-related and which were not.
- There were many findings in the infusion sites (vena cava or jugular vein). The most common finding was minimal to severe chronic-active inflammation which

was frequently observed with thrombus formation, abscesses, bacterial colonies, mineralization, and hemorrhage. The severity of the findings was generally greater in the early death animals, particularly those with evidence of secondary septicemia or disseminated intravascular coagulation.

- The infusion site-related inflammation and bacterial infection resulted in the complications of septicemia, urinary tract infections, vegetative valvular endocarditis, and disseminated intravascular coagulation and there were many microscopic findings related to these conditions. These findings were observed more frequently or only in defibrotide-treated animals; however, there was often not a clear dose response for the findings.
    - In the kidneys, findings of bacterial colonies, pyelitis and pyelonephritis, infarction, thrombosis, and abscess formation were likely related to septicemia and disseminated intravascular coagulation.
    - In the liver, findings related to systemic inflammation, septicemia, or disseminated intravascular coagulation included sinusoidal leukocytosis, bacterial colonies, infarction, abscess formation, and thrombosis.
    - Findings of lymphoid depletion (generalized, cortical, and marginal zone) or necrosis were observed in multiple lymphoid tissues (thymus, spleen, Peyer's patches, and lymph nodes) and were attributed to stress secondary to infusion site-related inflammation and secondary complications.
    - Several findings related to septicemia or vegetative valvular endocarditis including bacterial colonies, abscesses, acute, subacute, chronic, or chronic-active inflammation, vascular leukocytosis, focal necrosis, and infarcts were observed in a variety of tissues including the adrenal glands, brain, eyes, lungs, and heart.
    - Thrombus and hemorrhage related to disseminated intravascular coagulation were also observed in a variety of tissues including the adrenal glands, brain, heart, and lungs in addition the liver and kidney listed above.
- Defibrotide-related findings that were dose-dependent were identified by the Applicant in the kidneys, liver, mandibular and mesenteric lymph nodes, and spleen and are presented in the table below. The finding of hypertrophy/hyperplasia in the adrenal glands is also listed below.

Table 18: Microscopic findings in 13-week rat study

Treatment-Related Microscopic Findings			No. of animals affected							
			Males				Females			
Dose (mg/kg/day)			0	240	1200	4800	0	240	1200	4800
Number of animals examined			2*/13	13*/2	10*/5	9*/6	2*/13	14*/1	5*/10	5*/10
Organ	Finding									
Adrenal glands	Hypertrophy/ hyperplasia, diffuse cortical	Total	-	12*/NE	10*/NE	8*/3	1*/0	8*/NE	5*/NE	5*/6
		Minimal	-	5*/NE	-	1*/1	-	-	-	0/3
		Mild	-	6*/NE	3*/NE	2*/2	1*/0	6*/NE	2*/NE	1*/3
		Moderate	-	1*/NE	4*/NE	4*/0	-	2*/NE	3*/NE	4*/0
		Severe	-	-	3*/NE	1*/0	-	1*/NE	-	-
Kidneys	Basophilic granules, tubular cell	Total	-	-	4*/3	9*/6	-	-	3*/5	5*/10
		Minimal	-	-	-	2*/1	-	-	1*/2	-
		Mild	-	-	4*/1	1*/4	-	-	2*/3	3*/4
		Moderate	-	-	0/2	6*/1	-	-	-	2*/6
	Tubular degeneration/ regeneration	Total	-	1*/1	1*/5	3*/5	-	1*/0	0/7	1*/9
		Minimal	-	-	-	0/1	-	-	0/1	-
		Mild	-	0/1	1*/0	2*/3	-	-	0/3	0/6
		Moderate	-	1*/0	0/5	1*/1	-	-	0/2	1*/3
		Severe	-	-	-	-	-	1*/0	0/1	-
	Glomerulonephropathy	Total	-	1*/0	0/5	7*/4	-	-	0/3	2*/9
		Minimal	-	-	0/1	2*/0	-	-	0/3	0/1
		Mild	-	1*/0	0/3	3*/1	-	-	-	1*/2
		Moderate	-	-	0/1	2*/3	-	-	-	1*/6
	Lymphohistiocytic infiltration	Total	-	3*/0	7*/5	6*/5	-	-	0/5	4*/10
		Minimal	-	-	-	3*/0	-	-	-	-
		Mild	-	2*/0	2*/1	1*/1	-	-	0/2	3*/0
		Moderate	-	1*/0	5*/4	2*/4	-	-	0/3	0/9
		Severe	-	-	-	-	-	-	-	1*/1
Liver	Basophilic granules, kupffer cell	Total	-	-	0/2	1*/6	-	-	0/6	1*/10
		Minimal	-	-	0/2	-	-	-	0/5	0/1
		Mild	-	-	-	1*/4	-	-	0/1	1*/7
		Moderate	-	-	-	0/2	-	-	-	0/2
	Extramedullary hematopoiesis	Total	1*/0	6*/0	7*/2	5*/1	0/1	4*/0	3*/6	4*/1
		Minimal	1*/0	3*/0	7*/2	2*/1	0/1	3*/0	2*/5	3*/1
		Mild	-	3*/0	-	3*/0	-	1*/0	1*/1	1*/0
	Hypertrophy/ hyperplasia, kupffer cell	Total	2*/4	13*/2	8*/5	9*/6	0/3	10*/0	4*/9	5*/10
		Minimal	1*/3	0/2	1*/0	-	0/2	2*/0	0/1	1*/0
		Mild	0/1	8*/0	3*/2	5*/4	0/1	5*/0	2*/4	2*/5
		Moderate	1*/0	5*/0	1*/3	4*/2	-	3*/0	2*/4	2*/5
		Severe	-	-	3*/0	-	-	-	-	-
Lymph node, mandibular	Vacuolated macrophages	Total	-	-	0/2	5*/5	-	1*/0	2*/2	3*/8
		Minimal	-	-	0/2	4*/4	-	1*/0	1*/2	0/4
		Mild	-	-	-	1*/1	-	-	1*/0	3*/4
Lymph node, mesenteric	Vacuolated macrophages	Total	-	-	8*/5	9*/6	-	4*/0	5*/10	5*/10
		Minimal	-	-	1*/0	-	-	-	-	-
		Mild	-	-	7*/1	3*/2	-	3*/0	3*/8	3*/1
		Moderate	-	-	0/4	5*/4	-	1*/0	2*/2	2*/9
		Severe	-	-	-	1*/0	-	-	-	-
Spleen	Vacuolated macrophages	Total	-	-	0/3	8*/6	-	-	0/7	3*/10
		Minimal	-	-	-	3*/1	-	-	0/4	2*/3
		Mild	-	-	0/3	5*/5	-	-	0/3	1*/7

Number of animals examined and affected: Early deaths*/Terminal necropsy
- = no test-article related changes
NE= Not examined, tissue was not examined in this group at terminal necropsy

Special Evaluation

Functional Observational Battery

- Rearing and general arousal were decreased prior to necropsy in females treated with 1200 mg/kg/day defibrotide compared to controls.
- Hindlimb grip strength was decreased on Day 2 in males treated with 1200 mg/kg/day and both males and females treated with 4800 mg/kg/day compared to controls and hindlimb splay was decreased on Day 2 in both males and females treated with 4800 mg/kg/day compared to controls.

Table 19: Findings in functional observation battery in 13-week rat study

Index	Mean		Mean (Percentage deviation from Control)					
	Control 0 mg/kg/day		240 mg/kg/day		1200 mg/kg/day		4800 mg/kg/day	
	Males	Females	Males	Females	Males	Females	Males	Females
Activity/Arousal measurements								
Rearing								
Pretest	4.6	7.6	-	-	-	7.5	-	-
Day 2	3.9	8.3	-	-	-	-	-	-
Prior to necropsy	8.2	18.1	-	-	-	5.0* (↓72)	-	-
General arousal								
Pretest	2.7	3.0	-	-	-	3.0	-	-
Day 2	2.0	2.7	-	-	-	-	-	-
Prior to necropsy	2.4	3.2	-	-	-	2.3* (↓28)	-	-
Neuromuscular measurements								
Hindlimb grip strength (kg)								
Pretest	0.2398	0.2562	-	-	0.2316	-	0.3497	0.2409
Day 2	0.2112	0.1549	-	-	0.1105** (↓48)	-	0.1461** (↓31)	0.0929** (↓40)
Prior to necropsy	0.1955	0.1799	-	-	-	-	0.1791 (↓8)	0.1491 (↓17)
Hindlimb splay (mm)								
Pretest	108.77	98.53	-	-	-	-	123.57	105.10
Day 2	126.23	115.27	-	-	-	-	110.37* (↓13)	99.47 (↓14)
Prior to necropsy	145.77	116.85	-	-	-	-	-	-

↓= decrease - = no test-article related changes
*= P≤0.05, Significantly different from control
**= P≤0.01, Significantly different from control

Toxicokinetics

In rats, the toxicokinetics of defibrotide (240, 1200, and 4800 mg/kg/day) were evaluated on Day 1 with samples collected at predose and 0.5, 1, 2, 3, 12, and 24 hours after dosing and on Day 65 with samples collected immediately prior to the end of infusion.

- No measurable concentrations of defibrotide were detected in controls on Day 1, but a low concentration of defibrotide was detected in 1 of 6 plasma samples from control rats on Day 65.
- Due to the high lower limit of quantification (LLOQ; 50 µg/mL) used in this study, 12 male samples and 5 female samples in the 240 mg/kg/day group had defibrotide levels lower than the LLOQ.

- Following the start of the infusion, defibrotide was detected in the samples from the 1200 and 4800 mg/kg/day groups except for 3 male samples at 4800 mg/kg/day and one female sample at 1200 mg/kg/day that were below the LLOQ.
- Overall, exposure to defibrotide increased with an increase in dose from 240 to 4800 mg/kg/day on Day 1.
- Exposures (AUC_{0-24} and C_{max}) were slightly higher in females than males on Day 1.
- On Day 65, plasma concentrations prior to the end of the infusion were similar at 240 and 1200 mg/kg/day, but were higher at 4800 mg/kg/day.

Table 20: Toxicokinetics of defibrotide in rats administered 24-hour intravenous infusion

(excerpted from study report for 1529-001)

Dose (mg/kg/day)	Sex	AUC_{0-24} (hr•µg/mL)	AUC_{0-24}/Dose ((hr•µg/mL)/mg/kg)	C_{max} (µg/mL)	C_{max}/Dose ((µg/mL)/mg/kg)	T_{max} (hr)
240	Male	807	3.36	51.8	0.216	24.0
	Female	1110	4.62	71.4	0.298	24.0
	Mean	957	3.99	61.6	0.257	24.0
1200	Male	4430	3.69	267	0.223	24.0
	Female	5100	4.25	394	0.328	24.0
	Mean	4770	3.97	330	0.275	24.0
4800	Male	23300	4.85	1670	0.347	24.0
	Female	30100	6.28	2310	0.481	24.0
	Mean	26700	5.56	1990	0.414	24.0

Table 21: Mean plasma concentrations of defibrotide in rats prior to the end of the continuous intravenous infusion on Day 65

(excerpted from study report for 1529-001)

Sex	Dose (mg/kg/day)	Concentration (µg/mL)
Male	0	0±0
Female		21.7±37.5
Combined		10.8±26.5
Male	240	69.4±60.7
Female		47.3±82.0
Combined		58.4±65.6
Male	1200	97.2±99.6
Female		57.4±49.9
Combined		77.3±73.7
Male	4800	1790±342
Female		1920±139
Combined		1850±245

Dosing Solution Analysis

The results for all active formulation samples were expressed as mean % recovery of the nominal concentration and ranged from 92.0 to 99.9%. Defibrotide was detected in control samples at average concentrations between 0.0323 and 0.1090 mg/mL.

Study title: A 13-week intravenous toxicity study of defibrotide in dogs

Study no.:	1529-002
Study report location:	eCTD 4.2.3.2.
Conducting laboratory and location:	(b) (4)
Date of study initiation:	January 28, 2008
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Defibrotide, lot # 2070020006

Key Study Findings

- Coagulation changes included increases in activated partial thromboplastin time.
- The liver was the main organ of toxicity as indicated by increased liver weights and microscopic findings of basophilic granules and hypertrophy in Kupffer cells, acute inflammation, and necrosis of individual hepatocytes.
- Toxicities associated with trauma and irritation of infusions sites were observed in both control and defibrotide-treated groups.

Methods

Doses:	0, 60, 300 or 1600 mg/kg/day (0, 15, 75, or 400 mg/kg/dose)
Frequency of dosing:	Infusion four times daily for 13 consecutive weeks
Route of administration:	Intravenous infusion (2-hours) via venous access port at an infusion rate of 2.5 mL/kg/hour followed by 4-hour treatment free period
Dose volume:	20 mL/kg/day (5 mL/kg/dose)
Formulation/Vehicle:	0.9% sodium chloride for injection
Species/Strain:	Beagle dogs
Number/Sex/Group:	4/sex/group
Age:	5-5.5 months at receipt
Weight:	Males: 8.18-9.71 kg Females: 6.31-7.79 kg
Satellite groups:	None

Observations and times:

Mortality:	Twice daily
Clinical signs:	Cage side observations: Twice daily Detailed examinations: Daily beginning Day -1
Body weights:	Weekly beginning on Day -8
Food consumption:	Daily beginning on Day -8
ECG:	Prior to treatment, during Week 6, and prior to the necropsy
Ophthalmoscopy:	Once prior to treatment, during Week 6, and prior to the necropsy
Hematology:	Week -1, during Week 5, and prior to the necropsy
Clinical chemistry:	Week -1, during Week 5, and prior to the necropsy
Coagulation:	Week -1, during Week 5, and prior to the necropsy
Urinalysis:	Week -1, during Week 5, and prior to the necropsy
Gross pathology:	At necropsy
Organ weights:	At necropsy
Histopathology:	At necropsy
Immunogenicity:	Day 93
Toxicokinetics:	On Days 1 and 91 at 10, 20, and 30 minutes, and 2 and 4 hours following the end of the fourth infusion

Results

Mortality

One female treated with the low dose of 60 mg/kg/day (Animal # 2501) was euthanized *in extremis* on Day 44 based on signs of systemic infection.

- Clinical signs observed in this animal included sporadic episodes of decreased activity and/or impaired/lost limb function between Days 19 and 44. On Day 44, the animal was lethargic, its right tibiotarsal joint was moderately swollen, and its right limb presented a non-weight bearing lameness. Clinical pathology from blood samples collected prior to euthanasia showed increases in total leukocytes, neutrophils, and monocytes, prolonged APTT, increased globulins, and decreased albumin levels, suggesting an inflammatory response.
- A fluid sample from the right tibiotarsal joint was negative for bacteria. Blood culture samples collected directly from the infusion catheter and the jugular vein showed the presence of Enterobacter cloacae, Pseudomonas aeruginosa, and possible Escherichia coli at the infusion catheter and presence of Pseudomonas aeruginosa at the jugular vein. The presence of bacteria in the blood confirms bacteremia; however, there were no microscopic or supportive clinical pathology to suggest bacterial septicemia.
- Microscopic findings in the tibiotarsal and tibiofemoral joints included hemorrhage, subacute/chronic inflammation, and synovial hyperplasia/hypertrophy.
- Based on all the information available, the cause of mortality was determined to be secondary to incidental joint inflammation and unrelated to the administration of defibrotide.

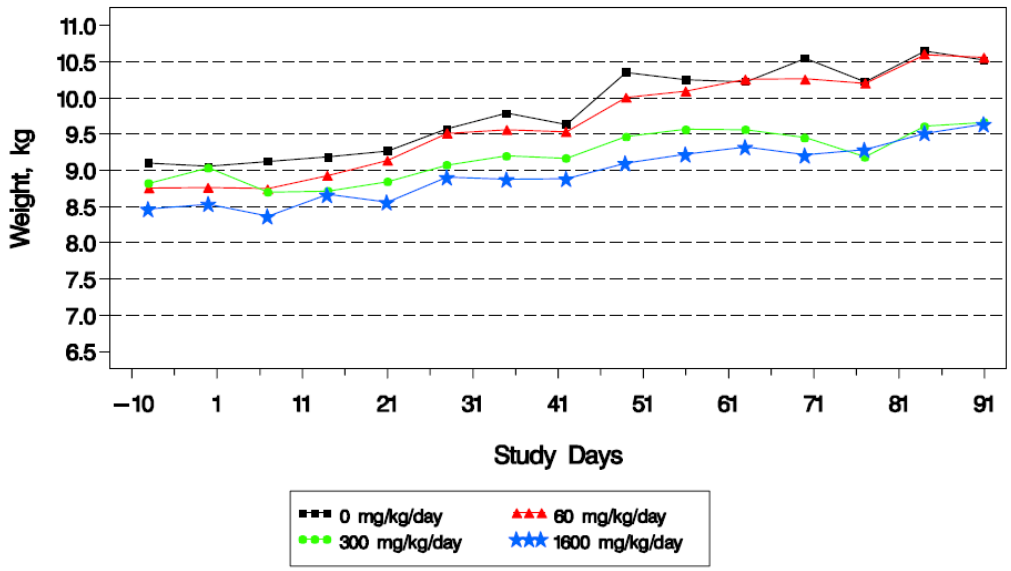
Clinical Signs

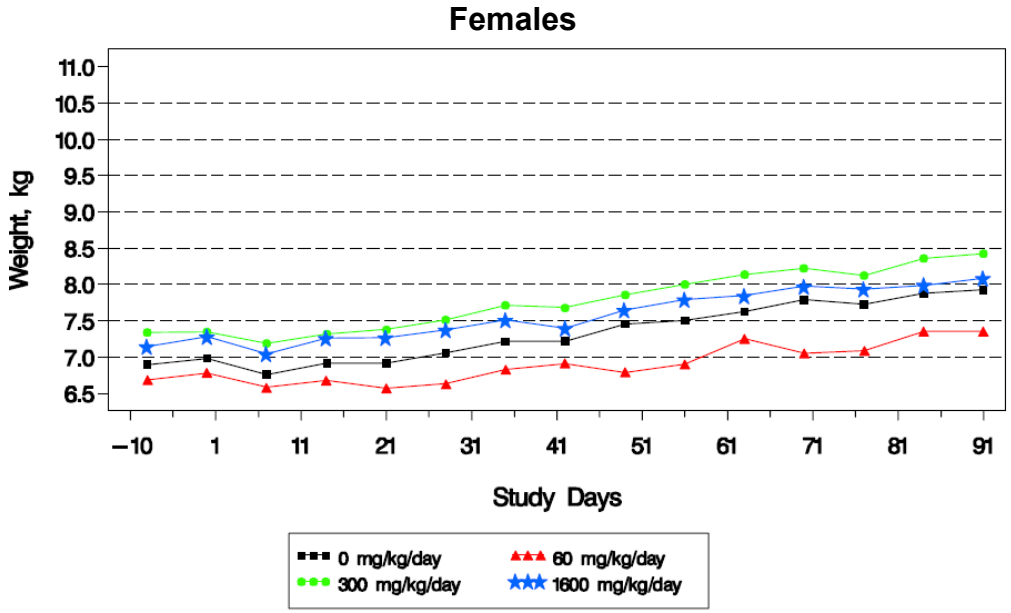
- There were no clinical signs that could clearly be attributed to the administration of defibrotide.
- Thin body condition was observed sporadically in individual animals and this change often correlated with body weight loss. While the number of animals affected appears greater at 1600 mg/kg/day compared to the other treatment groups, the duration and severity of these changes did not exhibit a dose-dependent relationship.
- Other sporadic signs observed included increased incidence of swelling observed in the females treated with 1600 mg/kg/day. Increased incidence of skin discolored in females treated with 300 mg/kg/day was observed during particular periods during the study (Days 24-35 and Days 56-66); however, the control group showed skin discoloration at in all animals at other time points.

Body Weights

- Body weights were lower in males treated with 1600 mg/kg/day with statistical significance on Days 49 and 70.

Figure 24: Mean body weights in 13-week dog study
(excerpted from study report for 1529-002)





Food Consumption

- There were no clear defibrotide-related changes in food consumption. Occasionally, mean food consumption for defibrotide-treated groups were statistically higher or lower than controls, but there was no clear trend or dose-dependent relationship.

Ophthalmoscopy

Unremarkable

ECG

Unremarkable

Hematology and coagulation

Hematology

- There were no clear defibrotide-related changes in hematology parameters.
- Erythrocytes (RBC), hemoglobin, and hematocrit were decreased in females treated with 60 mg/kg/day and in males treated with 300 mg/kg/day at Week 5 and prior to necropsy compared to controls. Platelets were also decreased in males treated with 300 mg/kg/day at Week 5 compared to controls. These changes were not observed at the high dose of 1600 mg/kg/day.

Coagulation

- Activated partial thromboplastin time (APTT) was increased at 300 and 1600 mg/kg/day at Week 5 compared to controls; APTT levels were comparable to controls prior to necropsy.

Table 22: Hematology and coagulation changes in the 13-week dog study

Index	Mean		Mean (Percentage deviation from Control)					
	Control 0 mg/kg/day		60 mg/kg/day		300 mg/kg/day		1600 mg/kg/day	
	Males	Females	Males	Females	Males	Females	Males	Females
RBC (10^6/µL)								
Pretest	6.220	6.563	-	6.683	6.118	-	-	-
Week 5	6.730	6.850	-	5.790 (↓15)	5.873* (↓13)	-	-	-
Prior to necropsy	6.745	6.768	-	5.030* (↓26)	5.325 (↓21)	-	-	-
Hemoglobin (g/dL)								
Pretest	13.55	14.53	-	14.80	13.40	-	-	-
Week 5	15.08	15.73	-	13.08 (↓17)	13.05* (↓13)	-	-	-
Prior to necropsy	15.68	15.85	-	12.43 (↓22)	12.18* (↓22)	-	-	-
Hematocrit (%)								
Pretest	40.95	44.08	-	44.33	40.90	-	-	-
Week 5	41.60	42.95	-	37.75 (↓12)	37.88 (↓9)	-	-	-
Prior to necropsy	41.15	40.80	-	31.53* (↓23)	32.38 (↓21)	-	-	-
Platelets (10^3/µL)								
Pretest	260.0	174.5	-	-	261.5	-	-	-
Week 5	221.0	180.0	-	-	143.3 (↓35)	-	-	-
Prior to necropsy	156.3	134.5	-	-	-	-	-	-
APTT (sec)								
Pretest	10.98	11.60	-	-	11.30	11.88	11.73	12.10
Week 5	10.78	11.35	-	-	13.43** (↑25)	14.63 (↑29)	26.65** (↑147)	27.63** (↑143)
Prior to necropsy	9.75	10.10	-	-	-	-	-	-

↓= decrease ↑=increase - = no test-article related changes
*= P≤0.05, Significantly different from control
**= P≤0.01, Significantly different from control

Clinical Chemistry

- While there were no clear defibrotide-related changes in clinical chemistry parameters, calcium and phosphorus (females only) were increased at 1600 mg/kg/day at Week 5 compared to controls. These changes were not observed prior to necropsy.
- Albumin was decreased in females at 60 and 1600 mg/kg/day and in males at 300 mg/kg/day at Week 5 and prior to necropsy compared to controls.

Table 23: Clinical chemistry changes in the 13-week dog study

Index	Mean		Mean (Percentage deviation from Control)					
	Control 0 mg/kg/day		60 mg/kg/day		300 mg/kg/day		1600 mg/kg/day	
	Males	Females	Males	Females	Males	Females	Males	Females
Calcium (mg/dL)								
Pretest	10.88	10.70	-	-	-	-	10.90	10.70
Week 5	11.20	11.00	-	-	-	-	12.45** (↑11)	11.98** (↑9)
Prior to necropsy	10.65	10.53	-	-	-	-	-	-
Phosphorus (mg/dL)								
Pretest	7.30	6.95	-	-	-	-	-	7.35
Week 5	7.20	6.48	-	-	-	-	-	7.83** (↑21)
Prior to necropsy	5.80	5.13	-	-	-	-	-	-
Albumin (g/dL)								
Pretest	2.95	3.08	-	2.95	2.93	-	-	3.08
Week 5	3.23	3.30	-	2.40** (↓27)	2.55* (↓21)	-	-	2.93 (↓11)
Prior to necropsy	3.20	3.33	-	2.27 (↓32)	2.38 (↓26)	-	-	2.93* (↓12)

↓= decrease ↑=increase - = no test-article related changes
*= P≤0.05, Significantly different from control
**= P≤0.01, Significantly different from control

Urinalysis

There were no clear defibrotide-related changes in urinalysis parameters. One male treated with 300 mg/kg/day (Animal # 3004) showed mild glucosuria, ketonuria, bilirubinuria, and proteinuria at Week 5, but these findings were resolved in this male prior to necropsy and were not observed in males or females at 1600 mg/kg/day.

Gross Pathology

Unremarkable

Organ Weights

Table 24: Organ weight changes in 13-week dog study

Group and Dose		Mean		Percentage deviation from Control					
		Control 0 mg/kg/day		60 mg/kg/day		300 mg/kg/day		1600 mg/kg/day	
Sex		Males	Females	Males	Females	Males	Females	Males	Females
Number of animals examined		4	4	4	3	4	4	4	4
Liver	Absolute (g)	270.703	194.875	-	↑26*	↑28*	↑17	↑13	↑29**
	Relative (BW, %)	2.6638	2.5547	-	↑37**	↑44*	↑10	↑25	↑28*

BW= Body weight
↑= increase - = no test-article related changes
* P≤0.05 ** P≤0.01

Histopathology

Adequate Battery: Yes

Peer Review: No

Histological Findings

- The main defibrotide-related microscopic findings were toxicities in the liver.
- Treatment (intravenous infusion)-related effects associated with trauma and irritation of infusions sites were observed in control and defibrotide-treated

groups. Findings included perivascular hemorrhage, endothelial cell hypertrophy or hyperplasia, neovascularization, adventitial mononuclear infiltration, and thrombus formation at distal infusion sites.

- While thrombus formation in the distal infusion sites occurred only in defibrotide-treated males and females, thrombus was observed in the lung of one control male and three control females. The pulmonary thrombi may be reflective of thrombus embolization from the infusion sites. The Applicant interpreted the thrombus formation to be resultant of the infusion process and not directly related to defibrotide treatment based on a lack of dose dependency.
- Other findings considered secondary to thrombus formation at infusion sites and not directly related to defibrotide treatment were cardiac atrial infarction and kidney lesions (renal tubular degeneration with or without acute inflammation and glomerulopathy). Two males treated with 300 mg/kg/day had cardiac infarctions with subacute inflammation in the heart.

Table 25: Microscopic findings in 13-week dog study

Treatment-Related Microscopic Findings			No. of animals affected							
			Males				**Females**			
Dose (mg/kg/day)			0	60	300	1600	0	60	300	1600
Number of animals examined			0/4	0/4	0/4	0/4	0/4	1*/3	0/4	0/4
Organ	Finding									
Liver	Basophilic granules, kupffer cell	Total	-	-	0/1	0/4	-	0/1	0/3	0/4
		Minimal	-	-	0/1	-	-	0/1	0/3	0/2
		Mild	-	-	-	0/3	-	-	-	0/1
		Moderate	-	-	-	0/1	-	-	-	0/1
	Hypertrophy, kupffer cell	Total	-	-	0/2	0/4	-	1*/2	0/3	0/4
		Minimal	-	-	0/1	-	-	0/2	0/3	0/2
		Mild	-	-	0/1	0/4	-	1*/0	-	0/1
		Moderate	-	-	-	-	-	-	-	0/1
	Subacute inflammation	Minimal	-	-	0/1	0/2	0/1	1*/0	-	-
	Necrosis of individual hepatocytes	Minimal	-	-	-	0/2	-	-	-	-
Lymph node, mandibular	Histiocytosis, sinus	Minimal	-	-	0/1	0/1	-	1*/0	-	0/3
Lymph node, mesenteric	Erythrocytosis/ erythrophagocytosis, sinus	Total	-	-	0/3	0/2	0/1	-	-	0/1
		Minimal	-	-	0/2	0/2	0/1	-	-	0/1
		Mild	-	-	0/1	-	-	-	-	-
Salivary gland, parotid	Lymphocytic infiltration	Total	-	0/2	0/2	0/3	-	0/1	0/2	0/2
		Minimal	-	0/2	0/2	0/2	-	0/1	0/2	0/2
		Mild	-	-	-	0/1	-	-	-	-

Number of animals examined and affected: Early deaths*/Terminal necropsy
- = no test-article related changes

Toxicokinetics

In dogs, the toxicokinetics of defibrotide (60, 300, and 1600 mg/kg/day) were evaluated on Days 1 and 91 with samples collected at 10, 20, and 30 minutes, and 2 and 4 hours following the end of the fourth infusion.

- The plasma samples from the control group did not contain detectable concentrations of defibrotide.
- Plasma concentrations of defibrotide at 60 mg/kg/day were below the limit of quantitation (50.0 µg/mL) on both Days 1 and 91.

- C_{max} increased with increased dose from 300 to 1600 mg/kg/day.
- Exposures to defibrotide were similar in males and females at 1600 mg/kg/day. At 300 mg/kg/day, C_{max} values were similar between males and females; however, due to low exposure in males at 300 mg/kg/day AUC values were not calculated on Day 91 in males, but females treated with 300 mg/kg/day showed an extremely high $AUC_{(0-\infty)}$ value (much higher than observed at 1600 mg/kg/day) on Day 91. AUC for these females was not as high when calculated other ways (see table below).
- The half-life ($t_{1/2}$) is less than one hour, indicating that the drug is rapidly eliminated from the body.

Table 26: Toxicokinetics for defibrotide following the fourth of four daily infusions on Days 1 and 91 in 13-week dog study

(excerpted from study report for 1529-002)

Day	Dose (mg/kg/day)	Sex	$AUC_{0-\infty}$ (hr•µg/mL)	$AUC_{0-\infty}$/Dose ((hr•µg/mL)/mg/kg)	AUC_{0-6} (hr•µg/mL)	AUC_{0-6}/Dose ((hr•µg/mL)/mg/kg)	$AUC_{0-tlast}$ (hr•µg/mL)	$AUC_{0-tlast}$/Dose ((hr•µg/mL)/mg/kg)
1	60	Male	0±0	0±0	0±0	0±0	0±0	0±0
		Female	0±0	0±0	0±0	0±0	0±0	0±0
		Combined	0±0	0±0	0±0	0±0	0±0	0±0
	300	Male	0±0	0±0	0±0	0±0	0±0	0±0
		Female	NC	NC	NC	NC	NC	NC
		Combined	0±0	0±0	0±0	0±0	0±0	0±0
	1600	Male	723±107	1.81±0.269	626±297	1.57±0.741	562±288	1.40±0.720
		Female	633±156	1.58±0.389	670±165	1.68±0.411	609±151	1.52±0.376
		Combined	672±135	1.68±0.338	648±223	1.62±0.558	585±214	1.46±0.535
91	60	Male	0±0	0±0	0±0	0±0	0±0	0±0
		Female	0±0	0±0	0±0	0±0	0±0	0±0
		Combined	0±0	0±0	0±0	0±0	0±0	0±0
	300	Male	0	0	0	0	0	0
		Female	9630±4560	128±60.8	522±132	6.96±1.75	522±132	6.96±1.75
		Combined	7220±6090	96.3±81.1	418±260	5.57±3.46	418±260	5.57±3.46
	1600	Male	731±136	1.83±0.340	774±149	1.94±0.372	704±124	1.76±0.311
		Female	585±27.1	1.46±0.0678	619±30.4	1.55±0.0760	563±22.6	1.41±0.0566
		Combined	658±120	1.65±0.299	697±130	1.74±0.324	633±112	1.58±0.279

NC = not calculated.

Day	Dose (mg/kg/day)	Sex	C_{max} (µg/mL)	C_{max}/Dose ((µg/mL)/mg/kg)	CL (mL/min/kg)	$t_{1/2}$ (hr)	V_{ss} (mL/kg)
1	60	Male	0±0	0±0	NC	NC	NC
		Female	0±0	0±0	NC	NC	NC
		Combined	0±0	0±0	NC	NC	NC
	300	Male	152±217	2.02±2.90	NC	NC	NC
		Female	60.8±6.19	0.811±0.0826	NC	NC	NC
		Combined	106±150	1.42±2.00	NC	NC	NC
	1600	Male	481±175	1.20±0.437	37.4±5.76	0.182±0.0269	2370±359
		Female	495±124	1.24±0.309	44.6±13.5	0.205±0.0158	2840±874
		Combined	488±140	1.22±0.351	41.5±10.8	0.195±0.227	2640±698
91	60	Male	0±0	0±0	NC	NC	NC
		Female	0±0	0±0	NC	NC	NC
		Combined	0±0	0±0	NC	NC	NC
	300	Male	69.0±48.2	0.920±0.643	NC	NC	NC
		Female	146±27.4	1.95±0.365	0.598±0.254	46.2±13.3	2690±199
		Combined	108±54.9	1.43±0.732	0.598±0.254	46.2±13.3	2690±199
	1600	Male	570±93.1	1.43±0.233	37.6±7.91	0.195±0.0203	2380±476
		Female	457±16.8	1.14±0.0421	45.6±2.13	0.200±0.0298	2900±115
		Combined	513±86.6	1.28±0.217	41.6±6.88	0.197±0.0237	2640±424

NC = not calculated.

Dosing Solution Analysis

Results from the analysis of the concentration samples indicated that the dose formulations prepared at concentrations of 0, 3, 15, and 80 mg/mL were acceptable with values within 9% of nominal concentrations. Results from the analysis of the homogeneity samples indicated that the dose formulations were homogeneous with calculated concentrations between 85.1% and 102% of nominal concentrations.

7 Genetic Toxicology

7.1 *In Vitro* Reverse Mutation Assay in Bacterial Cells (Ames)

Study title: Defibrotide: Bacterial reverse mutation assay

Study no.:	0245-2010
Study report location:	eCTD 4.2.3.3.1.
Conducting laboratory and location:	(b) (4)
Date of study initiation:	July 16, 2010
GLP compliance:	Signed and Included
QA statement:	Signed and Included
Drug, lot #, and % purity:	Defibrotide, Lot # 1070010073, purity 95.4%

Key Study Findings

- Defibrotide was negative in the bacterial reverse mutation assay in the tester strains of *S. typhimurium* or *E.coli* in the presence and absence of S-9 mix up to 5000 µg/plate, under the conditions tested.

Methods

Strains:	*Salmonella typhimurium* : TA98, TA100, TA1535 and TA1537; *Escherichia coli:* WP2 uvrA
Concentrations in definitive study:	312.5 µg/plate to 5000 µg/plate.
Basis of concentration selection:	Up to the limit dose (5000 µg/plate) recommended by regulatory guidelines.
Negative control:	Dimethyl sulfoxide (DMSO)
Positive control:	With S9: 2-Aminoanthracene Without S9: 2-nitroflourene, sodium Azide, 9-aminoacridine, Methyl-methane sulfonate
Formulation/Vehicle:	Phosphate Buffer Saline (PBS) pH 7.2 without Ca++ and Mg++
Incubation & sampling time:	Preincubation for 30 minutes at 37±1°C: 3 days at 37±1°C.

After the preincubation period, the content of each tube was combined with 2 mL of top agar (45ºC) containing 50 µM of histidine and tryptophan, then poured onto a 90-mm plate containing histidine/tryptophan-deficient base agar and was incubated at 37±1ºC for 3 days.

Analysis:

After incubation for 3 days, colonies were counted using automatic colony counter (Sorcerer - Perceptive) with an associated Ames data collection validated software program (Ames Study Manager) to generate a study-specific electronic file containing the individual plate counts and the means and standard deviations for each treatment group. Observations on toxicity and precipitation in the incubated plates were recorded.

Criteria for Positive Response:

Results were considered positive if the data showed a dose dependent response at two consecutive dose-levels or at the highest practicable dose-level with at least 2 times the concurrent vehicle control.

Study Validity

- The vehicle and the positive control values (± S9 mix) for each tester strain were within the laboratory historical ranges.
- Vehicle control plates displayed normal growth (ie, normal background lawn) in the ± S9 mix.
- Selection of the tester strains was adequate based upon Guideline for Industry: Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals (ICHS2A, April 1996).
- The highest concentration tested was 5000 µg/plate, which allowed maximum exposure.
- The appropriate positive control compounds (± S9 mix) produced clear, increases in the number of revertant colonies.
- Tester strains demonstrated their appropriate genetic markers for strain integrity.

Results

- Defibrotide was not cytotoxic (i.e., no appreciable decrease in spontaneous revertants and/or background lawn) to all strains tested.
- Defibrotide concentrations ≤5000 µg/plate showed no signs of bacterial mutagenicity.

Table 27: Summary of results for bacterial reverse mutation assay with S9 activation

(excerpted for study report for 0245-2010)

	TA100		TA1535		TA1537		TA98		WP2 uvrA	
Dose µg/plate	R.F.	Exp. I	R.F.	Exp. I	R.F.	Exp. I	R.F.	Exp. I	R.F.	Exp. I
5000	0.8	1.0	0.6	1.0	1.1	1.2	0.9	0.9	1.2	1.2
2500	1.1	1.1	0.8	1.0	1.3	1.5	1.1	1.0	1.4	1.0
1250	1.0	1.1	1.1	0.9	0.9	1.2	0.8	1.0	1.0	1.2
625	1.0	1.0	0.9	0.9	0.7	1.3	0.8	1.0	1.1	1.0
312.5	1.0	1.0	1.2	1.0	1.0	1.3	0.6	0.9	0.9	1.1
156.25	1.0	NT	1.1	NT	1.1	NT	0.9	NT	1.0	NT
78.12	1.0	NT	0.9	NT	0.8	NT	1.0	NT	1.0	NT
39	0.9	NT	0.9	NT	0.9	NT	1.0	NT	1.1	NT
19.5	0.9	NT	0.6	NT	1.0	NT	1.1	NT	1.1	NT
9.76	0.8	NT	0.7	NT	0.8	NT	1.0	NT	1.0	NT
Positive Control	22.3	25.7	12.0	15.4	7.3	6.4	63.4	74.7	3.1	2.7

R.F. : Range Finding assay

Exp. I : I° Experiment NT : Not tested

Table 28: Summary of results for bacterial reverse mutation assay without S9 activation
(excerpted for study report for 0245-2010)

	TA100		TA1535		TA1537		TA98		WP2 uvrA	
Dose µg/plate	R.F.	Exp. I	R.F.	Exp. I	R.F.	Exp. I	R.F.	Exp. I	R.F.	Exp. I
5000	0.9	1.1	1.7	0.8	1.5	0.7	0.9	1.0	1.1	1.1
2500	0.9	1.0	0.8	1.0	1.2	0.8	1.0	0.9	0.7	1.0
1250	1.1	1.2	1.7	0.9	1.5	0.7	0.9	1.2	0.8	1.1
625	1.0	1.1	1.0	0.9	0.8	0.8	0.8	0.9	0.9	1.1
312.5	1.0	1.2	1.3	1.2	0.7	0.7	0.8	1.1	0.8	1.1
156.25	0.9	NT	1.1	NT	1.1	NT	1.3	NT	1.0	NT
78.12	0.9	NT	1.2	NT	1.0	NT	0.9	NT	1.2	NT
39	0.9	NT	1.3	NT	1.0	NT	0.6	NT	1.2	NT
19.5	0.9	NT	0.8	NT	1.0	NT	0.8	NT	0.9	NT
9.76	0.9	NT	1.2	NT	0.7	NT	1.2	NT	1.0	NT
Positive Control	2.9	2.8	19.7	22.5	13.0	4.7	10.8	9.3	3.4	5.6

R.F. : Range Finding assay
Exp. I : I° Experiment NT : Not tested

7.2 *In Vitro* Assays in Mammalian Cells

Study title: Evaluation of in vitro chromosome aberrations in Chinese hamster ovary cells with defibrotide

Study no.: A0076
Study report location:
Conducting laboratory and location: (b) (4)

Date of study initiation: September 15, 2014
GLP compliance: Signed and Included
QA statement: Signed and Included
Drug, lot #, and % purity: Defibrotide, Lot# 1130030096, purity 98.9%

Key Study Findings

- Defibrotide was negative for inducing structural or numerical aberrations in Chinese hamster ovary cells with and without metabolic activation, under the conditions tested.

Methods

Cell line:	Chinese hamster ovary (CHO) cells
Concentrations in definitive study:	First main experiment: 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 250, and 500 µg/mL in the 3 hour treatment with and without S9 mix and 1.95, 3.9, 7.81, 15.6, 31.3, 62.5, 250, and 500 µg/mL in the 20 hours continuous treatment without S9.
Basis of concentration selection:	Up to the limit dose (1 millimolar (mM) or 0.5 milligram (mg)/milliliter (mL), whichever is lower) recommended by regulatory guidelines.
Negative control:	Physiological saline solution
Positive control:	Cyclophosphamide for +S9 3 hour treatment (first main experiment)[1] Mitomycin C for –S9 20 hour treatment (second main experiment)
Formulation/Vehicle:	Physiological saline solution
Incubation & sampling time:	3 hour treatment with 16 hour recovery for -S9/+S9[2]. 20 hour treatment with no recovery for –S9

[1] The positive control (cyclophosphamide) with metabolic activation was used concurrently with the nonactivated treatment in the first experiment to demonstrate the activity of the metabolic activation and the responsiveness of the test system. There were no concurrent positive control cultures with mitomycin-c for the nonactivated 3 hour treatment.

[2] The recovery time was 17 hours per protocol; the change was documented in a protocol deviation. This deviation did not affect the integrity of the study

Analysis:

Approximately 3 hours prior to harvest, Colcemid® (0.2 µg/mL, final concentration in culture) was added to each culture to arrest cells in metaphase. Cells were trypsinized, treated with hypotonic solutions and fixed with 3:1 methanol: glacial acetic acid. Slides were prepared, and stained for analysis.

Cytotoxicity:

The highest dose level was selected for scoring on the basis of the cytotoxicity calculated by reduction in population doubling (PD). Population doubling is the log of the ratio of the final cell count (N) to the starting (baseline) count (X0) divided by the log of 2:

$$PD = \frac{\log (N/X_0)}{\log 2}$$

Baseline count is obtained at the beginning of treatment from two additional control cultures at time zero. The reduction in PD is approximately 50% of the concurrent negative control.

Chromosome aberrations:

Coded slides were analyzed for structural chromosomal aberrations from a total of 200 metaphase cells and numerical aberrations from each concentration.

Study Validity:

- The frequency of cells with structural chromosome aberrations in the vehicle control groups were within the historical range for vehicle controls.
- The percentage of cells with chromosome aberrations in each positive control group were statistically increased ($p \leq 0.001$, Fisher's Exact test), relative to the appropriate vehicle controls.

Criteria for Positive Response:

- Statistically significant increases in the proportion of cells with aberrations (excluding gaps) over the concurrent controls observed at one or more concentrations in both replicates and exceed the historical control range.

Results

- Substantial toxicity (at least 50% reduction in population doubling relative to the vehicle control) was not observed in any treatment conditions.
- Defibrotide did not induce a statistically significant increase in the number of structural aberrations (excluding gaps) with or without S9 mix up to 500 µg/mL.
- Defibrotide did not induce a statistically significant increase in cells with numerical aberrations (polypoloidy (PP) or endoreduplications (ER)).

Table 29: Summary of in vitro chromosome aberration assay: 3-hour treatment without S9 activation

(excerpted for study report for A0076)

Treatment	Dose level (µg/mL)	Culture No.	Cells Scored	Gaps	Chromatid Del	Chromatid Exch	Chromosome Del	Chromosome Exch	Isolocus	Other	Tot.abs (+gaps)	Tot.abs (-gaps)	Cells with abs (+gaps)	Cells with abs (-gaps)
Solvent	1%	1	100	2	0	0	1	0	0	0	3	1	2	1
		2	100	0	0	0	0	0	0	0	0	0	0	0
			200	2	0	0	1	0	0	0	3	1	2	1
Defibrotide	125	7	100	3	2	0	0	0	0	2PP	5	2	4	2
		8	100	1	0	0	0	0	0	0	1	0	1	0
			200	4	2	0	0	0	0	2PP	6	2	5	2
Defibrotide	250	5	100	3	0	0	0	0	0	0	3	0	2	0
		6	100	4	1	0	1	0	0	1PP	6	2	4	2
			200	7	1	0	1	0	0	1PP	9	2	6	2
Defibrotide	500	3	100	3	2	0	1	0	0	0	6	3	5	3
		4	100	0	0	0	0	0	1	0	1	1	1	1
			200	3	2	0	1	0	1	0	7	4	6	4

No positive control cultures were set up for the 3 hour nonactivated treatment.

Table 30: Summary of in vitro chromosome aberration assay: 3-hour treatment with S9 activation

(excerpted for study report for A0076)

Treatment	Dose level (µg/mL)	Culture No.	Cells Scored	Gaps	Chromatid Del	Chromatid Exch	Chromosome Del	Chromosome Exch	Isolocus	Other	Tot.abs (+gaps)	Tot.abs (-gaps)	Cells with abs (+gaps)	Cells with abs (-gaps)
Solvent	1%	21	100	1	0	0	0	0	0	0	1	0	1	0
		22	100	2	2	1	0	0	0	2ER	5	3	4	3
			200	3	2	1	0	0	0	2ER	6	3	5	3
Defibrotide	125	27	100	1	0	0	0	0	0	1ER,1PP	1	0	1	0
		28	100	0	0	0	0	0	0	1PP	0	0	0	0
			200	1	0	0	0	0	0	2PP, 1ER	1	0	1	0
Defibrotide	250	25	100	3	0	0	0	0	0	1PP	3	0	3	0
		26	100	1	0	0	0	0	0	1PP,1ER	1	0	1	0
			200	4	0	0	0	0	0	2PP,1ER	4	0	4	0
Defibrotide	500	23	100	4	1	0	0	0	1	1PP,1ER	6	2	5	1
		24	100	2	0	0	0	0	0	0	2	0	2	0
			200	6	1	0	0	0	1	1PP,1ER	8	2	7	1
Cyclophosphamide	15.0	41	100	3	5	31	0	0	1	2PP	40	37	27	27
		42	100	3	6	29	0	1	0	1PP	39	36	29	29
			200	6	11	60	0	1	1	3PP	79	73	56	56

Cyclophosphamide: Individual and pooled cultures were statistically significant at $p<0.001$

Table 31: Summary of in vitro chromosome aberration assay: 20-hour treatment without S9 activation
(excerpted for study report for A0076)

Treatment	Dose level (µg/mL)	Culture No.	Cells Scored	Gaps	Chromatid Del	Chromatid Exch	Chromosome Del	Chromosome Exch	Isolocus	Other	Tot.abs (+gaps)	Tot.abs (-gaps)	Cells with abs (+gaps)	Cells with abs (-gaps)
Solvent	1%	45	100	1	0	0	0	0	0	0	1	0	1	0
		46	100	2	0	0	0	0	0	0	2	0	2	0
			200	3	0	0	0	0	0	0	3	0	3	0
Defibrotide	125	51	100	1	0	0	0	0	0	0	1	0	1	0
		52	100	1	0	0	1	0	0	0	2	1	2	1
			200	2	0	0	1	0	0	0	3	1	3	1
Defibrotide	250	49	100	1	1	0	0	0	0	1PP	2	1	2	1
		50	100	0	0	0	0	0	0	0	0	0	0	0
			200	1	1	0	0	0	0	1PP	2	1	2	1
Defibrotide	500	47	100	0	0	0	0	0	0	0	0	0	0	0
		48	100	1	1	0	0	0	0	0	2	1	2	1
			200	1	1	0	0	0	0	0	2	1	2	1
Mitomycin-C	0.100	63	100	0	6	39	13	1	0	2H	59	59	38	38
		64	100	3	7	24	5	2	2	1H	43	40	31	29
			200	3	13	63	18	3	2	3H	102	99	69	67

Mitomycin-C: Individual and pooled cultures were statistically significant at P<0.001
H = Heavily damaged cells (more than 5 aberrations/cell)

7.3 *In Vivo* Clastogenicity Assay in Rodent (Micronucleus Assay)

Study title: In vivo micronucleus test in rats with intravenous administration of defibrotide (multiple dosing)

Study no:	A0077
Study report location:	eCTD 4.2.3.3.2.
Conducting laboratory and location:	(b) (4)
Date of study initiation:	September 17, 2014
GLP compliance:	Signed and Included
QA statement:	Signed and Included
Drug, lot #, and % purity:	Defibrotide, Lot# 1130030096, purity 98.9%

Key Study Findings

• Defibrotide was not clastogenic in the micronucleus test in the bone marrow cells of male rats up to 1600 mg/kg, under the conditions tested.

Methods

Doses in definitive study:	400, 800, and 1600 mg/kg
Frequency of dosing:	Once daily for two days
Route of administration:	Intravenous slow injection 0.5 mL/min.
Dose volume:	20 mL/kg; Mitomycin-C at 10 mL/kg
Formulation/Vehicle:	Sodium citrate dihydrate in water for injection
Species/Strain:	Sprague-Dawley SD rats
Number/Sex/Group:	5 male rats/group at 400 and 800 mg/kg, and 8 male rats at 1600 mg/kg
Satellite groups:	Preliminary dose range finding (DRF) studies at 1000 and 2000 mg/kg dosed once daily for 2 days and 1600 mg/kg dosed once[1]; 8 males and 8 females. Tolerability and bone marrow toxicity was assessed.
Basis of dose selection:	Dose selection for the micronucleus assay was based on the range finding study.
Negative control:	Vehicle: Sodium citrate dihydrate in water for injection
Positive control:	Mitomycin-C at 2 mg/kg administered intraperitoneally once.

[1] Group 3 (16 mg/kg) from DRF received a single treatment instead of two. This deviation was not considered to have affected the integrity of the study.

Table 32: Treatment scheme for definitive assay of in vivo micronucleus test in rats
(excerpted for study report for A0077)

Group	Treatment mg/kg/day	Animal Numbers Males	Sampling time after last dosing
1	Vehicle 0.0	2-10	24 h
2	Defibrotide 400	12-20	24 h
3	Defibrotide 800	22-30	24 h
4	Defibrotide 1600	32-40 52-56[a]	24 h
5	Mitomycin-C 2.00	42-50	24 h

[a] Reserve group;
Animal numbers even only; n=5 per main study group; n=3 reserve group

Analysis:

- All animals were sacrificed 24 hours after the last dosing. Bone marrow smear slides were prepared and stained. One thousand polychromatic erythrocytes per animal and corresponding number of normochromatic erythrocytes were recorded to evaluate toxicity in the target tissue. Two thousand polychromatic erythrocytes (PCEs) per animal were examined for the presence of micronuclei.

Criteria for Positive Response:

A statistically significant increase in the micronucleated polychromatic erythrocytes (at $p<0.05$) observed in any treatment group.

Study Validity

The incidence of micronucleated PCEs of the vehicle control group were within the historical negative control range. The positive control induced a significant increase in the frequency of micronucleated PCEs. At least 5 animals per group were available for slide analysis.

Results

- Clinical signs at 1600 mg/kg observed on both days were: Hind limb, fore limb and ear redness, ataxia, reduced activity, piloerection, swollen muzzle, semi closed eyes and lethargy. Animals from the 800 mg/kg mid-dose level showed reduced activity and piloerection.

- No inhibitory effect on erythropoietic cell division was observed at any defibrotide dose level based on the ratio of polychromatic to total erythrocytes (PCE:NCE ratio) compared to control groups.
- Defibrotide did not produce an increase in the percent mean number of micronucleated polychromatic erythrocytes (%MN-PCEs) compared to the vehicle control group.

Table 33: Results from dose range finding assay for micronucleus test
(excerpted for study report for A0077)

Group No.	Dose mg/kg/day	Animal No./sex	Surviving rats/ dosed rats	PCE/ (NCE+PCE) ratio	Clinical signs			
					During treatment/shortly after	1 h post dose	End of day	Day of sacrifice
1	2000	58 M 60 M	2/2	0.50 0.50	Convulsions during treatment or shortly after, tremors, ataxia, difficulty in breathing and piloerection	Reduced activity, piloerection and swollen muzzle (marked)	Piloerection and swollen muzzle (marked/slight)	NAD
		57 F 59 F	2/2	0.51 0.50				
2	1000	62 M 64 M	2/2	0.53 0.54	Reduced activity	Piloerection	Piloerection	NAD
		61 F 63 F	2/2	0.51 0.53				
3	1600	66 M 68 M	2/2	ND	Ataxia and piloerection Convulsions shortly after treatment, ataxia, piloerection and reduced activity	Reduced activity, piloerection and swollen muzzle (marked)	Piloerection and swollen muzzle (slight)	Piloerection
		65 F 67 F	2/2	ND				

M: male animals
F: female animals
PCE/(PCE+NCE): ratio values for individual animals as an indicator of bone marrow toxicity
ND: not determined
NAD: no abnormality detected

Table 34: Survival and clinical signs in definitive micronucleus assay
(excerpted for study report for A0077)

Group No.	Dose mg/kg/day	Animal No.	Surviving rats/ dosed rats	Clinical signs			
				During treatment/shortly after	1 h post dose	End of day	Day of sacrifice
1	0 Vehicle	2-10	5/5	NAD	NAD	NAD	NAD
2	400	12-20	5/5	NAD	NAD	NAD	NAD
3	800	22-30	5/5	NAD	Reduced activity and piloerection	NAD	NAD
4	1600	32-40	5/5	Hind /fore limb and ear redness, ataxia, reduced activity and lethargy	Lethargy, piloerection and swollen muzzle and semi closed eyes	Piloerection and swollen muzzle (slight)	Piloerection
4	1600	52-56	3/3	Hind /fore limb and ear redness, ataxia, reduced activity and lethargy	Lethargy, piloerection and swollen muzzle and semi closed eyes	Piloerection and swollen muzzle (slight)	Piloerection

NAD: no abnormality detected
Animal numbers even only.

Table 35: Summary of incidence of micronucleated cells
(excerpted for study report for A0077)

M A L E S

INCIDENCE OF MICRONUCLEATED CELLS/1000 CELLS

Dose level mg/kg/day	Scored cells PCE	NCE	NCE/PCE Ratio	PCE/(PCE+NCE) Ratio	% over the mean control value	Polychromatic Mean	SE	Min	Max	Normochromatic Mean	SE	Min	Max
0.00	10000	9906	0.99	0.50	100	1.3	0.4	0.0	2.0	0.0	0.0	0.0	0.0
400	10000	9350	0.94	0.52	103	1.1	0.3	0.0	2.0	0.0	0.0	0.0	0.0
800	10000	9963	1.00	0.50	100	2.1	0.6	0.5	4.0	0.0	0.0	0.0	0.0
1600	10000	8980	0.90	0.53	105	1.3	0.4	0.0	2.0	0.0	0.0	0.0	0.0
Mitomycin C													
2.00	10000	11003	1.10	0.48	95	22.9	2.6	13.5	29.0	0.0	0.0	0.0	0.0

8 Carcinogenicity

Adequacy of Carcinogenicity Studies

- The Carcinogenicity Assessment Committee (CAC) was not consulted on the study design of the dietary carcinogenicity studies in mice.
- It is noted that for both dietary carcinogenicity studies, histopathology examination was conducted for all animals in the control groups and 2000 mg/kg/day defibrotide group and for only the animals sacrificed or found dead during the treatment period for the 100 and 400 mg/kg/day defibrotide groups. Since body weight was lower in the high-dose group (2000 mg/kg/day) than the controls, histopathology should have been examined for the low-dose (100 mg/kg/day) and mid-dose (400 mg/kg/day) groups at the terminal necropsy.
- The chair of the Executive Carcinogenicity Assessment Committee was consulted on the adequacy of the studies, and based on the feedback provided

the pharmacology/toxicology team determined that the study was inadequate based on the lack of histopathology data for the 100 and 400 mg/kg/day groups.

- (b) (4)

Appropriateness of Test Models

- The study evaluated three doses of defibrotide and had two control groups.
- The mean achieved intake of defibrotide calculated using individual body weight gain and food consumption data was generally similar to the target dose based on the means in both studies; however, the ranges of the mean achieved dosages for each group indicate that the dosages did vary throughout the treatment period.
- While traditional toxicokinetic analyses were not conducted, separate sets of mice and rats (15/sex/group) were administered defibrotide (100, 400, or 2000) in the diet and a group fed an untreated diet served as the control group to evaluate the absorption of defibrotide after dietary administration to mice and rats. Blood samples were analyzed for tissue plasminogen activator.

Study title: Defibrotide: Dietary oncogenicity study in mice

Study no.:	253-094-024
Study report location:	eCTD 4.2.3.4.3.
Conducting laboratory and location:	(b) (4)
Date of study initiation:	June 6, 1990
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Defibrotide, lot # 167, 16065, and 16162
CAC concurrence:	N/A; No ECAC meeting was held

Key Study Findings

- Survival in the defibrotide-treated groups was similar to that of controls.
- Non-neoplastic findings included distension in the gall bladder and urinary bladder, cortical cyst, mineralization, and pyelonephritis in the kidneys, acute inflammation in the seminal vesicles, and granulopoiesis in the spleen.
- The incidences of N-metastatic tumor in the brain were increased in females treated with 2000 mg/kg/day defibrotide (6%) compared to controls (2%).

Methods

Doses:	0, 100, 400, or 2000 mg/kg/day
Frequency of dosing:	Daily for at least 650 days in males and 730 days in females; dosing was continued until the day before necropsy for males and the day of necropsy for females

Route of administration:	Oral as an admixture with the diet; controls received the normal untreated diet
Formulation/Vehicle:	Test substance (defibrotide) was formulated using a powdered diet by initial preparation of a pre-mix followed by dilution with further quantities of diet and mixing.
Basis of dose selection:	Results of a previous study (239-094-022) not submitted with NDA
Species/Strain:	CD-1 mouse of Swiss origin (Crl:CD-1 (ICR)BR)
Number/Sex/Group:	50/sex/group
Age:	27-29 days old at arrival
Animal housing:	Males: 5/cage, after allocation Females: 5/cage, after allocation
Paradigm for dietary restriction:	Water and food (a commercially available rodent diet) were offered *ad libitum* throughout the study
Dual control employed:	There are two control groups; there appears to be no difference in the treatment of the two groups
Interim sacrifice:	None
Satellite groups:	Veterinary controls: 10/sex were housed and fed in same manner as controls and were not examined Proof of absorption: 15/sex/group

Observations and times:

Mortality:	Twice daily
Clinical signs:	For signs of reaction to treatment, observations were conducted daily from Day 2 during the first 3 weeks of treatment and weekly thereafter Physical examination and check for palpable masses conducted once weekly
Body weights:	On day before allocation to treatment, day prior to start of treatment, weekly during treatment, and prior to necropsy
Food consumption and intake of test material:	Weekly; weight of food consumed by each cage of mice was measured and intake of test material was calculated from body weight and food consumption data
Hematology:	During Weeks 93/94 for males and Week 103 for females
Urinalysis:	Not conducted
Gross pathology:	At necropsy*
Histopathology:	At necropsy*
Proof of absorption:	In main study animals: Once during Week 1 and Weeks 13, 26, and 52 for both males and females with urine

	samples collected from first 5 complete cages of 5 animals/cage (25 animals/sex); further urine samples were collected and pooled within each group during Week 93 for males and during Week 103 for females; samples were not analyzed In proof of absorption animals: Once during Week 1 and Weeks 13, 26, 53, 85, and 104; blood samples collected from all surviving animals in each group
Formulation analysis:	Samples of diet prepared and analyzed in Weeks 1, 13, 26, 39, 52, 65, 78, 91, and 104

*Males were terminally sacrificed after 650 days of treatment due to the survival in most groups approaching the 20% limit and the females were terminally sacrificed after 730 days of treatment. Necropsies were completed within 5 working days of the first necropsy for males and within 8 days of the first necropsy for females with approximately equal numbers of animals from each group killed on non-consecutive days.

Results

Mortality

- The incidence of mortality was higher for males than females and lead to an earlier termination of treatment in males.
- Overall, there was not a defibrotide-related effect on mortality.
    - In males, mortality was similar between the second control group and the defibrotide treated groups. It is noted that the mortality was slightly higher in the males treated with defibrotide than the control groups early in the study.
    - In females, mortality was slightly higher in the second control group and the 100 mg/kg/day defibrotide group than the remaining groups.

Table 36: Summary of cumulative mortality in dietary carcinogenicity study in mice

	Animals dead									
	Males					Females				
Dose (mg/kg/day)	0 Control	0 Control	100	400	2000	0 Control	0 Control	100	400	2000
Animals initially in study	50	50	50	50	50	50	50	50	50	50
Day 140										
Number	0	0	1	1	1	0	0	0	1	0
%	0	0	2	2	2	0	0	0	2	0
Day 224										
Number	1	0	3	1	5	0	1	1	1	1
%	2	0	6	2	10	0	2	2	2	2
Day 280										
Number	1	4	4	3	8	2	1	2	1	3
%	2	8	8	6	16	4	2	4	2	6
Day 364										
Number	4	9	12	8	13	3	2	2	4	3
%	8	18	24	16	26	6	4	4	8	6
Day 448										
Number	6	17	17	11	17	5	7	4	5	5
%	12	34	34	22	34	10	14	8	10	10
Day 504										
Number	10	21	22	21	19	8	9	9	6	6
%	20	42	44	42	38	16	18	18	12	12
Day 560										
Number	13	26	25	26	24	9	11	11	9	7
%	26	52	50	52	48	18	22	22	18	14
Day 616										
Number	19	34	32	31	34	10	18	16	12	9
%	38	68	64	62	68	20	36	32	24	18
Day 644										
Number	21	34	35	34	37	11	18	19	13	11
%	42	68	70	68	74	22	36	38	26	22
Day 672										
Number	NA	NA	NA	NA	NA	15	21	22	17	14
%	NA	NA	NA	NA	NA	30	42	44	34	28
Day 700										
Number	NA	NA	NA	NA	NA	15	26	25	19	18
%	NA	NA	NA	NA	NA	30	52	50	38	36
Day 728										
Number	NA	NA	NA	NA	NA	18	31	30	20	22
%	NA	NA	NA	NA	NA	36	62	60	40	44

NA= Data not available for males at this time point; males were terminally sacrificed after 650 days of treatment

Clinical Signs

- No defibrotide-related clinical signs were observed.
- Multiple clinical signs were observed in both the control and defibrotide groups with similar incidences including nodule tail, hairloss, scab/abrasion/excoriation, staining, hunched/prostate/prone, and swollen ventrum/abdomen/thorax, hindlimbs/forelimbs, and perigenital area.

Palpable masses

- No treatment-related differences in the number of animals with palpable masses, the total number of masses, or the mean day of onset of masses were observed between the control and defibrotide groups.

Table 37: Palpable masses in dietary carcinogenicity study in mice

Palpable masses	Males					Females				
Dose (mg/kg/day)	0 Control	0 Control	100	400	2000	0 Control	0 Control	100	400	2000
Number of animals examined	50	50	50	50	50	50	50	50	50	50
Number of animals with one or more masses	20	27	20	16	16	7	7	5	6	3
Number of animals with multiple masses										
1	6	15	10	10	10	6	6	5	2	3
2	9	11	6	2	2	1	1	0	3	0
3	3	1	3	3	3	0	0	0	0	0
4	0	0	1	1	1	0	0	0	1	0
5	1	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0
7 or more	1	0	0	0	0	0	0	0	0	0
Total number of palpable masses per group	45	40	35	27	27	8	8	5	12	3
Mean onset time (day)	401	323	421	386	376	500	647	502	650	603

Masses detected at necropsy only have not been included

Table 38: Incidences of mice with palpable masses confirmed as tumors, non-neo-plastic lesion, or no significant lesion

Palpable masses	Males					Females				
Dose (mg/kg/day)	0 Control	0 Control	100**	400**	2000	0 Control	0 Control	100**	400**	2000
Number of animals examined	50	50	50	50	50	50	50	50	50	50
Number of animals affected										
Malignant tumor	2	2	4	3	2	5	7	4	1	2
Non-neoplastic lesion	7	11	5	1	8	0	0	0	1	0
No specimen/ no significant lesion	11	14	6	5	6	2	0	0	1	1
Day										
Mean onset time of first mass confirmed as tumor	458	518	553	395	490	544	647	443	617	566

Masses detected at necropsy only have not been included
If more than one lesion was present, the animals were classified by the most severe
** Refers to unscheduled deaths only

Body Weights

- Body weight and body weight gain were slightly lower in males treated with 400 and 2000 mg/kg/day defibrotide.

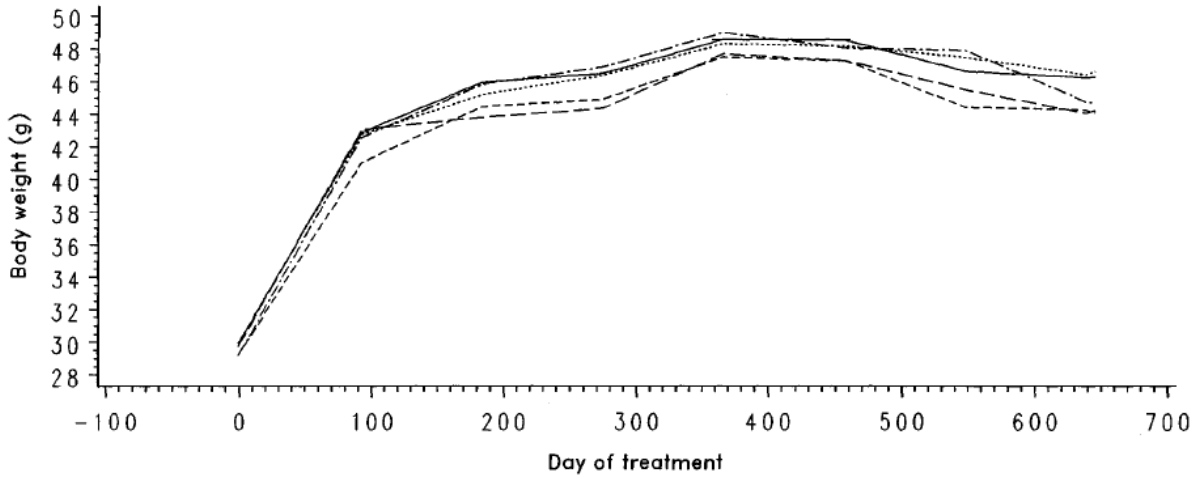
Table 39: Body weight gain in dietary carcinogenicity study in mice

Interval	Mean body weight gain (g)			
	Males			
Dose (mg/kg/day)	0 Control‡	100	400	2000
Days -1 to 365	18.64	19.84	18.62	18.28
Days 365 to 645	-2.79	-1.75	-2.94	-3.56
Days -1 to 645	16.26	15.59	15.17	14.99

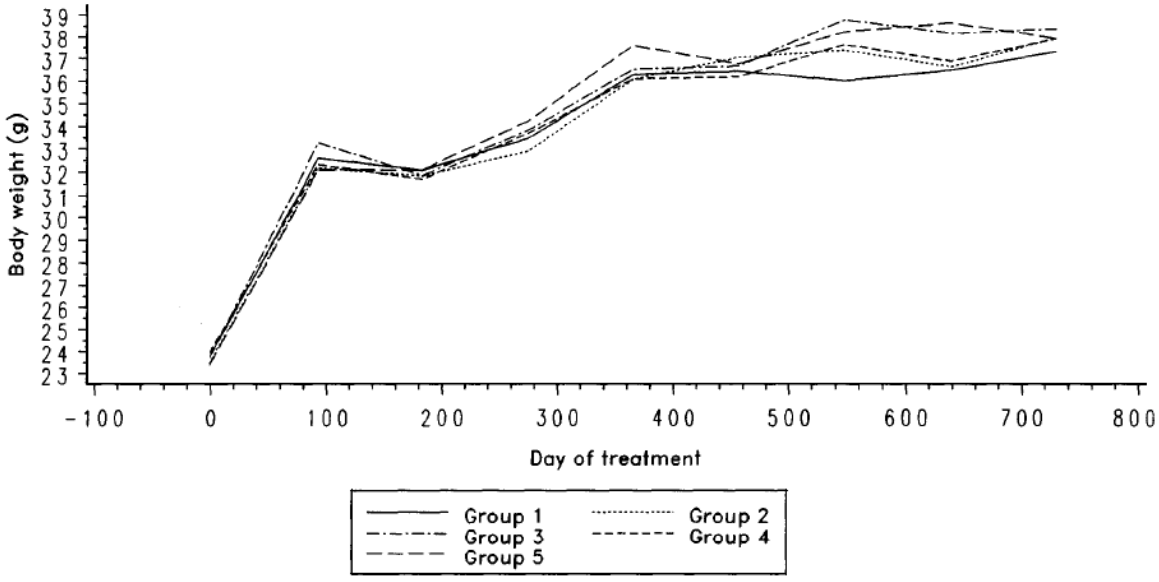
‡ Data for the control groups was combined

Figure 25: Body weights in dietary carcinogenicity study in mice
(excerpted from study report for 253-094-024)

Males



Females



Group 1 and Group 2= Control
Group 3= 100 mg/kg/day defibrotide
Group 4= 400 mg/kg/day defibrotide
Group 5= 2000 mg/kg/day defibrotide

Food Consumption

Unremarkable

Hematology

- Red blood cell count, hematocrit, hemoglobin, and mean corpuscular hemoglobin were significantly increased in males treated with 2000 mg/kg/day compared to controls at the end of treatment on Weeks 93/94.

Table 40: Hematology findings in males on Weeks 93/94

Parameter	Mean	Percentage deviation from Control		
	Control‡ 0 mg/kg/day	100 mg/kg/day	400 mg/kg/day	2000 mg/kg/day
RBC count (10'6/cmm)	8.790	-	-	↑12**
Hematocrit (%)	36.07	-	-	↑8*
Hemoglobin (g/dL)	12.98	-	-	↑14**
Mean corpuscular hemoglobin concentration (g/dL)	35.91	-	-	↑6**

‡ Data for the control groups was combined
↑= increase - = no test-article related changes
* Significantly different from control ($p<0.05$)
** Significantly different from control ($p<0.01$)

Gross Pathology

Unremarkable

Histopathology

Peer Review: No

Tissues were examined for all animals in the control groups and 2000 mg/kg/day defibrotide group and for animals sacrificed or found dead during the treatment period from the 100 and 400 mg/kg/day defibrotide groups.

Neoplastic

- While the Applicant considers there to be no defibrotide-related neoplastic findings, the incidences of N-metastatic tumor in the brain were increased in females treated with 2000 mg/kg/day defibrotide. N-metastatic tumor was observed in 2 of 99 control females (2%) and 3 of 50 females treated with 2000 mg/kg/day defibrotide (6%).

Table 41: Neoplastic microscopic findings in dietary carcinogenicity study in mice

Neoplastic microscopic findings		No. of animals affected									
		Males					Females				
Dose (mg/kg/day)		0 Control	0 Control	100	400	2000	0 Control	0 Control	100	400	2000
Number of animals examined		25*/24	34*/14	39*/NE	38*/NE	40*/10	17*/32	31*/19	29*/NE	21*/NE	23*/27
Brain	N-metastatic tumor	-	-	-	1*/NE	-	1*/0	0/1	1*/NE	1*/NE	3*/0

Number of animals examined and affected: Early deaths*/terminal necropsy
- = no test-article related changes
NE= Not examined

Non-Neoplastic

Table 42: Non-neoplastic microscopic findings in dietary carcinogenicity study in mice

Non-neoplastic microscopic findings		No. of animals affected									
		Males					Females				
Dose (mg/kg/day)		0 Control	0 Control	100	400	2000	0 Control	0 Control	100	400	2000
Number of animals examined		26*/24	36*/14	39*/NE	38*/NE	40*/10	18*/32	31*/19	30*/NE	21*/NE	23*/27
Gall bladder	Distension	0/4	0/1	-	1*/NE	1*/3	4*/0	4*/1	5*/NE	3*/NE	2*/7
Kidneys	Cortical cyst	1*/3	0/2	1*/NE	1*/NE	7*/2	1*/3	2*/2	1*/NE	1*/NE	1*/2
	Mineralization	2*/0	1*/0	7*/NE	2*/NE	5*/2	-	-	1*/NE	-	-
	Pyelonephritis	-	3*/0	4*/NE	4*/NE	7*/0	-	-	-	-	-
Seminal vesicles	Acute inflammation	1*/0	2*/0	3*/NE	2*/NE	7*/0	NA	NA	NA	NA	NA
Spleen	Granulopoiesis	1*/0	2*/0	8*/NE	4*/NE	6*/0	-	-	1*/NE	1*/NE	1*/1
Urinary bladder	Distension	4*/4	6*/1	10*/NE	12*/NE	13*/1	2*/0	1*/0	-	-	-

Number of animals examined and affected: Early deaths*/terminal necropsy
- = no test-article related changes
NA= Not applicable, issue not present in this sex
NE= Not examined

Proof of Absorption

To evaluate the absorption of defibrotide after dietary administration to mice, a separate set of animals (15/sex/group) were administered defibrotide (100, 400, or 2000) in the diet for at least 743 days (106 weeks) and a group fed an untreated diet served as the control group. This was referred to as a separate study (2 year oral treatment for proof of absorption determination in mice) with a separate report (Report # 254-094-028) present in the same PDF file as study # 253-094-024.

Blood samples were collected from all surviving animals in each group once during Week 1 and Weeks 13, 26, 53, 85, and 104 and analyzed for tissue plasminogen activator (t-PA).

- Oral administration of defibrotide to mice in the diet induced increases in plasma t-PA levels. The largest increases in t-PA were observed in the 100 and 400 mg/kg/day groups. Smaller increases were observed in males treated with 2000 mg/kg/day and no consistent changes in t-PA plasma activity were observed in the females treated with 2000 mg/kg/day.

Table 43: Proof of absorption of dietary administration of defibrotide in mice

Week of study	Mean t-PA (IU/mL)							
	Control 0 mg/kg/day		100 mg/kg/day		400 mg/kg/day		2000 mg/kg/day	
	Males	Females	Males	Females	Males	Females	Males	Females
1	1.80	3.39	2.89**	4.23*	2.68**	3.46	2.33*	3.45
13	2.17	2.97	2.93**	4.25**	2.83**	3.33	2.50	2.97
26	1.98	2.87	3.25**	4.56**	2.74**	3.67**	2.37**	3.03
53	2.28	2.48	3.72**	3.71**	3.55**	2.92	2.72	2.59
85	2.25	3.30	3.14	4.45*	1.83	2.93	2.50	2.64
104	2.88	3.10	3.97	4.14*	NA	3.02	NA	3.56

* Significantly different from control ($p<0.05$)
** Significantly different from control ($p<0.01$)
NA= Data not available for this group at this time point

Achieved Dosage

The mean achieved intake of defibrotide calculated using individual body weight gain and food consumption data was generally similar to the target dose based on the means presented in the table below. The ranges of the mean achieved dosages for each group indicate that the dosages did vary throughout the treatment period.

Table 44: Mean achieved dosage of defibrotide in dietary carcinogenicity study in mice

Achieved dosage	Dosage (mg/kg/day)					
	Males			Females		
Dose (mg/kg/day)	100	400	2000	100	400	2000
Range	88-155	303-559	1676-2984	71-156	260-653	1288-3078
Mean	112.4	428.6	2211.4	105.4	406.0	2000.1

Study title: Defibrotide: Dietary oncogenicity study in rats

Study no.: 237-094-021
Study report location: eCTD 4.2.3.4.3.
Conducting laboratory and location: (b) (4)
Date of study initiation: November 2, 1989
GLP compliance: Yes
QA statement: Yes
Drug, lot #, and % purity: Defibrotide, lot # 157, 16065, and 16162
CAC concurrence: N/A; No ECAC meeting was held

Key Study Findings

- Survival in the defibrotide-treated groups was similar to that of controls.
- Non-neoplastic findings included hemorrhage in the brain and spinal cord, tubular dilatation/protein casts, mineralization, and chronic interstitial nephritis in the kidneys, vascular mineralization in the lungs, colloid depletion in the seminal vesicles, and atrophy in the testes.
- The incidences of B-astrocytoma in the brain and B-lipoma in the skin were increased in males treated with 2000 mg/kg/day defibrotide. B-astrocytoma was observed in 1% control males and 6% of males treated with 2000 mg/kg/day defibrotide.

Methods

Doses:	0, 100, 400, or 2000 mg/kg/day
Frequency of dosing:	Daily for at least 732 days; dosing was continued until day of necropsy
Route of administration:	Oral as an admixture with the diet; controls received the normal untreated diet
Formulation/Vehicle:	Test substance (defibrotide) was formulated using a powdered diet by initial preparation of a pre-mix followed by dilution with further quantities of diet and mixing.
Basis of dose selection:	Results of a previous study (57-0-094-006) not submitted with NDA
Species/Strain:	Sprague Dawley rat
Number/Sex/Group:	50/sex/group
Age:	27-29 days old at arrival
Animal housing:	Males: 5/cage, after allocation Females: 5/cage, after allocation
Paradigm for dietary restriction:	Water and food (a commercially available rodent diet) were offered *ad libitum* throughout the study
Dual control employed:	There are two control groups; there appears to be no difference in the treatment of the two groups
Interim sacrifice:	None
Satellite groups:	Veterinary controls: 15/sex were housed and fed in same manner as controls and were not examined Proof of absorption: 15/sex/group

Observations and times:

Mortality:	Twice daily
Clinical signs:	Daily observations for gross reaction to treatment Physical examination and check for palpable masses conducted once weekly
Body weights:	On day of allocation to treatment, day prior to start of treatment, weekly during treatment, and prior to necropsy
Food consumption and intake of test material:	Weekly; weight of food consumed by each cage of rats was measured and intake of test material was calculated from body weight and food consumption data
Hematology:	During Weeks 103/104
Urinalysis:	Not conducted
Gross pathology:	At necropsy*
Histopathology:	At necropsy*
Proof of absorption:	In main study animals: Once during Week 1/2 and Weeks 13, 26, 52, and 103/104; overnight urine samples

	collected from the first 15 surviving main study animals per sex in each group In proof of absorption animals: Once during Week 1 and Weeks 13, 26, 52, and 104; blood samples collected from all surviving animals in each group
Formulation analysis:	Samples of diet prepared and analyzed in Weeks 1, 13, 27, 39, 52, 65, 78, 91, and 104

*Necropsies were completed within 12 working days of the first necropsy with approximately equal numbers of animals from each group killed on non-consecutive days.

Results

Mortality

- No remarkable differences in mortality between the control and defibrotide groups were observed during the study.

Table 45: Summary of cumulative mortality in dietary carcinogenicity study in rats

	Animals dead									
	Males					Females				
Dose (mg/kg/day)	0 Control	0 Control	100	400	2000	0 Control	0 Control	100	400	2000
Animals initially in study	50	50	50	50	50	50	50	50	50	50
Day 224										
Number	0	0	0	1	0	0	1	0	1	0
%	0	0	0	2	0	0	2	0	2	0
Day 448										
Number	2	5	2	5	1	3	2	2	5	2
%	4	10	4	10	2	6	4	4	10	4
Day 560										
Number	6	12	11	9	8	9	5	4	7	5
%	12	24	22	18	16	18	10	8	14	10
Day 616										
Number	11	14	14	15	10	10	7	4	10	7
%	22	28	28	30	20	20	14	8	20	14
Day 672										
Number	18	16	17	18	16	15	11	11	12	10
%	36	32	34	36	32	30	22	22	24	20
Day 700										
Number	21	18	21	24	18	17	13	15	14	15
%	42	36	42	48	36	34	26	30	28	30
Day 728										
Number	23	22	27	25	21	20	14	18	18	16
%	46	44	54	50	42	40	28	36	36	32

Clinical Signs

- Multiple clinical signs were observed in both the control and defibrotide groups with similar incidences including nodule tail, hairloss, scab/abrasion/excoriation, generalized staining, and swollen hindlimbs/forelimbs.

- Convulsions/tremors, abnormal breathing/rales, and nervous/aggressive were observed with a slightly higher incidence in defibrotide treated animals than controls in one or both sexes.

Table 46: Clinical signs in dietary carcinogenicity study in rats

Clinical signs	Number of animals affected									
	Males					Females				
Dose (mg/kg/day)	0 Control	0 Control	100	400	2000	0 Control	0 Control	100	400	2000
Number of animals examined	50	50	50	50	50	50	50	50	50	50
Convulsions/tremors	-	1	1	-	3	-	-	1	-	4
Abnormal breathing/rales	1	3	4	2	2	1	-	1	1	5
Nervous/aggressive	7	10	9	16	7	5	9	4	6	16

- = Clinical sign not observed in this group

Palpable masses

- No treatment-related differences in the number of animals with palpable masses, the total number of masses, or the mean day of onset of masses were observed between the control and defibrotide groups.
- The total number of palpable masses was slightly increased in both males and females in the second control group.

Table 47: Palpable masses in dietary carcinogenicity study in rats

Palpable masses	Males					Females				
Dose (mg/kg/day)	0 Control	0 Control	100	400	2000	0 Control	0 Control	100	400	2000
Number of animals examined	50	50	50	50	50	50	50	50	50	50
Number of animals with one or more masses	34	32	26	26	29	33	37	29	33	35
Number of animals with multiple masses										
1	22	13	15	13	15	16	16	13	12	16
2	7	8	6	6	7	9	10	9	11	10
3	5	6	2	2	7	4	4	3	5	7
4	0	1	3	3	0	3	5	3	4	2
5	0	2	0	2	0	0	1	1	1	0
6	0	2	0	0	0	1	0	0	0	0
7 or more	0	0	0	0	0	0	1	0	0	0
Total number of palpable masses per group	51	73	45	53	50	64	80	57	70	65
Mean onset time (day)	535	520	566	597	557	575	566	618	588	603

Masses detected at necropsy only have not been included

Table 48: Incidences of rats with palpable masses confirmed as tumors, non-neoplastic lesion, or no significant lesion

Palpable masses	Males					Females				
Dose (mg/kg/day)	0 Control	0 Control	100**	400**	2000	0 Control	0 Control	100**	400**	2000
Number of animals examined	50	50	50	50	50	50	50	50	50	50
Number of animals affected										
Malignant tumor	3	3	5	2	2	6	11	1	2	2
Benign tumor	14	14	3	6	16	23	21	8	5	25
Non-neoplastic lesion	4	10	2	1	5	1	3	0	1	3
No specimen/ no significant lesion	13	5	2	0	6	3	2	0	1	2
Day										
Mean onset time of first mass confirmed as tumor	596	601	575	504	622	582	596	536	533	612

Masses detected at necropsy only have not been included
If more than one lesion was present, the animals were classified by the most severe
** Refers to unscheduled deaths only

Body Weights

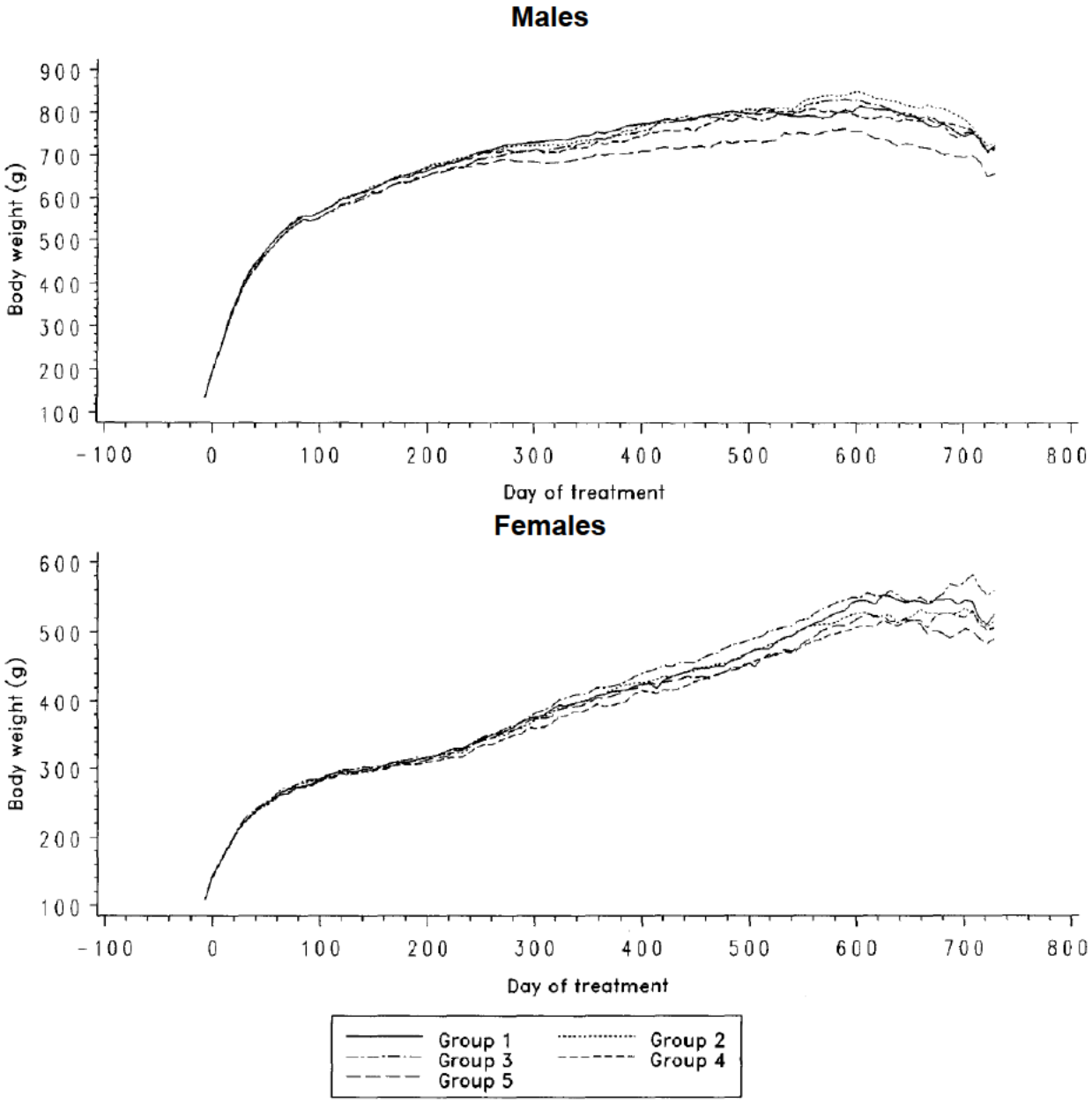
- Body weights were consistently lower in males treated with 2000 mg/kg/day defibrotide than controls throughout the treatment period with statistically significant differences at most time points starting on Day 281. Decreased body weight gain was also observed.
- Body weights were also lower in females treated with 2000 mg/kg/day defibrotide than controls; however, the differences were not statistically significant.
- At 400 mg/kg/day, body weights were slightly lower than controls, but not statistically significant.

Table 49: Body weight gain in dietary carcinogenicity study in rats

Interval	Mean body weight gain (g)							
	Males				Females			
Dose (mg/kg/day)	0 Control‡	100	400	2000	0 Control‡	100	400	2000
Days -1 to 365	556.56	552.12	537.93	514.80*	269.27	278.90	253.97	264.10
Days 365 to 729	7.13	-7.16	17.40	-10.84	112.52	148.63*	114.54	101.05
Days -1 to 729	540.15	529.49	531.12	472.93	380.49	419.70	368.40	352.43

‡ Data for the control groups was combined
* Significantly different from control ($p<0.05$)

Figure 26: Body weights in dietary carcinogenicity study in rats
(excerpted from study report for 237-094-021)



Group 1 and Group 2= Control
Group 3= 100 mg/kg/day defibrotide
Group 4= 400 mg/kg/day defibrotide
Group 5= 2000 mg/kg/day defibrotide

Food Consumption

- Decreased food consumption was observed in defibrotide-treated animals, particularly in males at 2000 mg/kg/day and females at 400 and 2000 mg/kg/day. While the decreases were occasionally statistically significant in females, the decreases in food consumption were not consistent throughout the study.

Hematology

- Prothrombin time was significantly increased in both males and females treated with 2000 mg/kg/day compared to controls.

Table 50: Hematology findings on Weeks 103/104

Parameter	Mean		Percentage deviation from Control					
	Control‡ 0 mg/kg/day		100 mg/kg/day		400 mg/kg/day		2000 mg/kg/day	
	Males	Females	Males	Females	Males	Females	Males	Females
Prothrombin time (sec)	13.45	13.43	-	-	-	-	↑9*	↑5**

‡ Data for the control groups was combined
↑= increase - = no test-article related changes
* Significantly different from control ($p<0.05$)
** Significantly different from control ($p<0.01$)

Gross Pathology

Table 51: Macroscopic findings in dietary carcinogenicity study in rats

Macroscopic findings		No. of animals affected									
		Males					Females				
Dose (mg/kg/day)		0 Control	0 Control	100	400	2000	0 Control	0 Control	100	400	2000
Number of animals examined		23*/27	24*/26	28*/22	26*/24	22*/28	21*/29	16*/34	18*/32	18*/32	17*/33
Testes	Abnormal consistency (flaccid)	5*/5	2*/4	7*/2	6*/3	4*/10	NA	NA	NA	NA	NA
Uterus	Abnormal consistency	NA	NA	NA	NA	NA	0/1	-	0/1	1*/2	0/3

Number of animals examined and affected: Early deaths*/terminal necropsy
- = no test-article related changes
NA= Not applicable, issue not present in this sex

Histopathology

Peer Review: No

Tissues were examined for all animals in the control groups and 2000 mg/kg/day defibrotide group and for animals sacrificed or found dead during the treatment period from the 100 and 400 mg/kg/day defibrotide groups.

Neoplastic

- While the Applicant considers there to be no defibrotide-related neoplastic findings, the incidences of B-astrocytoma in the brain and B-lipoma in the skin were increased in males treated with 2000 mg/kg/day defibrotide.
- B-astrocytoma was observed in 1 of 100 control males (1%) and 3 of 50 males treated with 2000 mg/kg/day defibrotide (6%). The Applicant did not consider the B-astrocytoma to be related to defibrotide due to the lack of consistent increase in the incidence in treated animals, a shift to less differentiated tumor types, the occurrence of preneoplastic lesions, or a decline in survival. Additionally, two M-meningiosarcomas were detected in a control group female.

Table 52: Neoplastic microscopic findings in dietary carcinogenicity study in rats

Neoplastic microscopic findings		No. of animals affected									
		Males					Females				
Dose (mg/kg/day)		0 Control	0 Control	100	400	2000	0 Control	0 Control	100	400	2000
Number of animals examined		23*/27	24*/26	28*/NE	26*/NE	22*/28	21*/29	16*/34	18*/NE	18*/NE	17*/33
Brain	B-astrocytoma	0/1	-	-	-	2*/1	-	-	-	1*/NE	-
Skin	B-lipoma	0/2	1*/3	1*/ NE	-	2*/5	0/1	0/2	-	1*/NE	0/1

Number of animals examined and affected: Early deaths*/terminal necropsy
- = no test-article related changes
NE= Not examined

Non-Neoplastic

Table 53: Non-neoplastic microscopic findings in dietary carcinogenicity study in rats

Non-neoplastic microscopic findings		No. of animals affected									
		Males					Females				
Dose (mg/kg/day)		0 Control	0 Control	100	400	2000	0 Control	0 Control	100	400	2000
Number of animals examined		23*/27	24*/26	28*/NE	26*/NE	22*/28	21*/29	16*/34	18*/NE	18*/NE	17*/33
Adrenals	Cortical telangiectasis	1*/3	0/1	2*/NE	0/NE	2*/5	11*/25	4*/31	10*/NE	10*/ NE	10*/31
	Congestion	0/0	1*/1	0/NE	0/NE	3*/7	1*/2	1*/1	-	2*/NE	0/2
Brain	Hemorrhage	0/1	1*/0	1*/NE	2*/NE	3*/2	1*/0	1*/0	-	1*/NE	2*/3
Kidneys	Tubular dilatation/ protein casts	4*/10	5*/4	9*/NE	12*/NE	5*/12	4*/8	3*/5	3*/NE	1*/NE	3*/11
	Mineralization	2*/1	2*/0	4*/NE	1*/NE	3*/4	3*/11	8*/17	10*/NE	7*/NE	4*/12
	Chronic interstitial nephritis	0/3	4*/2	3*/NE	3*/NE	5*/7	-	0/2	1*/NE	-	1*/8
Lungs	Vascular mineralization	19*/16	11*/14	19*/NE	19*/NE	16*/24	9*/13	6*/11	9*/NE	9*/NE	14*/28
Seminal vesicles	Colloid depletion	4*/4	2/3	5*/NE	3*/NE	7*/5	NA	NA	NA	NA	NA
Spinal cord	Hemorrhage	0/3	0/2	-	2*/NE	1*/7	-	1*/1	-	1*/NE	1*/1
Testes	Atrophy	3*/2	3*/4	6*/NE	4*/NE	5*/8	NA	NA	NA	NA	NA

Number of animals examined and affected: Early deaths*/terminal necropsy
- = no test-article related changes
NA= Not applicable, issue not present in this sex
NE= Not examined

Proof of Absorption

To evaluate the absorption of defibrotide after dietary administration to rats, a separate set of animals (15/sex/group) were administered defibrotide (100, 400, or 2000) in the diet for at least 748 days (107/108 weeks) and a group fed an untreated diet served as the control group. This was referred to as a separate study (2 year oral treatment for proof of absorption determination in rats) with a separate report (Report # 238-094-023) present in the same PDF file as study # 237-094-021.

Blood samples were collected from all surviving animals in each group once during Week 1 and Weeks 13, 26, 52, and 104 and analyzed for tissue plasminogen activator (t-PA).

- Oral administration of defibrotide to rats in the diet induced increases in plasma t-PA levels over a prolonged period of time at all doses tested.

Table 54: Proof of absorption of dietary administration of defibrotide in rats

Week of study	Mean t-PA (IU/mL)							
	Control 0 mg/kg/day		100 mg/kg/day		400 mg/kg/day		2000 mg/kg/day	
	Males	Females	Males	Females	Males	Females	Males	Females
1	1.99	4.27	2.75*	6.18*	2.92*	7.18*	2.97**	5.47
13	2.11	2.12	2.87**	3.03*	2.90	3.45**	2.95**	3.26*
26	2.01	2.13	2.80**	3.28**	3.23**	3.35**	2.91**	2.91*
52	2.07	2.41	2.56	2.84	2.81*	3.27*	2.33	2.34
104	3.44	3.07	2.65	3.93	3.08	3.37	3.00	3.39

* Significantly different from control ($p<0.05$)
** Significantly different from control ($p<0.01$)

Achieved Dosage

The mean achieved intake of defibrotide calculated using individual body weight gain and food consumption data was generally similar to the target dose based on the means presented in the table below. The ranges of the mean achieved dosages for each group indicate that the dosages did vary throughout the treatment period.

Table 55: Mean achieved dosage of defibrotide in dietary carcinogenicity study in rats

Achieved dosage	Dosage (mg/kg/day)					
	Males			Females		
Dose (mg/kg/day)	100	400	2000	100	400	2000
Range	86-125	335-523	1758-2625	73-129	276-505	1494-2590
Mean	103.2	404.9	2012.0	101.6	400.5	1986.0

9 Reproductive and Developmental Toxicology

9.1 Fertility and Early Embryonic Development

The fertility study submitted was a non-GLP study conducted with intramuscular administration of defibrotide; therefore, the study was not reviewed.

9.2 Embryonic Fetal Development

Study title: Defibrotide preliminary intravenous infusion embryo-fetal development study in rats

Study no.:	79820EXT
Study report location:	eCTD 4 2 3 5 2
Conducting laboratory and location:	(b) (4)
Date of study initiation:	Exact date not provided; the first batch of animals arrived on December 22, 2009
GLP compliance:	No
QA statement:	No
Drug, lot #, and % purity:	Defibrotide, lot # 2080010002

Key Study Findings

- Intravenous administration of defibrotide as a continuous infusion (240, 1200, or 4800 mg/kg/day) or 2-hour infusion 4 times daily (60, 120, or 240 mg/kg/day) to pregnant rats resulted in severe maternal toxicity that lead to early euthanization of the majority of the defibrotide-treated females.
- The majority of the early death females showed a red discharge on the urogenital region, which was related to imminent abortion.
- Only one female in each of the 120 and 240 mg/kg/day groups administered defibrotide as a 2-hour infusion 4 times daily had live fetuses on gestational day 20; therefore, meaningful comparisons could not be made for parameters regarding body weight, the cesarean section, or offspring.
- Since resorptions were observed in the two females with live fetuses and were not observed in controls, defibrotide appears to have increased resorptions.

Methods

Doses:	Continuous infusion: 0, 240, 1200, or 4800 mg/kg/day Four times daily: 60, 120, or 240 mg/kg/day
Frequency of dosing:	Continuous infusion or infusion four times daily gestational days (GD) 6-15
Route of administration:	Intravenous infusion; continuous infusion over an infusion period of 24 hours or 2-hours followed by 4-hour treatment free period at approximate rate of 2.5 mL/kg/hour
Formulation/Vehicle:	Sterile 0.9% sodium chloride for injection
Species/Strain:	Sprague-Dawley rat
Number/Sex/Group:	6-8 females/group; see table with study design below
Satellite groups:	None
Study design:	Pregnant female rats were dosed with vehicle or

240, 1200, or 4800 mg/kg/day defibrotide administered as a continuous intravenous infusion on GD 6-15. Due to excessive toxicity observed at these doses, these groups were euthanized early and 3 additional groups of pregnant female rats were dosed at 60, 120, and 240 mg/kg/day defibrotide administered as a 2-hour infusion 4 times a day on GD 6-15. All surviving females were euthanized on GD 20 and examined. The day successful mating was detected was considered GD 0. See table of the study design below.

Parameters and endpoints evaluated: Females: Mortality, body weight, uterine weights, clinical signs, necropsy, number of corpora lutea, number of implantations, early and late resorptions, and live and dead fetuses
Fetuses: Fetal weight and fetal exam (external abnormalities)

Table 56: Study design for preliminary embryo-fetal development study in rats

Group	Number of mated females	Dose level		Concentration	Dosing regimen of intravenous infusion
		mg/kg/day	mg/kg/hour	mg/mL	
1	8	0	0	0	Continuous
2	6	240	10	4	Continuous
3	6	1200	50	20	Continuous
4	7	4800	200	80	Continuous
5	8	60	7.5	3	2 hour infusion 4 times/day
6	8	120	15	6	2 hour infusion 4 times/day
7	8	240	30	12	2 hour infusion 4 times/day

Results

Mortality

During the treatment phase, 40 out of 51 animals were euthanized early for humane reasons or absence of patency of the intravenous catheter.

- Absence of patency: One female treated with 1200 mg/kg/day as a continuous infusion (Group 3; Animal # 41) was euthanized on GD 7 and one control (Animal # 5) and one female treated with 60 mg/kg/day as a 2-hour infusion 4 times daily (Group 5; Animal # 89) were euthanized on GD 12.
- The remaining 37 females euthanized early were treated with defibrotide and were euthanized between GD 10 and GD 15. Most of them showed red staining on urogenital region, which was related to an imminent abortion.

Table 57: Mortality and fate of females in preliminary embryo-fetal development study in rats

Group	Infusion type and Dose (mg/kg/day)	Number of mated females	Number of unscheduled deaths	Number of unscheduled death females pregnant	Number of females at final sacrifice (GD 20)	Number of females at final sacrifice (GD 20) with live fetuses
	Continuous infusion					
1	0 (Control)	8	1	1	7	7
2	240	6	6	6	None	NA
3	1200	6	6	4	None	NA
4	4800	7	7	7	None	NA
	4 times/day					
5	60	8	7	7	1	None
6	120	8	7	7	1	1
7	240	8	6	6	2	1

NA=Not applicable

Clinical Signs

Table 58: Clinical signs in preliminary embryo-fetal development study in rats

Clinical signs	Number of animals affected						
	Continuous infusion				4 times/day		
Dose (mg/kg/day)	0 Control	240	1200	4800	60	120	240
Number of animals examined	8	6	6	7	8	8	8
Red staining, vagina	-	3	2	4	6	6	6
Tachycardia	-	-	2	7	-	-	-
Scabs, dorsal region or neck	-	-	1	1	-	5	4
Hairloss, dorsal region or neck	-	-	-	-	-	3	2

- = Clinical sign not observed in this group

Body Weight

- Body weight and body weight gain were comparable between control and defibrotide-treated groups until GD 12. Due to the toxicity observed, the majority of defibrotide-treated animals were euthanized early and the available data for GD 15, GD 18, and GD 20 were not sufficient to compare the groups for body weight and body weight gain.
- Since most of the defibrotide-treated animals were euthanized before the end of gestation, the data for uterus weight and absolute weight gain were also not sufficient to compare the groups.

Food Consumption

Not conducted

Toxicokinetics

Not conducted

Necropsy

Macroscopic findings:

- Unscheduled deaths:
  - Swollen and enlarged spleens, dark/red fluid content in the uterus and/or in the abdominal cavity, and red staining on the skin of the urogenital region were observed during the *post mortem* examination in defibrotide-treated animals at all dose levels.
  - Females in the groups administered defibrotide (60, 120, and 240 mg/kg/day) as a 2-hour infusion 4 times daily also showed a thickened caudal vena cava. Edema with a single pale firm area and enlarged lumbar lymph nodes was observed in one female dosed at 240 mg/kg/day.
- Terminal necropsy:
  - Enlarged spleen and thickened caudal vena cava were detected in females treated with defibrotide at ≥120 mg/kg/day. No similar changes were observed in controls.

Microscopic findings:

Histopathological examination was conducted only on the abnormal spleens detected in the unscheduled deaths in the groups treated with continuous infusion of defibrotide (240, 1200, and 4800 mg/kg/day) and the spleens of controls from the terminal necropsy.

- Extramedullary hemopoiesis was observed in females treated with ≥ 240 mg/kg/day defibrotide with an increased incidence and severity compared to controls.
- The extramedullary hemopoiesis observed in defibrotide-treated animals was characterized by hyperplasia of erythroid and myeloid components, while the extramedullary hemopoiesis observed in controls was represented by a minimal proportion of erythroid, myeloid cell lines, and megakaryocytes in the red pulp.

Cesarean Section Data

- There is limited data from defibrotide-treated females since only one female in each of the 120 and 240 mg/kg/day groups administered defibrotide as a 2-hour infusion 4 times daily had live fetuses on GD 20. While meaningful comparisons cannot be made, the data available is presented below.

Table 59: Uterine examination data in rats

Dose	Mean Control 0 mg/kg/day	Values for the single female Defibrotide 2-hour infusion 4 times/day 120 mg/kg/day	240 mg/kg/day
Number of females	7	1	1
Corpora lutea	12.9	17	19
Implantations	11.7	16	18
Uterine deaths (resorptions)			
Early	0	3	0
Late	0	0	1
Total	0	3	1
Viable fetuses			
Males	6.6	5	7
Females	5.1	8	10
Total	11.7	13	17
Implantation loss (%)			
Pre	9.0	5.9	5.3
Post	0	18.8	5.6
Total	9.0	23.5	10.5

Offspring

- Only one female in each of the 120 and 240 mg/kg/day groups administered defibrotide as a 2-hour infusion 4 times daily had live fetuses on GD 20. While meaningful comparisons cannot be made, the data available for fetal sex ratio and fetal weights are presented below.

Table 60: Fetal sex ratio and weights

Dose	Mean Control 0 mg/kg/day	Values for the single female Defibrotide 2-hour infusion 4 times/day 120 mg/kg/day	240 mg/kg/day
Number of females	7	1	1
Fetal sex ratio (% males)	57.5	38.5	41.2
Litter weight (g)	44.39	46.99	58.95
Mean fetal weight (g)	3.72	3.614	3.467

- External examination of fetuses:
  - One fetus in the 120 mg/kg/day defibrotide group had generalized edema, which is considered a malformation.
  - No abnormalities were detected in the fetuses of the litter of the 240 mg/kg/day defibrotide group.

Study title: Defibrotide preliminary intravenous infusion embryo-fetal development study in rabbits

Study no.:	79840EXT
Study report location:	eCTD 4.2.3.5.2.
Conducting laboratory and location:	(b) (4)
Date of study initiation:	Exact date not provided; animals arrived on December 30, 2009
GLP compliance:	No
QA statement:	No
Drug, lot #, and % purity:	Defibrotide, lot # 2080010002

Key Study Findings

- Intravenous administration of defibrotide as a 2-hour infusion 4 times daily (30, 160, or 120 mg/kg/day) to pregnant rabbits resulted in severe maternal toxicity that lead to the mortality of the majority of the defibrotide-treated females.
- Total resorption was observed in 19 of the 30 (60%) pregnant females treated with defibrotide.
- Only one female in each of the 30 and 60 mg/kg/day groups and 2 females in the 120 mg/kg/day group had live fetuses on GD 29; therefore, meaningful comparisons could not be made for parameters regarding body weight, the cesarean section, or offspring.

Methods

Doses:	0, 30, 60, and 120 mg/kg/day
Frequency of dosing:	Infusion four times daily on GD 6-18
Route of administration:	Intravenous infusion; 2-hours followed by 4-hour treatment free period at approximate rate of 3 mL/kg/hour
Formulation/Vehicle:	Sterile 0.9% sodium chloride for injection
Species/Strain:	New Zealand White rabbit
Number/Sex/Group:	10 females/group
Satellite groups:	None
Study design:	Pregnant female rabbits were dosed with vehicle or 30, 60, or 120 mg/kg/day defibrotide administered as a 2-hour infusion 4 times a day on GD 6-18 and all surviving females were euthanized on GD 29 and examined. The day successful mating was detected was considered GD 0. See table of the study design below.
Parameters and endpoints evaluated:	Females: Mortality, body weight, uterine weights, clinical signs, necropsy, number of

	corpora lutea, number of implantations, early and late resorptions, and live and dead fetuses Fetuses: Fetal weight and fetal exam (external abnormalities)
Deviation from study protocol:	On first day of treatment, Animal # 1, 3, 21, 23, 25, 41, 43, 45, 61, 63, and 65 were accidentally administered at an infusion rate of 1.5 mL/kg/hour instead of 3 mL/kg/hour; therefore, they received half of the assigned dose for the day

Table 61: Study design for preliminary embryo-fetal development study in rabbits

Group	Number of mated females	Dose level		Concentration
		mg/kg/day	mg/kg/hour	mg/mL
1	10	0	0	0
2	10	30	3.75	1.25
3	10	60	7.5	2.5
4	10	120	15	5.0

Results

Mortality

- A total of 24 females were euthanized early or found dead prior to the end of the gestation period; see numbers for each group in the table below.

Table 62: Mortality and fate of females in preliminary embryo-fetal development study in rabbits

Group	Dose (mg/kg/day)	Number of females							
		Mated	Not pregnant	Euthanized early	Found dead	Total resorption	Unilateral implantation	Abortion	With live fetuses on GD 29
1	0	10	0	2	0	0	1	1	8
2	30	10	2	6	1	5	2	0	1
3	60	10	2	6	2	7	1	0	1
4	120	10	2	5	2	6	2	1	2

Clinical Signs

Table 63: Clinical signs in preliminary embryo-fetal development study in rabbits

Clinical signs	Number of animals affected			
Dose (mg/kg/day)	0 Control	30	60	120
Number of animals examined	10	10	10	10
Red staining on cage tray	-	6	6	4
Mucus on cage tray	-	3	2	2
Reduced feces	2	7	10	9
Soft feces	-	1	5	5
Decreased activity	-	5	2	1
Pale	-	2	4	1
Dyspnea	-	1	1	1

- = Clinical sign not observed in this group

Body Weight

- Due to the high mortality observed in the defibrotide-treated groups, available data were not sufficient to compare the groups for body weight and body weight gain.
- There is limited data for gravid uterus weights. While meaningful comparisons cannot be made, the data available is presented below.

Table 64: Uterus weights in preliminary embryo-fetal development study in rabbits

Dose (mg/kg/day)	Mean	Values for the individual females		
	0 (Control)	30	60	120
Number of females	8	1	1	2
Gravid uterus weight (g)	536.20	250.90	411.30	433.90
				235.90

Food Consumption

Not conducted

Toxicokinetics

Not conducted

Necropsy

Macroscopic findings:

- Incidences of the findings observed in the unscheduled deaths and at the terminal necropsy are presented in the table below. Most of the findings were observed in the unscheduled deaths.

Table 65: Macroscopic findings in preliminary embryo-fetal development study in rabbits

Macroscopic findings		No. of animals affected			
Dose (mg/kg/day)		0 (Control)	30	60	120
Number of animals examined		2*/8	7*/3	8*/2	7*/3
Gall bladder	Abnormal size	-	3*/0	2*/0	-
	Abnormal contents	-	1*/0	6*/0	3*/0
Jugular vein, left	Abnormal size	-	1*/0	-	3*/0
Jugular vein, right	Abnormal size	1*/1	5*/0	7*/1	6*/1
	Abnormal consistency	1*/0	1*/0	2*/0	3*/2
Kidneys	Abnormal color	-	1*/0	6*/0	4*/0
Liver	Abnormal color	-	4*/0	6*/0	6*/0
	Abnormal shape	-	-	1*/0	2*/0
	Abnormal consistency	-	3*/0	3*/0	1*/0
Lungs	Abnormal areas	-	1*/0	5*/0	2*/0
	Abnormal color	-	1*/0	3*/0	4*/1
	Incomplete collapse	-	-	3*/0	1*/0
Skin	Staining	-	-	2*/0	2*/0
	Abnormal consistency	-	2*/0	-	2*/0
Spleen	Abnormal shape	-	4*/0	5*/0	4*/1
	Abnormal size	-	3*/0	5*/0	2*/0
Thymus	Abnormal color	-	-	3*/0	1*/0
Uterus	Total resorption	-	3*/0	5*/1	4*/0
	Unilateral total resorption	-	2*/0	1*/0	1*/1
	Abortion	1*/0	-	-	1*/0
	Unilateral implantation	1*/0	-	-	-

Number of animals examined and affected: Unscheduled deaths*/terminal necropsy
- = no test-article related changes

Cesarean Section Data

- There is limited data from defibrotide-treated females since only one female in each of the 30 and 60 mg/kg/day groups and 2 females in the 120 mg/kg/day group administered defibrotide as a 2-hour infusion 4 times daily had live fetuses on GD 29. While meaningful comparisons cannot be made, the data available is presented below.

Table 66: Uterine examination data in rabbits

Dose	Mean	Values for individual females			
	Control	Defibrotide			
	0 mg/kg/day	30 mg/kg/day	60 mg/kg/day	120 mg/kg/day	
Number of females	8	1	1	2	
Corpora lutea	11.9	9	11	9	9
Implantations	10.3	9	7	9	9
Uterine deaths (resorptions)					
Early	0.9	5	0	0	2
Late	0.8	0	0	0	2
Total	1.7	5	0	0	4
Viable fetuses					
Males	4.5	1	7	4	0
Females	4.1	3	0	5	4
Total	8.6	4	7	9	5
Implantation loss (%)					
Pre	13.7	0	36.4	0	0
Post	16.9	55.6	0	0	44.4
Total	30.6	55.6	36.4	0	44.4

Offspring

- Only one female in each of the 30 and 60 mg/kg/day groups and 2 females in the 120 mg/kg/day group administered defibrotide as a 2-hour infusion 4 times daily had live fetuses on GD 29. While meaningful comparisons cannot be made, the data available for fetal sex ratio and fetal weights are presented below.

Table 67: Fetal sex ratio and weights

Dose	Mean	Values for individual females			
	Control	Defibrotide			
	0 mg/kg/day	30 mg/kg/day	60 mg/kg/day	120 mg/kg/day	
Number of females	8	1	1	2	
Fetal sex ratio (% males)	52.1	25	100	44.4	0
Litter weight (g)	359.90	152.60	295.18	302.67	128.84
Mean fetal weight (g)	42.63	38.15	42.17	33.63	25.77

- External examination of fetuses:
  - One fetus out of a total of 4 in the 30 mg/kg/day defibrotide group had a malformation of omphalocele.
  - Small fetus (body weight < 35.0 g) was observed in all groups with an increased incidence in the 30 mg/kg/day (2/4; 50%) and 120 mg/kg/day (8/14; 57%) groups compared to the control group (13/69; 19%).

Study title: Defibrotide embryo-fetal development study in rabbits by intravenous administration

Study no.:	95800
Study report location:	eCTD 4.2.3.5.2.
Conducting laboratory and location:	(b) (4)
Date of study initiation:	June 28, 2013
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Defibrotide, lot # 12G01

Maternal toxicity including mortality was observed in the non-GLP preliminary embryo-fetal development study (Study # 79840EXT) of defibrotide (0, 30, 60, and 120 mg/kg/day; infusion rate of 3 mL/kg/hour) administered by 2-hour intravenous infusion 4 times a day on gestational Days 6 through 18 in pregnant rabbits. Based on the results of that study, a modified design was used for the current study in an attempt to provide a tolerable dosing regimen in rabbits to increase the probability of evaluable litters at the end of the study. The study was conducted to determine the potential effects of defibrotide on embryo-fetal development when administered by the clinical dosing regimen during three different brief critical windows of organogenesis instead of a consecutive 13-day dosing period. Implantation and early embryonic development were covered with dosing from gestational Day (GD) 6 through GD 10, early embryonic development and organ formations were covered with dosing from GD 10 through GD 14, and completion of organ formations to the closure of the hard palate was covered with dosing from GD 14 through GD 18. A dose of 80 mg/kg (1.5-fold lower than the highest dose in the previous study) and a slower infusion rate were used for this study.

Key Study Findings

- A statistically significant lower gravid uterus weight in females treated with defibrotide compared to controls corresponded to an increased incidence of unilateral implantation noted in the defibrotide group (36% vs. 9% in controls).
- Two females treated with defibrotide had only one live fetus present; the data from these animals was excluded from the group mean calculations. In the data analyzed, the mean number of viable fetuses was significantly decreased in the defibrotide group compared to the control group.
- The mean number of implantations was lower and post-implantation loss was increased in the defibrotide group compared to the control group.
- No defibrotide-related teratogenicity was observed in this study.

Methods

Doses:	0 or 80 mg/kg/day (concentration 10 mg/mL)
Frequency of dosing:	Infusion four times daily on GD 6-10, GD 10-14, or GD 14-18
Route of administration:	Intravenous infusion (2-hours) at an infusion rate of 1.0 mL/kg/hour followed by 4-hour treatment free period
Formulation/Vehicle:	0.9% sodium chloride for injection
Species/Strain:	New Zealand White rabbit
Number/Sex/Group:	Planned numbers: 14 females/group for GD 6-10 and GD 10-14 administration subgroups; 10 females/group for GD 14-18 administration subgroups Due to low pregnancy rates the actual number of animals evaluated was lower; see note and table of the study design below.
Satellite groups:	None
Study design:	Pregnant female rabbits were dosed with vehicle or 80 mg/kg/day defibrotide administered as a 2-hour infusion of a concentration of 10 mg/mL at a rate of 1.0 mL/kg/hour 4 times a day on GD 6-10, GD 10-14, or GD 14-18 and were euthanized on GD 29. The day successful mating was detected or the day of artificial insemination was considered GD 0. See table of the study design below.
Parameters and endpoints evaluated:	Females: Body weight, uterine weights, clinical signs, food consumption, necropsy, exposure assessment, number of corpora lutea, number of implantations, early and late intrauterine deaths (resorptions), and live and dead fetuses Fetuses: Fetal weight and fetal exam (external and internal abnormalities)

Note: The first batch of matings (July 7-30, 2013) resulted in a low pregnancy rate in both the control (33%) and defibrotide (25%) groups. The cause of the low pregnancy rate was not determined. A second batch of matings (January 19-27, 2014) was conducted using another batch of animals and artificial insemination; this batch also had a low pregnancy rate (21% in controls and 36% in the defibrotide group). Of the 76 animals mated/inseminated (38 in each of the control and defibrotide groups), only 11 females in the control group and 11 females in the defibrotide group had live fetuses at GD 29.

Table 68: Study design for embryo-fetal development study in rabbits

Group/Subgroup		Treatment (mg/kg/day)	Concentration (mg/mL)	Dosing period (GD)	Number mated	Number with live fetuses on GD 29
1	1	0 (control)	0	6-10	14	4
	2			10-14	14	2
	3			14-18	10	5
2	1	80	10	6-10	14	4
	2			10-14	14	3
	3			14-18	10	4

Results

Mortality

No mortalities were observed in this study.

Clinical Signs

Unremarkable

Body Weight

- There were no defibrotide-related changes in body weight and body weight gain.
- The statistically significant lower gravid uterus weight in females treated with defibrotide compared to controls is likely due to the increased incidence of unilateral implantation noted in the defibrotide group (36% vs. 9% in controls).

Table 69: Terminal body weight, uterus weight, and absolute weight gain in female rabbits in embryo-fetal development study

Parameter	0 mg/kg/day Control	80 mg/kg/day Defibrotide
Body weight at GD 0 (kg)	3.3	3.2
Terminal body weight (kg)	4.07	3.85
Gravid uterus weight (g)	464.27	331.39*
Absolute weight gain[#]	263.91	323.97

[#] Absolute weight gain= Body weight at necropsy minus gravid uterus weight and body weight at GD 0
* Significantly different from control ($p<0.05$)

Food Consumption

Unremarkable

Toxicokinetics (exposure assessment)

Blood samples were collected on the last day of treatment at pre-dose and 0.5, 1, and 2 hours after the start of the infusion and 10, 20, 30 minutes and 1, 1.5, 2, 3, and 4 hours after the end of the infusion. A total of 66 samples from the pregnant females treated with defibrotide were analyzed; no samples from the control group were analyzed.

- All animals presented measureable plasma concentrations of defibrotide demonstrating that all treated rabbits were exposed to defibrotide.

- A higher concentration of defibrotide was observed in plasma collected from the start of the 2 hour infusion with a C_{max} of 46-50 µg/mL in samples collected at 1 hour from the start of the last infusion than in plasma collected after the end of the infusion. See figures below.
- Due to the sparse sampling, no formal toxicokinetics evaluation was performed.

Figure 27: Individual plasma concentrations at the start of the last 2-hour intravenous infusion of defibrotide to pregnant female rabbits
(excerpted from study report for 95800)

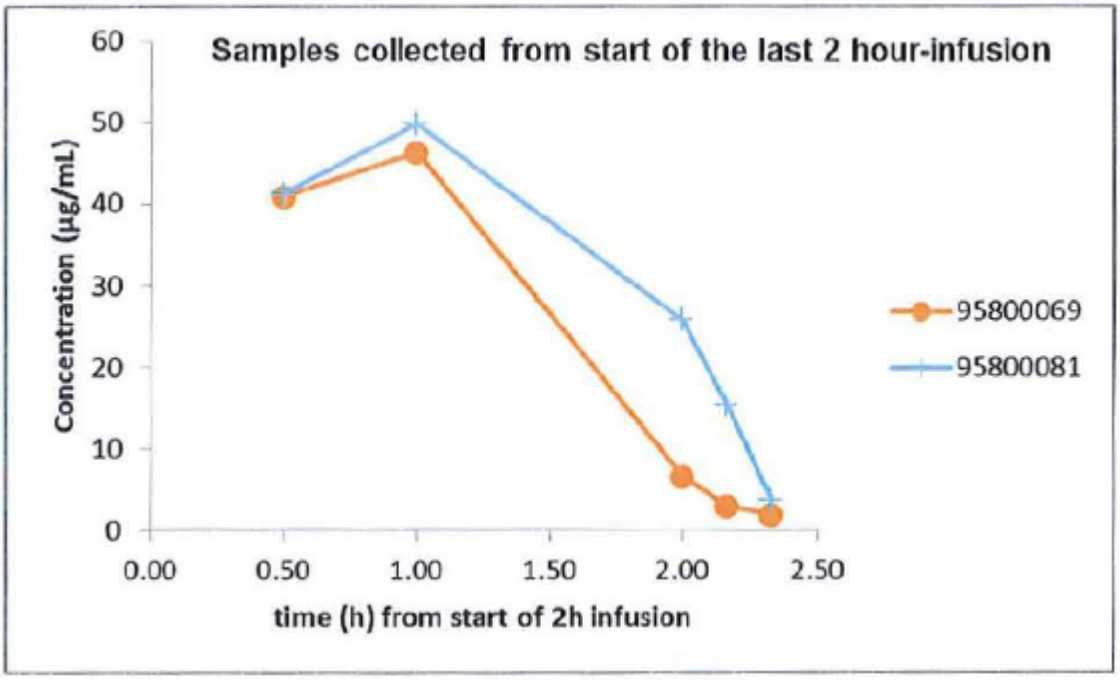
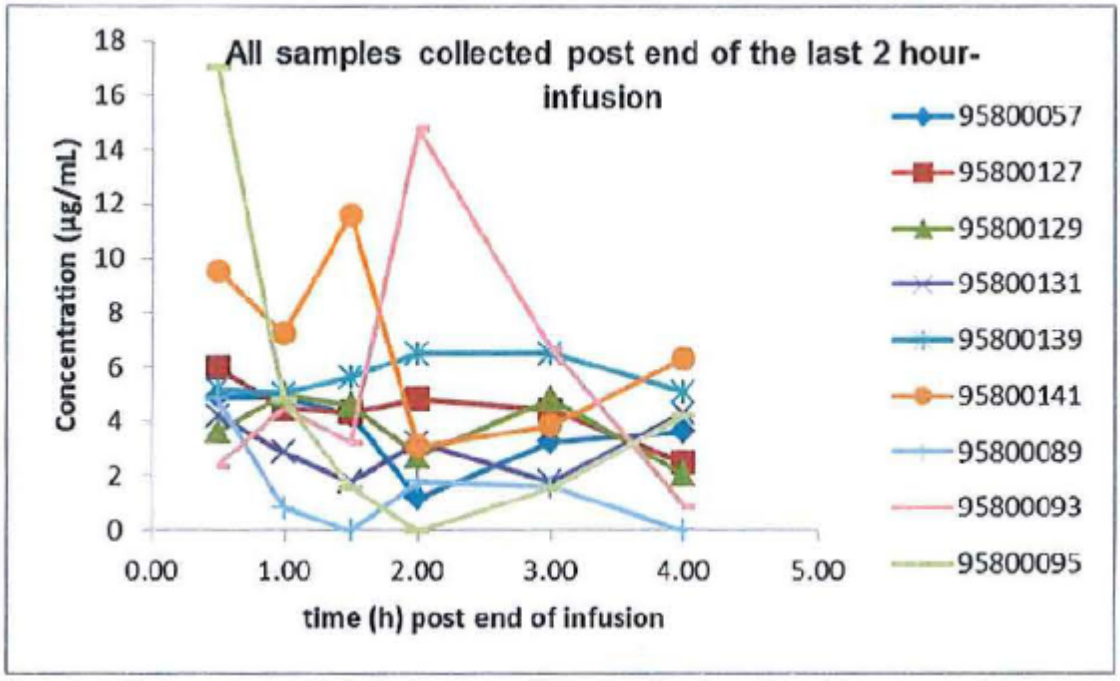


Figure 28: Individual plasma concentrations at the end of the last 2-hour intravenous infusion of defibrotide to pregnant female rabbits
(excerpted from study report for 95800)



Dosing Solution Analysis

Samples of the formulations prepared during the first and last weeks of treatment and analyzed to check the concentration were within the limit of acceptance (95-105%).

Necropsy

Unremarkable

Cesarean Section Data

- The total number of females with live fetuses at GD 29 was 11 in the control group and 11 in the defibrotide group. Of these, one female in the control group and 4 females in the defibrotide group showed unilateral implantation. Another female in the control group had a total resorption of a unilateral implantation.

- Due to the low number of pregnant females in each subgroup, the data is presented as the means for the control group and defibrotide group with subgroups combined. Two females treated with defibrotide had only one live fetus present; therefore, the data from these animals was excluded from the group mean calculations.

- The mean number of implantations was lower, the mean number of viable fetuses was significantly decreased, and post-implantation loss was increased in the defibrotide group compared to the control group.

Table 70: Uterine examination data in rabbits

Dose	0 mg/kg/day (Control)			80 mg/kg/day Defibrotide		
Dosing period	GD 6-10	GD 10-14	GD 14-18	GD 6-10	GD 10-14	GD 14-18
Number of pregnant females	5	2	5	4	3	4
Number of females with unilateral implantation	1	0	0	1	2	1
Number of females with total resorptions	1	0	0	0	0	0
Number of females with viable fetuses	4	2	5	4	3	4
Mean						
Dose	0 mg/kg/day (Control)			80 mg/kg/day Defibrotide		
Number of females	11			9[a]		
Corpora lutea	9.09			7.00		
Implantations	8.00			6.00		
Uterine deaths (resorptions)						
Early	0.09			0.22		
Late	0.18			0.33		
Total	0.27			0.56		
Viable fetuses						
Males	4.00			2.33		
Females	3.73			3.11		
Total	7.73			5.44*		
Implantation loss (%)						
Pre	10.80			15.92		
Post	2.36			10.29		
Total	12.88			25.29		

[a] Two females treated with defibrotide were excluded from the group mean calculations since only 1 fetus was present in the litter

* Significantly different from control ($p<0.05$)

Offspring

All fetuses were weighed and examined externally and internally including the thoracic and abdominal cavities. Head section was performed in fetuses selected for skeletal examination at necropsy and the head from approximately half of the fetuses in each litter was preserved in Bouin's solution for subsequent fixed examination of internal structures of the head (eye, brain, nasal passages and tongue). All fetuses were eviscerated, skinned, and fixed in 95% ethanol for subsequent staining and examination.

- A lower percentage of the fetuses in the defibrotide group were male compared to the control group.
- The incidences of the findings in the fetal examination were comparable between the control and defibrotide groups.

Table 71: Fetal sex ratio and weights

	Mean	
Dose	0 mg/kg/day (Control)	80 mg/kg/day Defibrotide
Fetal sex ratio (mean % males)	50.45	40.94
Mean litter weight (g)	327.7	259.8
Mean fetal weight (g)	44.95	48.26*

* Significantly different from control ($p<0.05$)

9.3 Prenatal and Postnatal Development

Study title: Effect of compound P on peri- and post-natal development of the rat

Study no.:	17/75802
Study report location:	eCTD 4.2.3.5.3.
Conducting laboratory and location:	(b) (4)
Date of study report:	October 24, 1975
GLP compliance:	No
QA statement:	No

This pre- and post-natal development study was a non-GLP study conducted with intramuscular administration of defibrotide; therefore, the study was not fully reviewed. Defibrotide (0, 3, 6, or 12 mg/kg; referred to as compound P) was administered daily by intramuscular injection to pregnant rats starting on GD 15 and continuing throughout lactation until Day 21 post-partum. Defibrotide did not have any adverse effects in the females rats treated with the drug as assessed by daily observation, body weight changes, pregnancy rate, and duration of gestation. Additionally, there were no effects of defibrotide on litter size, cumulative pup mortality, and litter and mean pup weights at birth and 4, 12 and 21 days post-partum and no treatment-related abnormalities were observed.

9.4 Juvenile Animal Development

Study title: Defibrotide 28 days intravenous repeated toxicity study in juvenile rats followed by a 28 days recovery period

Study no.:	82640
Study report location:	eCTD 4.2.3.5.4.
Conducting laboratory and location:	(b) (4)
Date of study initiation:	September 21, 2010
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Defibrotide, lot # 2080010002

Key Study Findings

- Hematology changes included increases in reticulocytes and lymphocytes and decreases in partial thromboplastin time.
- Liver toxicity was observed as indicated by increased liver enzymes (ALT and AST) and microscopic findings of centrilobular hepatocytic vacuolation and extramedullary hematopoiesis.
- The spleen was also an organ of toxicity with increased spleen weight, a macroscopic finding of abnormal shape, and a microscopic finding of germinal centre hyperplasia.

- Preputial separation was significantly delayed in males treated with defibrotide at all doses compared to controls, suggesting a delay in the onset of male puberty.

Methods

Doses:	0, 40, 150, or 320 mg/kg/day
Frequency of dosing:	Once daily for 28 days; 28-day recovery period
Route of administration:	Intravenous injection into the tail vein
Dose volume:	4 mL/kg
Formulation/Vehicle:	Sterile 0.9% sodium chloride for injection was used to dilute defibrotide to final concentrations and as the control.
Species/Strain:	Sprague-Dawley rat
Number/Sex/Group:	Main study: 10/sex/group Recovery: 5/sex/group for control and 320 mg/kg/day groups only
Satellite groups:	None
Study design:	Juvenile rats were administered vehicle or defibrotide (40, 150, or 320 mg/kg/day) by intravenous injection once daily for 4 weeks starting on Day 21 post-partum to at least Day 48 post-partum. Main study animals (10/sex/group) were dosed until the day before necropsy. Recovery animals (5/sex/group) in the control and 320 mg/kg/day groups were dosed for the 4-week period and received no treatment during the 4-week recovery period.
Parameters and endpoints evaluated:	Clinical signs, body weight, food consumption, post-weaning development (vaginal opening, testis descent and preputial separation, motor activity, water-filled Y-maze, and accelerating rota-rod), toxicokinetics, and clinical pathology were evaluated. Additionally, selected organs were collected, weighed, and examined for macroscopic and microscopic evaluation.

Observations and times:

Mortality:	Twice daily
Clinical signs:	At least once daily
Body weights:	On day of allocation to treatment group, on first day of treatment, twice weekly during study, and prior to necropsy
Food consumption:	Weekly starting on first day of treatment; weight of food consumed per cage of rats was recorded and group mean daily intake per rat was calculated
Vaginal opening:	Monitored from 28 days of age until 100% occurrence
Testis descent and	Testis descent and scrotal development were checked

preputial separation:	at Day 35 of age The cleavage of the balanopreputial gland of males was checked from Day 35 of age until 100% occurrence
Motor activity:	Once during Week 3 of treatment (from Day 16 to Day 20); the general activity of the rat within each cage was monitored electronically for 5 minutes using an automated activity recording
Water-filled Y-maze:	Once during Week 3 of treatment (from Day 15 to Day 16) and during the first week of recovery to assess learning ability; the time taken by each rat to swim through the maze in four successive trials was measured; a maximum of 60 seconds was allowed for each trial and any rat exceeding this time was removed and considered to have failed the test; maintained improvement in swimming time was considered an indication of learning
Accelerating rota-rod:	Once during Week 3 of treatment to assess motor coordination; the time each rat remained on the rota-rod in each of the three successive trials was recorded
Hematology:	At necropsy
Clinical chemistry:	At necropsy
Coagulation:	At necropsy
Gross pathology:	At necropsy
Organ weights:	At necropsy
Histopathology:	At necropsy
Toxicokinetics:	Day 28 of treatment at 3, 15, and 30 minutes and 1 and 4 hours after the end of injection; 6/sex/group at each time point with each rat sampled at a maximum of 3 alternating time points

Results

Mortality

No mortality occurred in this study.

Clinical Signs

Unremarkable

Body Weights

Unremarkable

Food Consumption

Unremarkable

Hematology

Table 72: Hematology changes in juvenile rat study

Index	Mean		Percentage deviation from Control					
	Control 0 mg/kg/day		40 mg/kg/day		150 mg/kg/day		320 mg/kg/day	
	Males	Females	Males	Females	Males	Females	Males	Females
Reticulocytes (10^9/L)								
Treatment period	336.50	285.60	↑23*	↑38	↑28*	↑47	↑36**	↑50
Recovery period	145.42	144.38	NE	NE	NE	NE	-	-
Lymphocytes (10^3/µL)								
Treatment period	6.966	6.802	↑12	-	↑18	-	↑33*	-
Recovery period	9.358	6.096	NE	NE	NE	NE	↓33**	-
PTT (sec)								
Treatment period	19.48	21.83	-	-	↓13*	-	↓13*	-
Recovery period	20.90	16.42	NE	NE	NE	NE	↓7*	-

↑= increase ↓= decrease - = no test-article related changes
* P≤0.05 ** P≤0.01
PTT= Partial thromboplastin time
NE= Not evaluated, no recovery animals in this group

Clinical Chemistry

- Liver enzyme (ALT and AST) levels were severely increased (17-fold and 15-fold, respectively) in one male treated with 320 mg/kg/day (Animal #82640084) and slightly increased (26-43% for ALT and 34-70% for AST) in three other males treated with 320 mg/kg/day (Animal #8640078, 82640082, and 82640086) compared to controls following the treatment period. The individual ALT and AST values for these animals along with the group means for the male control and 320 mg/kg/day groups are presented in the table below. The remaining males and the females in the 320 mg/kg/day group had values similar to the control group means. No changes in AST and ALT were observed following the recovery period.

Table 73: ALT and AST changes in males in juvenile rat study

Group	Mean or Individual rat	ALT (U/L)	AST (U/L)
Control (0 mg/kg/day)	Mean	36.46	89.79
320 mg/kg/day	Mean	93.97	220.24
	82640084	618.1	1318.4
	82640078	49.9	120.7
	82640082	46.0	134.8
	82640086	52.1	152.9

- Urea levels were slightly increased during the treatment period and significantly increased following the recovery period in both males and females treated with 320 mg/kg/day.

Table 74: Urea changes in juvenile rat study

Index	Mean		Percentage deviation from Control					
	Control 0 mg/kg/day		40 mg/kg/day		150 mg/kg/day		320 mg/kg/day	
	Males	Females	Males	Females	Males	Females	Males	Females
Urea (mg/dL)								
Treatment period	39.32	45.94	-	-	-	↑12	↑13	↑18
Recovery period	41.38	46.12	NE	NE	NE	NE	↑24**	↑25**

↑= increase - = no test-article related changes
** P≤0.01
NE= Not evaluated, no recovery animals in this group

Gross Pathology

Table 75: Macroscopic findings in juvenile rat study

Treatment-Related Macroscopic Findings		No. of animals affected							
		Males				**Females**			
Dose (mg/kg/day)		0	40	150	320	0	40	150	320
Number of animals examined		10/**5**	10/**NE**	10/**NE**	10/**5**	10/**5**	10/**NE**	10/**NE**	10/**5**
Organ	Finding								
Eyes	Abnormal areas	-	-	-	**-**	**-**	**-**	-	2/**0**
Spleen	Abnormal shape	0/**1**	3/**NE**	6/**NE**	7/**1**	1/**0**	5/**NE**	4/**NE**	6/**0**
	Abnormal size	-	-	-	1/**0**	-	-	-	**-**

Number of animals examined and affected: Terminal necropsy / **Recovery necropsy**
- = no test-article related changes
NE= Not evaluated, no recovery animals in this group

Organ Weights

Table 76: Organ weight changes in juvenile rat study

Group and Dose		Mean		Percentage deviation from Control					
		Control 0 mg/kg/day		40 mg/kg/day		150 mg/kg/day		320 mg/kg/day	
Sex		Males	Females	Males	Females	Males	Females	Males	Females
Number of animals examined		10/**5**	10/**5**	10/**NE**	10/**NE**	10/**NE**	10/**NE**	10/**5**	10/**5**
Spleen	Absolute (g)								
	Treatment necropsy	0.6558	0.5550	↑15	↑9	↑23*	↑16	↑38**	↑24*
	Recovery necropsy	0.8206	0.5908	NE	NE	NE	NE	↑12	↑7
	Relative (BW, %)								
	Treatment necropsy	0.3037	0.3362	↑16	↑12	↑26**	↑20*	↑41**	↑31*
	Recovery necropsy	0.2323	0.2874	NE	NE	NE	NE	↑15	↑9

BW= Body weight NE= Not evaluated, no recovery animals in this group
Number of animals examined: Terminal necropsy / **Recovery necropsy**
↑= increase - = no test-article related changes
* P≤0.05 ** P≤0.01

Histopathology

Microscopic examination was performed on all specified tissues from all animals in the control and 320 mg/kg/day groups and all abnormalities observed in all groups at the main study necropsy. Additionally, the spleen and liver (males only) were examined in all animals in the 40 and 150 mg/kg/day groups from the main study necropsy and all animals from the recovery necropsy.

Adequate Battery: Yes

Peer Review: No

Histological Findings

Table 77: Microscopic findings in juvenile rat study

Treatment-Related Microscopic Findings			No. of animals affected							
			Males				Females			
Dose (mg/kg/day)			0	40	150	320	0	40	150	320
Number of animals examined			10/**5**	10/**NE**	10/**NE**	10/**5**	10/**5**	10/**NE**	10/**NE**	10/**5**
Organ	Finding									
Eyes	Phthisis bulbi	Moderate	-	NE	NE	**-**	**-**	NE	NE	1/**0**
Liver	Centrilobular hepatocytic vacuola ion	Mild	-	-	-	2/**0**	-	NE	NE	-
	Extramedullary hematopoiesis	Minimal	2/**0**	4/**NE**	4/**NE**	6/**0**	**-**	NE	NE	3/**0**
Spleen	Germinal centre hyperplasia	Total	2/**4**	10/**NE**	10/**NE**	10/**5**	2/**5**	9/**NE**	9/**NE**	10/**4**
		Minimal	2/**3**	-	-	0/**3**	2/**2**	-	-	0/**1**
		Mild	0/**1**	6/**NE**	1/**NE**	2/**2**	0/**3**	4/**NE**	2/**NE**	2/**3**
		Moderate	-	4/**NE**	9/**NE**	8/**0**	-	5/**NE**	7/**NE**	8/**0**

Number of animals examined and affected: Terminal necropsy / **Recovery necropsy**
- = no test-article related changes
NA= Not applicable, organ not present in sex
NE= Not evaluated, tissue not examined in this group or no recovery animals in this group

Special Evaluation

Post-weaning development

Sexual maturation

- Vaginal opening: There was no difference in the mean day of vaginal opening between the control and defibrotide-treated females.
- Testis descent and preputial separation:
  - All males showed testis descent.
  - Preputial separation was significantly delayed (by ~3 days) in males treated with defibrotide at all doses compared to controls. In one male treated with 150 mg/kg/day (Animal # 82640060), the preputial separation was not detected until the day of the necropsy (Day 50 of age).

Table 78: Preputial separation in male rats

	Mean (% deviation from control)			
Dose (mg/kg/day)	0 (Control)	40	150	320
Days post-partum of preputial separation	39.00	42.50* (↓9)	41.67* (↓7)	42.20* (↓8)

* Significantly different from control ($p<0.05$)
↓=decrease

Neurobehavioral testing

There were no effects of defibrotide in the neurobehavioral tests.

- Motor activity: There were no significant differences in general motor activity between the control and defibrotide-treated groups.
- Water-filled Y-maze test: All groups demonstrated learning in the Y-maze test, and the mean time required for a successful trial was comparable between groups during the treatment and recovery periods.
- Accelerating rota-rod: There were no significant differences in rota-rod performance between the control and defibrotide-treated groups.

Toxicokinetics

The toxicokinetics of defibrotide (40, 150, and 320 mg/kg/day) were evaluated in the juvenile rats on Day 28 of treatment with samples collected at 3, 15, and 30 minutes and 1 and 4 hours after the end of injection.

- The plasma samples from the control group did not contain detectable concentrations of defibrotide.
- C_{max} and $AUC_{(0\text{-tlast})}$ values increased with increased dose; in general, increases were approximately dose proportional or slightly greater than dose proportional from 40 to 150 mg/kg/day and 150 to 320 mg/kg/day.
- The highest concentration of defibrotide in plasma occurred at the first sampling after dosing (T_{max}=3 minutes; 0.05 hours).
- Exposures to defibrotide were similar in males and females.
- The half-life ($t_{1/2}$) is less than one hour, indicating that the drug is rapidly eliminated from the body.

Table 79: Toxicokinetics of defibrotide in juvenile rat study
(excerpted from study report for 82640)

Males

Dose (mg/kg/day)	Group Sex	Day	C_{max} (µg/mL)	T_{max} (h)	$AUC_{0\text{-tlast}}$ (µg*h/mL)	$AUC_{0\text{-}\infty}$ (µg*h/mL)	$AUC_{0\text{-tlast}}$/dose	CL (L/h/kg)	V (L/kg)	t ½ (h)
40	2 M	28	488.8	0.05	143.47	150.81	3.59	0.27	0.31	0.82
150	3 M	28	1373.0	0.05	620.17	625.06	4.13	0.24	0.19	0.55
320	4 M	28	3666.0	0.05	1848.61	1857.48	5.78	0.17	0.13	0.54

Females

Dose (mg/kg/day)	Group Sex	Day	C_{max} (µg/mL)	T_{max} (h)	$AUC_{0\text{-tlast}}$ (µg*h/mL)	$AUC_{0\text{-}\infty}$ (µg*h/mL)	$AUC_{0\text{-tlast}}$/dose	CL (L/h/kg)	V (L/kg)	t ½ (h)
40	2 F	28	468.7	0.05	117.89	126.99	2.95	0.31	0.42	0.92
150	3 F	28	1655.0	0.05	556.13	562.23	3.71	0.27	0.22	0.58
320	4 F	28	3812.0	0.05	1550.11	1562.0	4.84	0.20	0.18	0.59

10 Special Toxicology Studies

No special toxicology studies were reviewed.

11 Integrated Summary and Safety Evaluation

Pharmacology

While the mechanism of action for defibrotide in the treatment of veno-occlusive disease is not fully understood, nonclinical pharmacology studies published in the literature or submitted in the NDA suggest that defibrotide protects endothelial cells by protecting cells from further damage including apoptosis and by producing fibrinolytic effects.

Pharmacology studies were conducted primarily with the human microvascular endothelial cell line CDC/EU.HMEC-1 (HMEC). The effects of defibrotide on endothelial cell damage induced by various types of stress were investigated in one study. Defibrotide protected HMEC cells from density stress and both reversed and prevented endothelial cell damage induced by serum free medium; however, defibrotide was not able to protect endothelial cells from oxidative stress from H_2O_2. Treatment with defibrotide significantly decreased caspase-3 gene expression in endothelial cells, suggesting that one way defibrotide may protect endothelial cells is through interfering with the apoptotic signaling pathway involving active caspase-3.

The effects of defibrotide on fludarabine-induced apoptosis and cellular changes in endothelial cells were investigated in three of the studies submitted using a variety of assays. Defibrotide was shown to decrease apoptosis induced by fludarabine in experiments with microscopic counting of DAPI-stained cells and in an apoptosis assay using flow cytometry. Cytometric bead arrays demonstrated that treatment of defibrotide with fludarabine protected cells from the fludarabine-induced increases in caspase-3 that lead to cell death. Defibrotide did not modulate Bcl-2. In microarray gene expression analysis in HMEC cells activated by fludarabine after 24 hours of incubation, the presence of defibrotide downregulated several genes induced by fludarabine, particularly those associated with apoptosis (PDCD6, FAS, APAF-1, MOAP-1, caspase-3, caspase-9, and BAD), angiogenesis/migration (heparanase, FGFR3, CCL5, MCAM (CD146), PLAU, VEGF, and IL-8) and inflammatory activation/innate immunity (IL-1β, IL-32, IL-11, TRL1, TRL3, ICAM1, and MHC Class II). Real time PCR revealed that fludarabine alone increased the gene expression of heparanase and IL-8 in HMEC cells and treatment of defibrotide with fludarabine decreased the gene expression of both heparanase and IL-8 compared to fludarabine alone. Defibrotide also significantly decreased the release of IL-8 by fludarabine observed in an ELISA assay. Finally, treatment of defibrotide with fludarabine reduced the rates of CD146-positive HMECs and the number of cells expressing MHC Class II molecules, which were increased in cells treated with fludarabine alone. Collectively, these data suggest that defibrotide protects endothelial cells from fludarabine-induced cytotoxicity through inhibition of factors of apoptosis, inflammation, and cell-adhesion processes, and that these inhibitory activities may be involved in the mechanism of the activity of defibrotide in reducing or preventing chemotherapeutic-related veno-occlusive disease.

In one study, degradation serum products of defibrotide were tested for their ability to decrease fludarabine-induced apoptosis in endothelial cells. Results indicate that the degradation products of defibrotide did not decrease fludarabine-induced apoptosis. This suggests that the loss of the defibrotide polymer structure results in the loss of the pharmacologic activity.

Based on literature and previous nonclinical studies demonstrating that defibrotide has pro-fibrinolytic and anti-thrombotic activities, one study investigated the effects of defibrotide on the thrombotic effects of thalidomide in HMEC cells. In ELISA assays, thalidomide significantly reduced the levels of tissue plasminogen activator (t-PA) antigen in HMEC cells supernatant following 24 hours of incubation and treatment of

defibrotide with the thalidomide significantly increased the levels of t-PA antigen compared to thalidomide alone. In experiments with real time PCR, thalidomide reduced the t-PA gene expression by 2.2 fold and increased the plasminogen activator inhibitor-1 (PAI-1) expression by 4 fold compared to control HMEC cells. Treatment of defibrotide with thalidomide reversed these effects by up-regulating the expression of t-PA and down-regulating the gene expression of PAI-1 compared to thalidomide alone. To assess the ability of defibrotide to modulate fibrinolytic activity to degrade a clot, a fibrin clot plate assay was conducted with thalidomide with and without defibrotide present. Thalidomide reduced the fibrinolytic activity of HMEC cells, and treatment of defibrotide with thalidomide significantly increased in a concentration-dependent manner the fibrinolytic ability of HMEC to degrade the fibrin clot compared to thalidomide alone. Defibrotide also demonstrated protective effects against the cytotoxic effect of thalidomide in MTT experiments and decreased thalidomide-induced apoptosis in HMEC cells. Results from this study indicate that defibrotide reduces the pro-thrombotic effects of thalidomide in endothelial cells by producing changes in levels and gene expression of the fibrinolytic proteins t-PA and PAI-1 and increasing the ability of endothelial cells to degrade clots.

General toxicology

General toxicology studies conducted to support the development of defibrotide include 13-week studies GLP studies with intravenous infusion of defibrotide in rats and dogs. In the 13-week study in rats, Sprague-Dawley rats were administered defibrotide (240, 1200, or 4800 mg/kg/day; 1440, 7200, or 28,800 mg/m^2/day) or vehicle as a continuous intravenous infusion at a rate of 2.5 mL/kg/hour for a planned duration of 13 consecutive weeks. Due to extensive mortality and dosing complications with the intravenous catheters, the study was terminated early following 9 weeks of treatment with defibrotide. A total of 89 rats were either found dead or euthanized *in extremis*. While the number of mortalities observed is not dose-dependent, proportionally there was more mortality in each of the defibrotide-treated groups than in the control group. The number of mortalities in the defibrotide-treated groups along with the information provided on the clinical signs, clinical pathology, and pathology findings suggests that the higher rates of mortality observed were related to the administration of defibrotide. The nature of the relationship is unclear. The exact cause of death or reason for euthanasia was not apparent and deemed undetermined in some cases. The most common cause of death was heart failure (usually associated with vegetative valvular endocarditis), septicemia, and kidney inflammation/necrosis. Other causes of death included disseminated intravascular coagulation, heart inflammation/necrosis, pulmonary thrombosis, pulmonary hemorrhage, and complications related to surgical procedures to repair and reposition of the catheter. In general, clinical signs were dose-dependent and included decreased activity, prostration, impaired limb function, red material around eyes, mouth, and nose, hunched posture, swelling, cold to touch, skin discoloration, unkempt appearance, and rapid breathing. Body weight gain and food consumption were decreased in defibrotide-treated groups compared to controls with the largest decreases of 37% observed in males at 1200 mg/kg/day (7200 mg/m^2/day). Hematology changes included increases in neutrophils, lymphocytes, and monocytes

indicating an inflammatory response and decreased red blood cell parameters indicating anemia. Decreased coagulation was observed with increases in activated partial thromboplastin time at 1200 and 4800 mg/kg/day (7200 and 28,800 mg/m^2/day) and prothrombin time at 4800 mg/kg/day (28,000 mg/m^2/day). Clinical chemistry changes included increases in total bilirubin, AST, ALT, urea nitrogen, creatinine, globulins, amylase, and lipase and decreases in albumin. These changes are compatible with renal failure and hepatic injury.

Due to the high mortality rate in the rat study and the complexity of the macroscopic and microscopic findings, it is difficult to interpret which findings were defibrotide-related and which were not. Many findings were observed in the infusion sites (vena cava or jugular vein). The most common finding was minimal to severe chronic-active inflammation which was frequently observed with thrombus formation, abscesses, bacterial colonies, mineralization, and hemorrhage. The infusion site-related inflammation and bacterial infection resulted in the complications of septicemia, urinary tract infections, vegetative valvular endocarditis, and disseminated intravascular coagulation and there were many microscopic findings related to these conditions. These findings were observed more frequently or only in defibrotide-treated animals; however, there was often not a clear dose response for the findings. Defibrotide-related findings that were dose-dependent were identified in the kidneys (basophilic granules in tubular cells, tubular degeneration/regeneration, glomerulonephropathy, and lymphohistiocytic infiltration), liver (basophilic granules and hypertrophy/hyperplasia in kupffer cells), and the mandibular and mesenteric lymph nodes and spleen (vacuolated macrophages). The functional observation battery conducted in this study demonstrated decreases in rearing and general arousal in females treated at 1200 mg/kg/day (7200 mg/m^2/day) and decreases in both hindlimb grip strength and hindlimb splay at 4800 mg/kg/day (28,000 mg/m^2/day) defibrotide.

In the 13-week study in dogs, defibrotide (60, 300, or 1600 mg/kg/day; 1200, 6000, or 32,000 mg/m^2/day; 15, 75, or 400 mg/kg/dose; 300, 1500, or 8000 mg/m^2/dose) or vehicle was administered as a 2-hour intravenous infusion at a rate of 2.5 mL/kg/hour four times daily for 13 consecutive weeks. Unlike the rat study, there was no defibrotide-related mortality observed in this study. Body weights were lower in males treated with 1600 mg/kg/day (32,000 mg/m^2/day) compared to controls. Many of the findings in the study could not be not clearly attributed to defibrotide due to a lack of dose-dependent relationship or consistency including changes in hematology. Red blood cells, hemoglobin, and hematocrit were decreased in females treated with 60 mg/kg/day (1200 mg/m^2/day) and in males treated with 300 mg/kg/day (6000 mg/m^2/day) at Week 5 and prior to necropsy compared to controls. These changes were not observed at the high dose of 1600 mg/kg/day (32,000 mg/m^2/day), but are consistent with decreases in red blood cell parameters observed in the rat study. As in the rat study, decreased coagulation was observed in dogs with increases in activated partial thromboplastin time at 300 and 1600 mg/kg/day (6000 and 32,000 mg/m^2/day) at Week 5 compared to controls. Toxicities associated with trauma and irritation of infusions sites were observed in both control and defibrotide-treated groups. The liver was the main organ of toxicity as indicated by increased liver weights and microscopic

findings of basophilic granules and hypertrophy in kupffer cells, acute inflammation, and necrosis of individual hepatocytes.

Genetic Toxicology

A standard battery of genetic toxicology studies was conducted to assess the potential for defibrotide to induce genotoxicity. In an in vitro bacterial reverse mutation (Ames) assay, defibrotide was negative for mutagenicity in the tester strains of *S. typhimurium* or *E.coli* in the presence and absence of S-9 mix up to 5000 µg/plate. An in vitro chromosome aberrations assay with defibrotide was conducted in Chinese hamster ovary cells. Defibrotide did not induce structural or numerical aberrations in Chinese hamster ovary cells with and without metabolic activation under the conditions tested. For in vivo assessment of clastogenicity, an in vivo micronucleus assay was conducted in bone marrow from male rats administered intravenous of defibrotide (0, 400, 800, or 1600 mg/kg; 2400, 4800, or 9600 mg/m^2) once daily for two days. Defibrotide did not produce an increase in the percent mean number of micronucleated polychromatic erythrocytes compared to the vehicle control group.

Carcinogenicity

Dietary carcinogenicity studies of defibrotide were conducted in CD-1 mice of Swiss origin (Crl:CD-1 (ICR)BR) and Sprague Dawley rats. In both studies, defibrotide (100, 400, or 2000 mg/kg/day; 300, 1200, or 6000 mg/m^2/day in mice and 600, 2400, or 12,000 mg/m^2/day in rats) was administered orally as an admixture with the diet once daily and two separate control groups received the normal untreated diet. Defibrotide was administered for at least 650 days in males and 730 days in females in mice and at least 732 days in rats. The mean achieved intake of defibrotide calculated using individual body weight gain and food consumption data was generally similar to the target dose based on the means; however, the ranges of the mean achieved dosages for each group indicate that the dosages did vary throughout the treatment period. To evaluate the absorption of defibrotide after dietary administration to mice and rats, separate sets of animals were treated with diet with the same doses of defibrotide and control diet as the main study and blood samples were analyzed for t-PA. Results showed that oral administration of defibrotide to mice and rats in the diet induced increases in plasma t-PA levels with the largest increases observed at 100 and 400 mg/kg/day, particularly in mice.

The survival of both mice and rats treated with defibrotide was similar to controls. In mice, the incidence of mortality was higher for males than females and lead to the earlier termination of treatment in males. No treatment-related differences in the number of animals with palpable masses, the total number of masses, or the mean day of onset of masses were observed between the control and defibrotide groups in either study. An increased number of convulsions/tremors were observed in male and female rats treated with 2000 mg/kg/day (12,000 mg/m^2/day). Body weight and body weight gain were slightly lower in male mice treated with 400 and 2000 mg/kg/day (1200 and 6000 mg/m^2/day) defibrotide, while body weights and body weight gain were

consistently and significantly lower in male rats treated with 2000 mg/kg/day (12,000 mg/m^2/day) defibrotide than controls throughout the treatment period. Body weights were also lower than controls but not statistically significant in male and female rats treated with 400 mg/kg/day (2400 mg/m^2/day) and female rats treated with 2000 mg/kg/day (12,000 mg/m^2/day) defibrotide. Hematology changes observed with the administration of 2000 mg/kg/day defibrotide included significant increases in prothrombin time in both male and female rats and significant increases red blood cell count, hematocrit, hemoglobin, and mean corpuscular hemoglobin in male mice. In mice, non-neoplastic findings included distension in the gall bladder and urinary bladder, cortical cyst, mineralization, and pyelonephritis in the kidneys, acute inflammation in the seminal vesicles, and granulopoiesis in the spleen. The non-neoplastic findings in rats included hemorrhage in the brain and spinal cord, tubular dilatation/protein casts, mineralization, and chronic interstitial nephritis in the kidneys, vascular mineralization in the lungs, colloid depletion in the seminal vesicles, and atrophy in the testes. While the Applicant considers there to be no defibrotide-related neoplastic findings, the incidences of B-astrocytoma in the brain and B-lipoma in the skin were increased in male rats treated with 2000 mg/kg/day (12,000 mg/m^2/day) defibrotide and the incidences of N-metastatic tumor in the brain were increased in female mice treated with 2000 mg/kg/day (6000 mg/m^2/day) defibrotide.

It is noted that for both dietary carcinogenicity studies, histopathology examination was conducted for all animals in the control groups and 2000 mg/kg/day defibrotide group and for only the animals sacrificed or found dead during the treatment period for the 100 and 400 mg/kg/day defibrotide groups. Since body weight was lower in the high-dose group (2000 mg/kg/day) than the controls, histopathology should have been examined for the low-dose (100 mg/kg/day) and mid-dose (400 mg/kg/day) groups at the terminal necropsy. (b) (4) Carcinogenicity studies are not required for the proposed duration of administration of defibrotide.

Reproductive and Developmental Toxicology

The embryo-fetal development effects of defibrotide were studied in the rat and rabbit. Preliminary studies with intravenous infusion of defibrotide in pregnant females were conducted in both species. In the rat study, defibrotide (0, 240, 1200, or 4800 mg/kg/day; 0, 1440, 7200, or 28,000 mg/m^2/day) or vehicle was initially administered as a continuous infusion to pregnant Sprague-Dawley rats on GD 6-15. Due to excessive maternal toxicity, the defibrotide-treated females were euthanized early and additional groups of pregnant female rats were administered defibrotide (60, 120, or 240 mg/kg/day; 360, 720, or 1440 mg/m^2/day) as a 2-hour infusion 4 times daily on GD 6-15. Surviving females were euthanized on GD 20. Severe maternal toxicity also lead to early euthanization of the majority of the defibrotide-treated females in these groups. The majority of the early death females showed a red discharge on the urogenital region, which was related to imminent abortion. Other clinical signs included tachycardia observed in females treated with a continuous infusion of defibrotide and

scabs or hairloss on the dorsal region or neck. Macroscopic findings for the early deaths included swollen and enlarged spleens and dark/red fluid in the uterus or in the abdominal cavity in all defibrotide-treated groups and thickened caudal vena cava in groups administered defibrotide as a 2-hour infusion 4 times daily. Only one female in each of the 120 and 240 mg/kg/day (720 and 1440 mg/m^2/day) groups administered defibrotide as a 2-hour infusion 4 times daily had live fetuses on GD 20; therefore, meaningful comparisons could not be made for parameters regarding body weight, the cesarean section, or offspring. Of note, since resorptions were observed in the two females with live fetuses and were not observed in control, defibrotide appears to have increased resorptions.

In the preliminary rabbit study, New Zealand White rabbits were administered defibrotide (30, 60, or 120 mg/kg/day; 360, 720, or 1440 mg/m^2/day) as a 2-hour infusion 4 times daily (infusion rate of 3 mL/kg/hour) on GD 6-18 and all surviving females were euthanized on GD 29. Intravenous administration of defibrotide as a 2-hour infusion 4 times daily pregnant rabbits resulted in severe maternal toxicity that lead to the mortality of the majority of the defibrotide-treated females. Clinical signs in defibrotide-treated females included red staining or mucus on cage tray, reduced feces, soft feces, decreased activity, pale, and dyspnea. Macroscopic findings were observed in the gall bladder, jugular vein, kidneys, liver, lungs, skin, spleen, and thymus. Total resorption was observed in 19 of the 30 (60%) pregnant females treated with defibrotide. Only one female in each of the 30 and 60 mg/kg/day (360 and 720 mg/m^2/day) groups and 2 females in the 120 mg/kg/day (1440 mg/m^2/day) group had live fetuses on GD 29; therefore, meaningful comparisons could not be made for parameters regarding body weight, the cesarean section, or offspring.

Based on the mortality observed in the preliminary study in rabbits, a modified design was used for the GLP embryo-fetal development study in rabbits in order to increase the probability of evaluable litters at the end of the study. A dose of 80 mg/kg/day (960 mg/m^2/day) defibrotide (1.5-fold lower than the highest dose in the previous study) or vehicle was administered to pregnant females by a 2-hour infusion 4 times daily at a slower infusion rate of 1 mL/kg/hour during three different brief critical windows of organogenesis (GD 6-10, GD 10-14, or GD 14-18) instead of a consecutive 13-day dosing period. Pregnant females were euthanized on GD 29. There was a low pregnancy rate in this study and of the 76 females mated/inseminated (38 in each of the control and defibrotide groups), only 11 females in the control group and 11 females in the defibrotide group had live fetuses at GD 29. There were no defibrotide-related changes in body weight and body weight gain. A statistically significant lower gravid uterus weight in females treated with defibrotide compared to controls corresponded to an increased incidence of unilateral implantation noted in the defibrotide group (36% vs. 9% in controls). Two females treated with defibrotide had only one live fetus present and the data from these animals was excluded from the group mean calculations. In the data analyzed, the mean number of viable fetuses was significantly decreased in the defibrotide group compared to the control group. Additionally, the mean number of implantations was lower and post-implantation loss was increased in the defibrotide

group compared to the control group. No defibrotide-related teratogenicity was observed in this study.

To evaluate the effects of defibrotide on the development of rats, a 28-day juvenile toxicology study with a 28-day recovery period was conducted in Sprague-Dawley rats. Defibrotide (0, 40, 150, or 320 mg/kg/day; 0, 240, 900, or 1920 mg/m^2/day) was administered by intravenous injection once daily for 28 days starting on Day 21 post-partum to at least Day 48 post-partum. No mortality was observed in the study and defibrotide had no effect on body weight or food consumption. The toxicities observed in this study were similar to the toxicities observed in the general toxicology studies. Hematology changes included significant increases in reticulocytes at all doses and a significant increase in lymphocytes in males treated at 320 mg/kg/day (1920 mg/m^2/day). Partial thromboplastin time was decreased in males at 150 and 320 mg/kg/day (900 and 1920 mg/m^2/day). Liver toxicity was observed as indicated by increased liver enzymes (ALT and AST) in individual males at 320 mg/kg/day (1920 mg/m^2/day) and microscopic findings of centrilobular hepatocytic vacuolation and extramedullary hematopoiesis. The spleen was also an organ of toxicity at all doses with increased spleen weight, a macroscopic finding of abnormal shape, and a microscopic finding of increased incidence and severity of germinal centre hyperplasia. In general, these toxicities were resolved or were resolving following the 28-day recovery period. Sexual maturation was evaluated in the study and there was no difference in the mean day of vaginal opening between the control and defibrotide-treated females. While all males showed testis decent, preputial separation was significantly delayed by approximately 3 days in males treated with defibrotide at all doses compared to controls, suggesting a delay in the onset of male puberty. Neurobehavioral tests including motor activity to test for general motor activity, accelerating rota-rod to assess motor coordination, and water-filled Y-maze to assess learning ability were conducted and there were no effects of defibrotide in any of the tests.

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