CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER:

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NON-CLINICAL REVIEW(S)

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:	209570
Supporting document/s:	SD #1
Applicant's letter date:	12/29/2016
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Product:	TRADENAME (benznidazole)
Indication:	Treatment of Chagas disease
Applicant:	Chemo Research
Review Division:	Division of Anti-infective Products
Reviewer:	James S. Wild, Ph.D.
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TABLE OF CONTENTS

Table of Tables

Table of Figures

1 Executive Summary

1.1 Introduction

Chagas disease is caused by a parasite, *Trypanosome cruzi*, which is widespread in Latin America and a growing presence in North America. There are no FDA approved drugs for the treatment of Chagas disease, but two drugs, benznidazole and nifurtimox have historically been used to treat Chagas disease.

In the early 1970s, Hoffman La-Roche (Roche) developed benznidazole for the treatment of Chagas disease. Roche obtained the registration of benznidazole in Brazil, Argentina, Bolivia, Uruguay, Peru and Nicaragua as Radanil®, Ragonil® or Rochagan®, which are products for oral administration formulated in 100 mg and 50 mg uncoated scored tablets. In 2003, Roche donated all commercial rights and transferred the technology to manufacture benznidazole to the Brazilian government as a generic version of Roche's product, and, at the same time, Roche also withdrew its registration.

The product was granted a marketing authorization by the Brazilian Drug Regulatory Authority (ANVISA) in November 2006. Since that time, Laboratorio Farmacéutico de Pemambuco (LAFEPE), a Brazilian government manufacturing facility, has provided benznidazole to patients in Latin America and also to the United States under an IND held by the CDC.

Another benznidazole product, Abarax®, 100 mg and 50 mg tablets for oral administration, manufactured by Laboratorio ELEA, Buenos Aires, Argentina, has been approved in Argentina, Bolivia, Paraguay, and Chile in recent years (2012-2014) and is available in Spain.

The latest formulation in development for this NDA is supplied in tablet strengths of 12.5 mg and 100 mg. The dosing regimen for the Chemo Research product is a (b) (4) (b) (4)

1.2 Brief Discussion of Nonclinical Findings

- Clinically relevant toxicities for benznidazole in nonclinical studies included teratogenicity in rats and rabbits, dose dependent testicular atrophy and aspermia in rats and mice, and genotoxicity.
- In a rat embryo-fetal study, a low numerical increase in malformations was observed in fetuses from pregnant females treated with 50 mg/kg/day (anasarca) and 150 mg/kg/day (anasarca, multiple skeletal abnormalities, anophthalmia, and microphthalmia) benznidazole compared to vehicle-control fetuses (no malformations). In rabbits, a serious visceral malformation, ventricular septal defect, occurred at a low incidence in mid- and high-dose fetuses and was considered related to benznidazole administration. In both rats and rabbits,

pregnant females experienced pronounced weight reductions that were benznidazole dose-dependent indicating a consistent potential to produce maternal toxicity.

- A clinically relevant toxicity associated with benznidazole treatment was dose-dependent testicular atrophy and inhibition or arrest of spermatogenesis in testes and epididymides in rats and mice. The toxicity was characterized by tubular atrophy, peritubular interstitial edema, and accumulation of syncytial/degenerate sperm in seminiferous tubules and epididymides. Questions remain about the reversibility of the testicular and fertility effects, and the clinical relevance of the finding particularly in developing children. Testicular and fertility adverse effects have not been reported in clinical studies with benznidazole in adults and children with Chagas disease.
- Benznidazole has been shown to be mutagenic in Ames assays and to increase the frequency of sister chromatid exchange and micronuclei *in vitro* in human cells, and *in vivo* in mice. However, benznidazole also produced negative clastogenic results in other chromosome aberration and micronucleus studies in mice and rats. In chagasic children, the incidence of micronucleated lymphocytes and chromosome aberrations both significantly increased approximately two fold after benznidazole treatment.
- In literature manuscripts, benznidazole in intraperitoneal doses of 8 mg/kg/day for 60 days was reported to increase the incidence of lymphomas in rabbits and mice. The clinical relevance of this finding is uncertain because the studies were not GLP-compliant, and the intraperitoneal route of administration does not match the oral clinical route of administration.
- Another serious nonclinical toxicity associated with chronic repeated dosing of benznidazole is severe neurotoxicity in dogs including seizure contractions, opisthotonos, and nystagmus proceeding to death. However similar findings were not observed in rabbits, guinea pigs, rats, or mice.
- Other dose-dependent toxicity findings reported in the 26-week toxicology study in rats with orally administered benznidazole included reversible centrolobular hepatocellular hypertrophy, eosinophilic droplets and karyomegaly in kidney tubular epithelial cells, vacuolation in the pars distalis in the pituitary gland, increased incidence of extramedullary hematopoiesis and hemosiderin deposits in the spleen, and minimal follicular hypertrophy of the thyroid gland. The clinical relevance of these findings is unknown.
- Benznidazole was shown to minimally inhibit hERG potassium channels *in vitro* at a concentration higher than the expected plasma concentration in patients.
- In pharmacokinetic studies, benznidazole did not accumulate with repeated dosing in rats, and distributed widely in tissues including placenta and fetuses in pregnant rats and testes in male rats. The plasma $t_{1/2}$ was approximately 1.5

hours in mice, 2-2.5 hours in rats, 4-5 hours in sheep, and 9-11 hours in dogs. Plasma protein binding in multiple animal species was approximately 50% and approximately 10% of the administered benznidazole dose was excreted in urine in rats. Benznidazole showed minimal metabolism in hepatic microsomes from test species and humans and there were no human specific metabolites. A primary metabolic pathway is nitroreduction via intracellular nitroreductases to reactive metabolites that covalently bind to tissues often with correlating toxicity.

- Benznidazole did not reduce the pregnancy rates in a female fertility study in rats, but findings associated with embryo patency were observed. Significant decreases in live embryos were associated with increased postimplantation loss in pregnant females administered the high dose of 150 mg/kg. Similar findings occurred in the rat embryo-fetal study with a high dose of 150 mg/kg/day and in the rat pre-postnatal study with a high dose of 75 mg/kg/day.
- In the pre-postnatal study in rats, caesarean section findings in the F_1 dams included higher pre-implantation loss and reduced numbers of corpora lutea, implantations, and live embryos. Other developmental toxicity results in the pre-postnatal study included reduced mean body weights for F_1 males at PND 21, and reduced testicular size and impaired spermatogenesis and mating in 5% of the mid- and high-dose F_1 males that were selected for mating. However, the testicular effects were not widespread and did not alter the mean fertility results or the mean results for testicular sperm counts in F_1 males or mean motility and progression data for epididymal sperm in F_1 males.

1.3 Recommendations

1.3.1 Approvability

This NDA is approvable from a Pharmacology/Toxicology perspective.

1.3.2 Additional Non Clinical Recommendations

In a previous pre-NDA meeting conducted at the FDA on April, 27th 2016 (meeting minutes submitted to DARRTS on 5/26/2016) for IND 118976, the Applicant agreed to conduct a post-marketing male fertility study in rats.

(b) (4)

1.3.3 Labeling

8 USE IN SPECIFIC POPULATIONS

Reviewer Proposed Language

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Risk Summary

(b) (4)

Based on findings from animal studies, TRADENAME (b) (4) cause fetal harm when administered to a pregnant woman (b) (4). Published postmarketing reports on benznidazole use during pregnancy are insufficient to inform a drug-associated risk of adverse pregnancy-related outcomes. There are risks to the fetus associated with Chagas Disease (*see Clinical Considerations*). In animal reproduction studies, benznidazole administered orally to pregnant rats and rabbits during organogenesis was associated with (b) (4) fetal malformations at doses approximately 1-3 times the (b) (4) (MRHD) in rats (anasarca, anophthalmia, and/or microphthalmia) and doses approximately 0.3-1 times the MRHD in rabbits (ventricular septal defect). (*see Data*). Advise pregnant women of the potential risk to a fetus.

The estimated background risk of major birth defects and miscarriage for the indicated population is unknown. All pregnancies have a background risk of birth defect, loss, or other adverse outcomes. In the U.S. general population, the estimated background risk of major birth defects and miscarriage in clinically recognized pregnancies is 2-4% and 15-20%, respectively.

Data

(b) (4)

(b) (4)

In an embryo-fetal toxicity study in pregnant rats, an oral dose of benznidazole of 150 mg/kg/day during organogenesis (days 6-17 of gestation) was associated with maternal weight loss, reduced fetal weights, and smaller litter sizes. Benznidazole was also associated with a low incidence of fetal malformations including anasarca at a dose of 50 mg/kg/day and anasarca and eye abnormalities (anophthalmia and microphthalmia) at a high dose of 150 mg/kg/day (approximately equivalent to 1 and 3 times the maximum recommended human dose based on body surface area comparisons). The NOAEL dose for maternal toxicity in this study, 50 mg/kg/day, is approximately equal to the maximum recommended dose (b) (4) humans based on body surface area comparisons. The NOAEL dose for fetal toxicity was 15 mg/kg/day which is approximately equivalent to 0.3 times the maximum recommended dose (b) (4) humans based on body surface area comparisons.

In an embryo-fetal study in pregnant rabbits, oral (gavage) administration of benznidazole during organogenesis (days 6 to 19 of gestation) at a high dose dose of 25 mg/kg/day was associated with maternal toxicity including reduced weight gain and food consumption and abortions in 2/20 females. Benznidazole was also associated with a low incidence of fetal abnormalities including ventricular septal defect at a doses of 7.5 and 25 mg/kg/day (approximately equivalent to 0.3 and 1 times the maximum recommended human dose based on body surface area comparisons). The NOAEL values for maternal and fetal toxicity in this study were 7.5 and 2.5 mg/kg/day respectively, which are equivalent to approximately 0.3 and 0.1 times the maximum recommended dose (b) (4) humans based on body surface area comparisons.

In a pre- postnatal study in rats, first generation (F_1) pups born to dams administered 15, 50, and 75 mg/kg/day benznidazole demonstrated normal pre-weaning behavior, physical and functional development, neurological findings, and reproductive parameters. However, cesarean section data for the pregnant first generation (F_1) females in the high-dose group included significantly higher pre-implantation loss and significantly lower mean values for corpora lutea counts, number of implantations, and number of live embryos. Also small testes and/or epididymides were observed in 1/20 and 2/20 first generation males in the

mid- and high-dose groups respectively, and two of the affected animals failed to mate or induce pregnancy. However, the mean values for mating performance, fertility index, testes weight, testes and epididymides sperm counts, and epididymal sperm motility and progression were not altered in any of the F_1 males in benznidazole treatment groups. The number of live second generation (F_2) fetuses born to F_1 dams was reduced in the high-dose group. The NOAEL value was considered to be 50 mg/kg/day which is approximately equal to the maximum recommended dose (b) (4) humans based on body surface area comparisons.

8.2 Lactation

Risk Summary

(b) (4)

Limited published literature based on breast milk sampling reports that benznidazole is present in human milk at infant doses of 5.5 to 17% of the maternal weight-adjusted dosage and a milk/plasma ratio ranging between 0.3-2.79. There are no reports of adverse effects on the breastfed infant and no information on the effects of benznidazole on milk production. Because of the potential for serious adverse reactions, (b) (4) transmission of Chagas disease, advise patients that breastfeeding is not recommended during treatment with TRADENAME.

(b) (4)

8.3 Females and Males of Reproductive Potential

Pregnancy Testing

Pregnancy testing is recommended for females of reproductive potential.

Contraception

(b) (4)

Females
TRADENAME can cause fetal harm when administered to a pregnant woman *[see Use in Specific Populations (8.1)]*. Advise females of reproductive potential to use effective contraception during treatment with TRADENAME and for 5 days after the final dose.

Infertility

(b) (4)

Males
Based on findings in rodents, TRADENAME may impair fertility in males of reproductive potential. It is not known whether effects on fertility are reversible. *[see Nonclinical Toxicology,* (b) (4) *(13)]*

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Carcinogenicity

(b) (4)

Long-term carcinogenicity studies for benznidazole have not been performed. (b) (4) nitroimidazole (b) (4) been reported to be carcinogenic in mice and rats. (b) (4)

(b) (4)

Genetic Toxicity

Genotoxicity of benznidazole has been demonstrated *in vitro* in several bacterial species and mammalian cell systems and *in vivo* in (b) (4).

Benznidazole was mutagenic in several strains of *S. typhimurium* (TA 100, 102 1535, 1537, 1538, 97, 98 99 53 and UTH8414), *E.coli*, and *K. pneumoniae*.

Benznidazole was genotoxic in several *in vitro* mammalian cell assays including a chromosome aberration assay in human lymphocytes and in sister chromatid exchange assays in human lymphocytes and human Hep G2 cells.

In vivo, benznidazole was shown to be positive for genotoxicity in a mouse bone marrow micronucleus assay, in mouse and human red blood cell micronucleus assays, in a mouse abnormal sperm head assay and in a human peripheral blood lymphocyte

assay. However in other micronucleus studies in mice and rats, oral doses of benznidazole did not cause a significant increase in the frequency of chromosomal aberrations in bone marrow cells or micronuclei in peripheral blood cells.

(b) (4)

Impairment of Fertility:

(b) (4)

In a 6-month, chronic repeated-dosing study with Wistar rats, benznidazole was shown to produce dose-dependent testicular and epididymal atrophy at a dose of 30 mg/kg/day (approximately equivalent to 0.6 times the maximum recommended human dose based on whole body surface area comparisons). Aspermia was also evident in affected rats, but fertility was not assessed in this study. The NOAEL value in this study was considered to be 10 mg/kg/day (5 mg/kg twice daily) in males (approximately 0.2-times the maximum recommended human dose based on body surface area comparisons). In literature reports, (b) (4) been shown to cause

testicular atrophy and (b) (4) inhibit spermatogenesis in pubertal and adult rats and mice (b) (4)

In a female fertility study, oral (gavage) administration of benznidazole to female Wistar rats twice daily for a 2-week pre-mating period, during mating, and through day 7 of gestation was associated with transient lower body weight gain and food consumption. There was no benznidazole-related effect on mating performance or fertility and no adverse macroscopic or reproductive organ weight changes. However, benznidazole (b) (4) was associated with a higher post-implantation loss with (b) (4) live litter size at a dose of 150 mg/kg/day (equivalent to approximately 3 times the maximum recommended human dose based on whole body surface area comparisons). The NOAEL value for this study was consider to be 50 mg/kg/day which is approximately equivalent to the maximum recommended human dose based on whole body surface area comparison.

13.2 Animal Toxicology and/or Pharmacology

(b) (4)

Single oral-dose toxicity studies in rats have established that benznidazole causes ultrastructural changes in the adrenal cortex, colon, esophagus, ovaries, and testis (b) (4) (b) (4) (b) (4) In these tissues, these changes were associated with the presences of nitro reductase activity, the production of reactive metabolites, and or covalent binding of metabolites.

(b) (4)

Neurotoxicity including brain axonal degeneration and Purkinje cell degeneration was observed with repeated-oral dosing in dogs without adverse changes in peripheral nerves (b) (4) Neurological signs included: apathy, hypertonia, hyperreflexia, ataxia, loss of balance, oscillarory movements of the trunk and head, strong contractions of the back and leg muscles, opisthotonus and nystagmus. In other test species, including mouse, rat, guinea pig, and rabbit, (b) (4)

2 Drug Information

2.1 Drug

CAS Registry Number: 22994-85-0

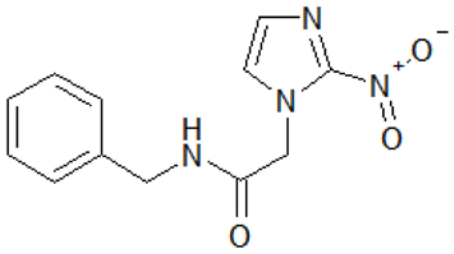
Generic Name: benznidazole

Code Name: none

Chemical Name: 2 N-benzyl-2-nitroimidazole-acetamide; N-benzyl-2-nitro-1-imidazole-acetamide

Molecular Formula/Molecular Weight: $C_{12}H_{12}N_4O_3$/260.25 Kd

Structure or Biochemical Description



Pharmacologic Class
Nitroimidazole antimicrobial

2.2 Relevant INDs, NDAs, BLAs and DMFs

IND 118976

2.3 Drug Formulation

The new benznidazole product will be provided in two different tablet sizes, a 12.5 mg tablet (Table 1), and a 100 mg tablet (Table 2).

Table 1: Formulation for the Benznidazole 12.5 mg Tablet. (Sponsor's Table)

Ingredients	Amount per tablet (mg)	Amount per tablet (% w/w)	Function	Reference to standards*
Active substance:				
Benznidazole	12.50	(b) (4)	Active substance	Drug substance manufacturer specifications
Excipients				
Pregelatinized Corn Starch	(b) (4)	(b) (4)	(b) (4)	NF
Monohydrate Lactose (b) (4)				NF
Sodium Croscarmellose				NF
Microcrystalline Cellulose (b) (4)				NF
Magnesium Stearate				NF
(b) (4)				NF
Tota				
Total weight				

*Current edition

(b) (4)

[2] http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm

Table 2: Formulation for the 100 mg Benznidazole Tablet. (Sponsor's Table)

Ingredients	Amount per tablet (mg)	Amount per tablet (% w/w)	Function	Reference to standards*
Active substance:				
Benznidazole	100.00	(b) (4)	Active substance	Drug substance manufacturer specifications
Excipients 1				
Pregelatinized Corn Starch	(b) (4)	(b) (4)	(b) (4)	NF
Monohydrate Lactose (b) (4)				NF
Sodium Croscarmellose				NF
Microcrystalline Cellulose (b) (4)				NF
Magnesium Stearate				NF
(b) (4)				NF
Totals				
Total weight				

*Current edition

(b) (4)

2.4 Comments on Novel Excipients

There are no novel excipients. A search of the FDA "Inactive Ingredients Search for Approved Drug Products indicated all of the excipients contained in the 12.5 and 100 mg benznidazole tablets have been used in greater amounts in previously approved oral products. Two of the excipients, microcrystalline cellulose (b) (4), have been used in any previously approved products, but are not listed in the Inactive Ingredients Database. However microcrystalline cellulose has been used in previously

approved products at much higher amounts than the amounts used in the 12.5 and 100 mg benznidazole tablets. The specific designations of [redacted]

Table 3: Benznidazole Product Excipients and Comparison of Tablet Amounts to Amounts Used in Previously Approved Products.

Excipient	12.5 mg tablet		100 mg tablet		Highest[a] Amount used in a Previously Approved Oral Products
	Amount (mg)	%	Amount	%	
Corn Starch	[redacted]				
Monohydrate Lactose					
Sodium Croscarmellose					
Microcrystalline Cellulose					
Magnesium Stearate					

[a] Data derived from FDA Inactive Ingredients Database.

2.5 Comments on Impurities/Degradants of Concern

The Sponsor identified several organic impurities including: [redacted] All of the organic impurities will be controlled at [redacted]

In addition, the Sponsor reports that [redacted] show alerts for genotoxic risk as assessed with DEREK software. However, the specifications for these impurities are not limited to the lower acceptable intake threshold for individual impurities [redacted] recommended in the Guidance for Industry: [redacted] Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk." The Sponsor's rationale for maintaining the specifications for these [redacted] impurities at the [redacted]% threshold is the following statement from the [redacted] Additionally, there may be some cases where a drug substance intended for other indications is itself genotoxic at therapeutic concentrations and may be expected to be associated with an increased cancer risk. Exposure to a mutagenic impurity in these cases would not significantly add to the cancer risk of the drug substance. Therefore, impurities could be controlled at acceptable levels for nonmutagenic impurities." Because benznidazole is considered genotoxic, it is considered acceptable that the potentially genotoxic impurities of benznidazole, [redacted] at the [redacted]% threshold.

Residual Solvents: The (b) (4) residual solvents listed in the benznidazole drug substance, (b) (4), are controlled at specifications that are equal to the concentration limits listed for these (b) (4) substances in the Guidance for Industry: "Q3C – Tables and List."

2.6 Proposed Clinical Population and Dosing Regimen

Clinical Population: Benznidazole is recommended for (b) (4) children, (b) (4).

Dosing Regimen

(b) (4)

2.7 Regulatory Background

This benznidazole product was submitted in IND 118976 and the application for NDA 209570 was submitted on 12/29/2016. No studies have been put on hold. At an EOP2 meeting with the Sponsor on April 27th, 2016 (meeting minutes submitted to DARRTS on 5/26/2016), the Agency agreed to allow a male fertility study in rats to be submitted as a postmarketing requirement.

3 Studies Submitted

3.1 Studies Reviewed

Safety Pharmacology

1. The Effect of Benznidazole on the hERG Tail Current in Stably Transfected HEK-293 Cells. (Study No.: 505539).

Pharmacokinetics

Absorption

1. Pharmacokinitics of EPL-BS0063 in Male Sprague Dawley Rats following IV and Oral Administration. (Report # CDCO_DNDi_09_032_PK).

Distribution

1. Preclinical Pharmacokinetics of Benznidazole. Workman et al., Br J Cancer, 50:291-303 (1984).
2. Administration of Benznidazole, a Chemotherapeutic Agent Against Chagas Disease, to Pregnant Rats. Covalent Binding of Reactive Metabolites to Fetal and Maternal Proteins. Diaz de Toranzo EG, Masana M, and Castro JA, Arch Int Pharmacodyn Ther, 272:17-23 (1984).

3. Differential Tissue Distribution of Benznidazole After Oral Administration to Male Rats. Diaz de Toranzo EG, Masana M, and Castro JA, Acta Bioquimica Clinica Latinoamericana, Vol. XX, 61-64 (1986).

Metabolism

1. Interspecies Comparison of In Vitro Metabolism of Benznidazole in Mouse, Rat, Rabbit, and Human Hepatocytes (Study No.: 505538).
2. Characterization of Human Cytochrome P450 Isoenzymes Involved in the *In Vitro* Metabolism of Benznidazole (Study No.: 513489)
3. *In Vitro* Determination of the Inhibitory Properties (Reversible or Time Dependent) of Benznidazole for the Human Cytochrome P450 Isoenzymes 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4, Using Human Liver Microsomes (Study No.: 513491).
4. Benznidazole Levels in Blood Vary with Age in Rats. Bulffer RF, Castro JA, Fanelli SL, Mem Inst Oswaldo Cruz, Rio de Janeiro, 106: 374-377 (2011).

General Toxicoclogy

Single-dose Toxicology

1. Ultrastructural Alterations in Ovaries from Nifurtimox or Benznidazole-Treated Rats: Their Relation to Ovarian Nitroreductive Biotransformation of Both Drugs. de Castro CR, de Toranzo EGD, Bernacchi AS, Carbone M, and Castro JA, Exp Mol Pathol, 50:385-397 (1989).
2. Benznidazole-induced Ultrastructural Alterations in Rat Adrenal Cortex. Mechanistic Studies. de Castro RC, Diaz de Toranzo EG, and Castro JA, Toxicology, 74:223-232 (1992).
3. Benznidazole-induced Ultrastructural and Biochemical Alterations in Rat Esophagus. de Castro RC, Montalto de Mecca M, Fanelli SL, de Ferreyra EC, Diaz, EG, Castro JA, Toxicology, 191:189-198 (2003).
4. Benznidazole-induced Ultrastructural and Biochemical Alterations in Rat Colon. Diaz EG, de Castro RC, Montalto de Mecca M, and Castro JA: Acta Pharmacol Sin, 21:961-966 (2000).
5. Benznidazole Biotransformation in Rat Heart Microsomal Fraction Without Observable Ultrastructural Alterations: Comparison to Nifurtimox-induced Cardiac Effects. Montalto de Mecca M, Bartel LC, Rodriguez de Castro C, Castro JA, Mem Inst Oswaldo Cruz, Rio de Janeiro, 103:549-553 (2008).

Repeat-dose Toxicology

1. Selective Purkinje Cell Damage in Dogs After Oral Administration of High Doses of Nitroimidazole Derivatives. Scharer K: Verhandlungen der Deutschen Gesellschaft fur Pathologie, 56:407-10 (1972).
6. Experimental Benznidazole Encephalopathy: I Clinical-Neurological Alterations. Flores-Vieira CLL and Barreira AA, J Neurol Sci, 150:3-11 (1997a).
7. Experimental Benznidazole Encephalopathy: II Electroencephalographic and Morphological Alterations. Flores-Vieira CLL, Chimelli, L, Fernandez RMF, and Barreira AA, J Neurol Sci, 150:13-25 (1997b).

8. Benznidazole - 26-week Oral (Twice Daily Gavage, 6 hours apart) Toxicity Study in the Wistar Rat Followed by a 4-week Treatment-free Period. (Study No.: AB20563).

Genetic Toxicology

1. Mutagenicity of Nifurtimox and Benznidazole in the Salmonella Microsome Assay. Ferreira RCC and Ferreira LCS: Braz. J. Med. Biol. Res., 19:19-25 (1986).
2. Mutagenicity of 2 Anit-Chagasic Drugs and Their Metabolic Deactivation. Nagel R and Nepomnaschy I: Mutation Research, 117:237-242 (1983).
3. Genotoxicity Studies with Two Antichagasic Drugs. Nagel R: Mut Res., 191, 17-20 (1987).
4. Screen the Mutagenic Activities of Commonly Used Antiparasitic Drugs by Simultest, A Simplified Salmonella/Microsome Plate Incorporation Assay. Melo MEB and Ferreira LCS: Rev Inst Med Trop Sao Paulo, 32:269-274 (1990).
5. Mutagenicity of Anti-trypanosomal Drug Ro 7-1051 in Escherichia coli. Ohnish T, Ohashi Y, Nozu K, and Inoki S: Jpn J Genet, 58:505-509 (1983).
6. The Mutagenic Action of Nitroimidazoles, II. Effects of 2-Nitroimidazoles. Voogd CE, Van Der Stel JJ, and Jacobs JJJAA: Mutation Research, 31:149-152 (1975).
7. Cytogenetic Effects of the Antichagasic Benznidazole on Human Cells *In Vitro*. Santos SS, Takahashi CS, and Natarajan AT: Mut. Res., 1994, 320:305-314.
8. Micronucleus Formation in Bone Marrow of Mice Treated with Nifurtimox or Benznidazole. Gorla NB and Castro JA: Toxicology Letters, 25:259-263 (1985).
9. Clastrogenic Activity of Two Antichagasic Drugs. Navarro ML, Dain L, Migliorini AM, and Nagel R, Comunicaciones Biologicas, 3:25-28 (1984).
10. Evaluation of the Mutagenic Potential of the Antichagasic Drug Rochagan in Healthy and Chagasic Rodents. Souza SC, Takahashi CS, and da Silva, JS: Mutat Res, 259: 139-45 (1991).
11. Assessment of Cytogenetic Damage in Chagasic Children Treated with Benznidazole. Gorla NB *et al.*: Mutat Res, 206:217-228 (1988).

Carcinogenicity

1. Chagas disease. Carcinogenic Activity of the Antitrypanosomal Nitroarenes in Mice. Teixeira *et al.*: Mutat Res, 305:189-196 (1994).
2. Treatment with Benznidazole in Association with Immunosuppressive Drugs in Mice Chronically Infected with Trypanosoma cruzi: Investigation into the Possible Development of Neoplasms. Andrade *et al.*: Rec Soc Bras Med Trop, 36:441-447 (2003).
3. Malignant Non-Hodgkins Lymphoma in Trypanosoma cruzi-Infected Rabbits Treated with Nitoarenes. Teixeira *et al.*: J Comp Path, 103:37-48 (1990a).
4. Chagas Disease Lymphoma Growth in Rabbits Treated with Benznidazole. Teixeira *et al.*: Am J Trop Med Hyg, 43:146-158 (1990b).

Reproductive and Developmental Toxicology

1. Effects of Nifurtimox or Benznidazole Administration on Rat Testes: Ultrastructural Observations and Biochemical Studies. Bernacchi AS, de Castro CR, de Toranzo EG, Castro JA, Exp Mol Pathol 45:245-256 (1986).

2. Testes Alterations in Pubertal Benznidazole-treated Rats. Vieira CL, Lamano-Carvalho TL, Favaretto AL, Valenca MM, Antunes-Rodridgues J, Barreira AA, Braz J Med Biol Res, 22:695-698 (1989).
3. Abnormal Sperm Induced in Mice by Oral Administration of Antichagasic Drugs. Navarro ML and Nagel R, Comunicaciones Biologicas, 8:251-258 (1990).
4. Pituitary-Testicular Axis in Benznidazole-treated Rats. Favaretto AL, Antunes-Rodriques J, Vieira CL, Lamano-Carvalho TL, Braz J Med Biol Res, 23:719-722 (1990).
5. Benznidazole – Fertility Toxicity Study by the Oral Route (Twice Daily Gavage) in the Female Rat (Segment I) Followed by a 13-week Treatment-free Period (Study No.: AB21207).
6. Benznidazole – Embryo-Foetal Toxicity Study by the Oral Route (Gavage) in the Rat (Segment II) (Study No.: AB20567).
7. Benznidazole –Embryo-fetal Toxicity Study by the Oral Route (gavage) in the Rabbit (Segment II) (Study No.: AB20565).
8. Benznidazole – Pre- and Post-natal Development Study by the Oral Route (Gavage) in the Wistar Rat (Segment III)(AB20568)

3.2 Studies Not Reviewed

1. Benznidazole – Analytical Method Validation to Determine Test Items Concentrations in Formulations (Study No.: AB20562).
2. Validation of an LC-MS/MS Procedure for the Quantification of Benznidazole in EDTA Rabbit Plasma (Study No.: 505623).
3. Validation of an LC-MS/MS Procedure for the Quantification of Benznidazole in EDTA Rat Plasma (Study No.: 505622).
4. Determination of Benznidazole as a Possible Substrate or Inhibitor of P-glycoprotein using the Bi-directional Transport Assay in Caco-2 Cells. (Study No.: 513493).
5. An *In Vitro* Investigation to Assess if Benznidazole is an Inhibitor of the Uptake Transporters (OATP1B1*1a, OOATPB13, OAT1, OAT3, OCT1, and OCT2) (Study No.: EXL/REP/01).
6. An *In Vitro* Investigation to Assess if Benznidazole is a Substrate of the Uptake Transporters (OATP1B1*1a, OATPB13, OAT1, OAT3, OCT1, and OCT2) (Study No.: EXL/REP/02).
7. An *In Vitro* Investigation to Assess if Benznidazole is an Inhibitor of the BCRP efflux Transporter (Study No.: EXL/REP/03).
8. An *In Vitro* Investigation to Assess if Benznidazole is a Substrate of the MDCK-BCRP efflux Transporter (Study No.: EXL/REP/04).
9. Benznidazole: Potential for Development of Resistance and Activity Against Different Lineages of T. cruzi Justification Report. (Study No.: LPRI747-DOC007).

3.3 Previous Reviews Referenced

None

4 Pharmacology

4.1 Primary Pharmacology

The primary pharmacology studies were reviewed by the microbiology reviewer, Dr. Shukal Bala.

4.2 Secondary Pharmacology

No secondary pharmacology studies were submitted by the Sponsor.

4.3 Safety Pharmacology

1. The Effect of Benznidazole on the hERG Tail Current in Stably Transfected HEK-293 Cells. (Study No.: 505539).

Methods

This GLP-compliant study including a quality assurance statement was conducted by (b) (4) in 2014. Benznidazole (Batch # 00013) was tested in concentrations of 30 and 100 mcM with HEK-293 cells stably transfected with hERG-1 cDNA. Potassium hERG tail currents were measured using the patch clamp technique in the whole-cell configuration. Cells were equilibrated then exposed to different concentrations of benznidazole for 5 minutes until an apparent steady-state effect was observed. One or more increasing concentrations of benznidazole were applied sequentially without washout steps to each cell. Five cells were tested for each benznidazole concentrations and the negative control (0.1% DMSO in Tyrode solution) and positive control (0.1 mcM E-4031) solutions were applied to nine and five cells respectively.

Results

The actual concentrations of the benznidazole stock solutions were within less than 10% of the nominal concentrations.

Benznidazole at concentrations of 30 and 100 mcM significantly inhibited hERG potassium currents by 7.9 and 17.0% compared to vehicle control values (Table 4). The highest concentration, 100 mcM (26.0 mcg/ml) is substantially higher than the highest expected plasma concentrations associated with the clinical dose (2.2 to 2.4 mcg/ml). An IC_{50} value was not estimated from this data. The positive control agent, 0.1 mcM E-4031, produced an average inhibition of 91.8% supporting the patency of the assay.

Table 4: The Effect of Benznidazole on hERG Potassium Channel Currents.
(Sponsor's Table)

	Peak amplitude (pA)	% inhibition			
	Tyrode	Vehicle	Test substance (μM)		E-4031 (μM)
		0.1% DMSO	30	100	0.1
Mean	3917	5.0	7.9*	17.0*	91.8*
SEM	468	1.1	1.7	2.7	1.2
N	9	9	5	5	5

(* significant effect compared to vehicle p<0.01, one-tailed paired t-test)

5 Pharmacokinetics/ADME/Toxicokinetics

5.1 PK/ADME

1. **Pharmacokinitics of EPL-BS0063 in Male Sprague Dawley Rats following IV and Oral Administration.** (Report # CDCO_DNDi_09_032_PK).

Methods
Experiment #1: Fresh heparinized whole blood and plasma from male Sprague Dawley rats was spiked with EPL-BS0063 (benznidazole) to a nominal concentration of 500 ng/ml. Samples of the spiked whole blood were incubated at 37°C for 2, 60, and 240 minutes. Subsequently, plasma and erythrocytes were separated and plasma concentrations of benznidazole were determined using a LC-MS method with a lower limit of quantification of 0.0019 M. Whole blood to plasma partitioning ratios were obtained by dividing the measured concentration in the control plasma sample by the concentration measured in plasma following centrifugation of spiked whole blood.

Experiment #2: Benznidazole was administered in single doses intravenously at a nominal concentration of 5.1 mg/kg or orally by gavage at a nominal concentration of 22 mg/kg (2 animals per group). Blood and urine samples were collected up to 24 hours after dosing.

Results
Benznidazole was stable in fresh rat blood and plasma for the maximum 4 hours of incubation. The results shown in Table 5 indicate approximately equal partitioning between plasma and whole blood.

Table 5: *In vitro* Stability of EPL-BS0063 in Freshly Collected Rat Plasma and Whole Blood. (Sponsor's Table)

Incubation Time (min)	% EPL-BS0063 Remaining*	
	Plasma	Whole Blood (Plasma Fraction)
2	100	100
60	102.0	104.6
240	99.3	95.5

* Calculated relative to the 2 minute sample

Benznidazole concentrations were measureable in plasma for the duration of the 24 hour sampling period allowing calculation of multiple pharmacokinetic parameters (Table 6). Following oral administration, benznidazole was approximately 100% bioavailable. Plasma $t_{1/2}$ values ranged from 2 to 2.5 hours and the volume of distribution was low (0.8 L/kg) suggesting limited distribution to tissues. LC-MS analysis of plasma and urine samples did not identify potential metabolites, and approximately 10% of the dose was recovered in urine during the 24 hour collection period.

Table 6: Pharmacokinetic Parameters for Benznidazole in Male Sprague Dawley Rats following IV and Oral Administration. (Sponsor's Table)

Parameter	IV administration		Mean	Oral administration		Mean
	A_2809	B_2809		C_2809	D_2809	
Measured dose (mg/kg)	5.1	5.1	5.1	22.3	21.3	21.8
Apparent $t_{1/2}$ (h)	2.1	2.0	2.1	2.5	2.2	2.4
Plasma CL_{total} (mL/min/kg)	4.3	4.3	4.3	---	---	---
B/P ratio	---	---	1.04	---	---	---
Blood CL_{total} (mL/min/kg)	4.2	4.1	4.2	---	---	---
V_z (L/kg)	0.8	0.8	0.8	---	---	---
V_{SS} (L/kg)	0.8	0.9	0.8	---	---	---
% Dose in urine[a]	9.7	excluded[b]	9.7	6.4	13.2	9.8
C_{max} (µM)	---	---	---	42.0	44.3	43.1
T_{max} (min)	---	---	---	150	150	150
BA (%)	---	---	---	98.1	107.4	102.7

[a] Unchanged EPL-BS0063 present in pooled urine (collected over 0-24 h)
[b] Value was excluded for this rat, as apparent urinary excretion (0.7 % of the dose) was inconsistent with the other animals in this study

Distribution

No original distribution studies with benznidazole were conducted by the Applicant for this NDA. However, a number of distribution studies are described in the literature.

These studies were summarized in a memo prepared by James Wild for IND 118976 (Submitted to DARRTS on 6/13/2016). The literature references and summary information for each reference are shown below.

1. **Preclinical Pharmacokinetics of Benznidazole.** Workman *et al.*, Br J Cancer, 50:291-303 (1984).

Summary Results: Pharmacokinetic behavior was linear except at high doses in mice. Absorption was fairly rapid and bioavailability was complete following both intraperitoneal administration in mice and oral administration in dogs. Plasma $t_{1/2}$ values were 90 minutes in mice, 4-5 hours in sheep and 9-11 hours in dogs. Plasma-protein binding for benznidazole was shown to be 38-39% in mice, 52-58% in dogs, and 41-42% in sheep. Distribution to nervous tissue was extensive with brain/whole plasma ratios averaged between 61 and 76% in mice and 42% in dogs, while peripheral nerve/whole plasma ratios in dogs averaged 74%. Mean liver/whole plasma ratios were 42% and 71% in BALB/c and C3H/He mouse strains respectively. Approximately 5% of the administered dose was excreted unchanged in the urine, indicating the likelihood of extensive metabolism.

2. **Administration of benznidazole, a chemotherapeutic agent against Chagas disease, to pregnant rats. Covalent binding of reactive metabolites to fetal and maternal proteins.** Diaz de Toranzo EG, Masana M, and Castro JA, Arch Int Pharmacodyn Ther, 272:17-23 (1984).

Summary Results: In pregnant Sprague-Dawley rats, ^{14}C-benznidazole at an oral dose of 100 mg/kg rapidly distributed to many tissues with the highest concentrations of radioactivity occurring in liver, kidney, heart, lung, blood, brain, and skeletal muscle (Table 7). Radioactivity also readily distributed to the placenta and fetus with concentrations similar to blood concentrations. Covalent binding of radioactivity deriving from ^{14}C-benznidazole occurred in many tissues with covalent binding to fetal proteins occurring at 1 hour after dosing which increased at 3 and 6 hours.

Table 7: Distribution and Covalent Binding of Radioactivity Derived from ^{14}C-Benznidazole in Tissues from Pregnant Rats. (Manuscript Table)

	Protein	BNZ Content [a]	BNZ Covalently Bound [b]
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3. **Differential Tissue Distribution of Benznidazole After Oral Administration to Male Rats.** Diaz de Toranzo EG, Masana M, and Castro JA, Acta Bioquimica Clinica Latinoamericana, Vol. XX, 61-64 (1986).

Summary Results: In male Sprague-Dawley rats, radioactivity derived from ^{14}C-benznidazole administered orally at a dose of 50 mg/kg distributed widely throughout the body with the greatest concentrations occurring in the GI tract, liver, kidney, blood, heart, lung, testicles, and brain (Table 8). During the measurement period from 1 to 24 hours after administration, there was a progressive decline in radioactivity concentrations in all the tissues except the large intestine.

Table 8: Distribution of Radioactivity from ^{14}C-Benznidazole in Rat Tissues at Different Times after Administration (n=3). (Manuscript Table)

	^{14}C from ^{14}C-BNZ (nmoles) / g tissue
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Metabolism

1. **Interspecies Comparison of In Vitro Metabolism of Benznidazole in Mouse, Rat, Rabbit, and Human Hepatocytes.** (Study No.: 505538)

Methods: This GLP-compliant study including a quality-assurance statement was conducted in the (b) (4) in 2014. Hepatocytes were incubated at 37°C for 1, 30, 60, 90, and 120 minutes with 0, 10, 50, 100, 200, 500, and 1000 mcM benznidazole.

Results: Benznidazole was highly stable in incubations with hepatocytes isolated from mice, rats, rabbits and human (Table 9). No metabolic degradation of parent benznidazole was observed in incubations with rat and human hepatocytes and only 7.3 and 11.7% metabolic degradation of benznidazole occurred in incubations with mouse and rabbit hepatocytes respectively.

Table 9: Remaining Average Percentage Benznidazole with Various Incubation Periods in Different Species. (Sponsor's Table)

Species	Remaining percentage Benznidazole (mean)				
	1±1 min	30±1 min	60±1 min	90±1 min	120±1 min
Mouse	100[1]	103	91.9	95.7	92.7
Rat	100[1]	103	103	101	105
Rabbit	100[1]	85.1	82.7	84.8	88.3
Human	100[1]	101	107	111	105

[1)] Set at 100%
[2)] based on a single measurement because the duplicate was rejected

In total, six benznidazole metabolites were detected in the incubations using hepatocytes from four different species (Table 10) and structures were proposed (Figure 1). No human-specific metabolites were detected.

Table 10: Benznidazole Metabolites Detected in Incubations with Hepatocytes from Mice, Rats, Rabbits and Humans. (Sponsor's Table)

m/z of $[M+H]^+$	Rt (min)[1)]	Mass Shift[2)]	Proposed reaction	Presence in incubation[3)]			
				mouse	rat	rabbit	human
231.124	10.6-10.7	-29.974	Nitro reduction	+	+	+	+
536.192	10.8	+275.093	Glutathione conjugation	+	+	-	-
536.192	11.4	+275.093	Glutathione conjugation	+	-	-	-
536.192	11.8	+275.093	Glutathione conjugation	+	-	-	-
277.093	13.8-13.9	+15.995	Oxidation	+	+	+	-
277.093	17.5	+15.995	Oxidation	-	-	+	-
261.098	20.2-20.4	0	**=Parent compound**	+	+	+	+

Rt: retention time; min: minutes
[1)] Range of retention time mentioned as observed in different runs and different species
[2)] Mass shift compared to parent compound
[3)] + present, - not present

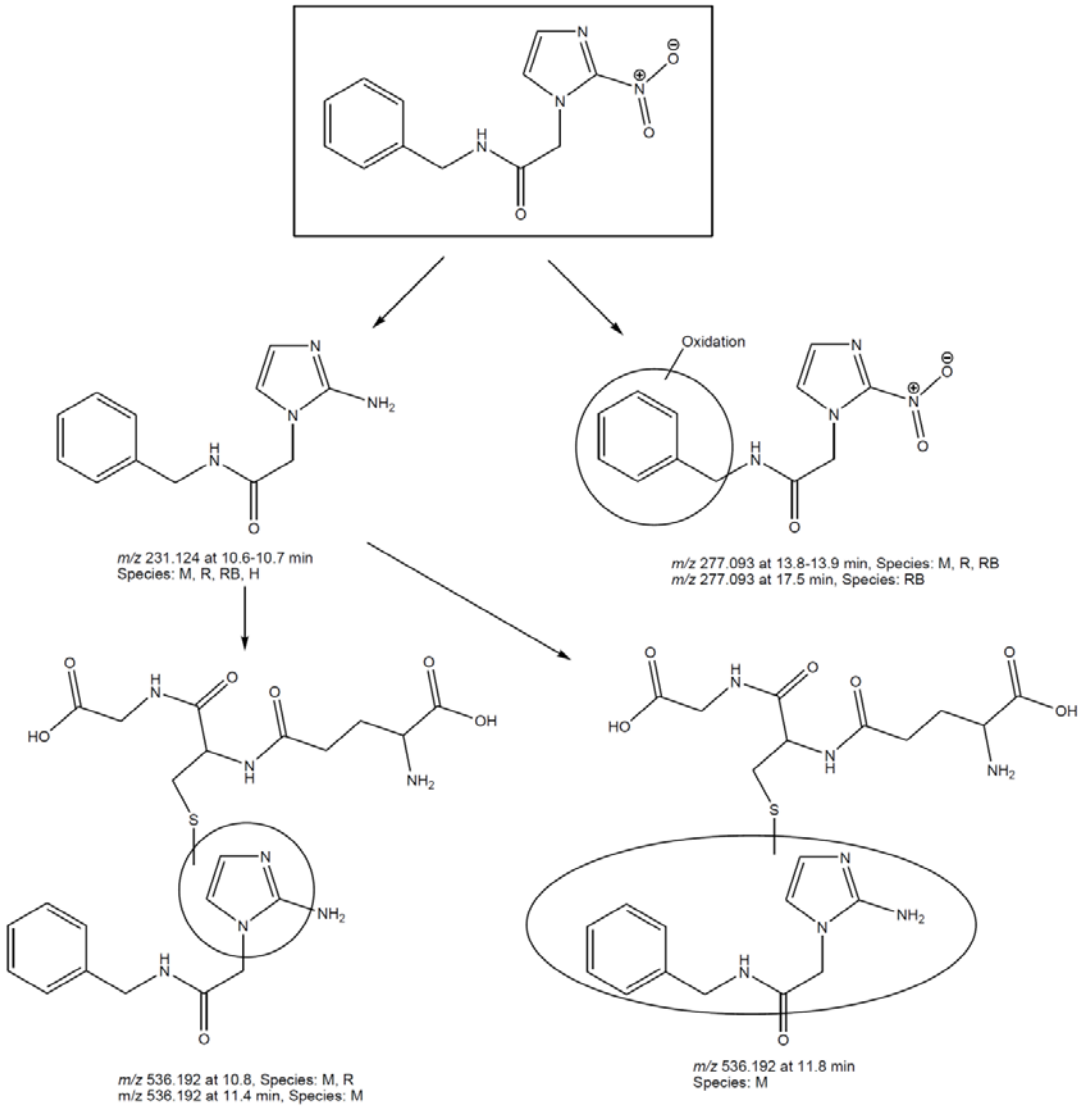

Figure 1: Proposed Metabolic Reactions for Benznidazole in Incubations with Dog, Rat, Rabbit and Human Hepatocytes. (Sponsor's Figure from the Study Report for Study No.: 505538)

2. **Characterization of Human Cytochrome P450 Isoenzymes Involved in the In Vitro Metabolism of Benznidazole.** (Study No. 513489)

Methods

This non-GLP study was conducted by (b) (4) (b) (4) beginning in June 2016. Benznidazole (10 mcM) was incubated with human liver microsomes isolated and pooled from 25 donors in incubations for 30 and 60 minutes. The concentration of benznidazole from incubation mixtures was measured

using a LC-PDA-MS technique and possible metabolites were measured using a UPLC-PDA-MS system.

Results
Benznidazole was poorly metabolized in human liver microsomes with 3% metabolism occurring in 30-minute incubations and 0-6% metabolism occurring in 60-minute incubations (Table 11).

Analysis of the limited benznidazole metabolites indicated that oxidation and S-cysteine conjugation were two possible contributing metabolic reactions (Table 12).

Table 11: Benznidazole Metabolism in Human Liver Microsomes in Incubations for 30 and 60 Minutes. (Sponsor's Table)

Benznidazole concentration	Protein concentration (mg/mL)	Replicate	MS peak area Benznidazole		
			t=0 min	t=30 min	t=60 min
10 µM	0.25	1	172500331	167958670	162069815
		2	172048373	165181082	162882255
		Average	172274352	166569876	162476035
%remaining			100%[1]	97%	94%
10 µM	0.5	1	163434604	159593441	164931891
		2	170309859	165343071	168293998
		Average	166872231	162468256	166612944
%remaining			100%[1]	97%	100%

[1] Set at 100%

Table 12: Possible Metabolic Reactions Involved in the Metabolism of Benznidazole in Human Liver Microsomes. (Sponsor's Table)

m/z value	Retention time (min)	Mass shift with benznidazole	Possible metabolic reaction
277.093	13.4	+15.995	Oxidation
380.102	17.6	+119.004	S-cysteine conjugation

3. ***In Vitro* Determination of the Inhibitory Properties (Reversible or Time Dependent) of Benznidazole for the Human Cytochrome P450 Isoenzymes 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4, Using Human Liver Microsomes.** (Study No.: 513491)

Methods
This non-GLP study was conducted by (b) (4) beginning in May 2016. Pooled human liver microsomes from 25 healthy donors were used in experiments intended to determine reversible or time-dependent CYP inhibition by benznidazole.

In order to test the reversible inhibition of multiple CYP450 enzymes (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4), microsomes were incubated with isozyme-specific substrates, and then tested for inhibition with known reversible inhibitors of specific isozymes or different concentrations of benznidazole (0, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 mcM). For the reversible inhibition experiment,

microsomes were pre-incubated with different concentrations of benznidazole or a specific positive control inhibitor for 5 minutes at 37°C, then combined with NADPH-generating reagents and an effective concentration of the correct isozyme-specific substrate for an additional 5-30 minutes at 37°C (depending on the isozyme) before stopping the reaction by placing the incubation solution to ice and adding acetic acid (Table 13 and Table 14).

Testing for time-dependent inhibition followed the same format except different positive control inhibitors were used and pre-incubations were performed for 30 minutes instead of 5 (Table 15).

Table 13: CYP Isozymes, Substrates, and Positive Control Inhibitors. (Table 2 from Section 3.5, Substrates and Positive Control Inhibitors, in the Study Report)

Human CYP isoenzyme	Substrate	Metabolite	Positive control inhibitor(s) (reversible)	Positive control inhibitor (time dependent)
CYP1A2	Phenacetin	Acetaminophen	Fluvoxamine	Furafylline
CYP2B6	Bupropion	Hydroxybupropion	Ticlopidine	Thiotepa
CYP2C8	Paclitaxel	6α-hydroxypaclitaxel	Ketoconazole	Isoniazid
CYP2C9	Diclofenac	4'-hydroxydiclofenac	Sulfaphenazole	Tienilic acid
CYP2C19	(S)-mephenytoin	(S)-4'-hydroxymephenytoin	Tranylcypromine	S-fluoxetine
CYP2D6	Bufuralol	1'-hydroxybufuralol	Quinidine	Paroxetine
CYP3A4	Midazolam Testosterone	1'-hydroxymidazolam 6β-hydroxytestosterone	Ketoconazole Ketoconazole	Mifepristone Mifepristone

Table 14: Positive Control Inhibitors and Reference IC_{50} Values for Reversible Inhibition (Table 3 from Section 3.5, Substrates and Positive Control Inhibitors, in the Study Report)

Human CYP isoenzyme	Positive control inhibitor	Concentrations used in the study (μM)	IC_{50} historical control values (μM)[a)]	
			min	max
CYP1A2	Fluvoxamine	0.003, 0.01, 0.03, 0.1, 0.3, 1, 3	0.02	0.34
CYP2B6	Ticlopidine	0.02, 0.06, 0.2, 0.6, 2, 6, 20	0.02	0.26
CYP2C8	Ketoconazole	0.1, 0.3, 1, 3, 10, 30, 50	3.3	23
CYP2C9	Sulfaphenazole	0.01, 0.03, 0.1, 0.3, 1, 3, 10	0.35	3.0
CYP2C19	Tranylcypromine	0.1, 0.3, 1, 3, 10, 30, 100	1.1	24
CYP2D6	Quinidine	0.01, 0.03, 0.1, 0.3, 1, 3, 10	0.02	0.14
CYP3A4 (substrate testosterone)	Ketoconazole	0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1	0.01	0.08
CYP3A4 (substrate midazolam)			0.01	0.07

[a)] based on values obtained at WIL Research Europe from 2005- 2014

Table 15: Concentrations of Positive Control Inhibitors Used for Time-dependent Inhibition (Table 4 from Section 3.5, Substrates and Positive Control Inhibitors, in the Study Report)

Human CYP isoenzyme	Positive control inhibitor (time dependent)	Concentration (µM)
CYP1A2	Furafyllline	5
CYP2B6	Thiotepa	5
CYP2C8	Isoniazid	200
CYP2C9	Tienilic acid	1
CYP2C19	S-Fluoxetine	30
CYP2D6	Paroxetine	0.5
CYP3A4	Mifepristone (testosterone) Mifepristone (midazolam)	1 1

Results

Benznidazole did not produce reversible inhibition of any of the test CYP450 isozymes at concentrations ≤ 100 mcM. This was also the result for time-dependent inhibition except for inhibition of CYP2C19 which benznidazole inhibited in a time-dependent manner with an IC_{50} of 51 mcM (Table 16).

Table 16: Summary of IC_{50} Values of Benznidazole for Tested CYP Isoforms. (Table 15 from Section 6, Conclusion, in the Study Report)

CYP isoform	Substrate	Reversible inhibition	Time dependent inhibition	
		IC_{50} Benznidazole (µM)	IC_{50} Benznidazole (µM)	IC_{50} shift
1A2	Phenacetin	No inhibition	>100	n.a.
2B6	Bupropion	>100	>100	n.a.
2C8	Paclitaxel	No inhibition	>100	n.a.
2C9	Diclofenac	No inhibition	No inhibition	n.a.
2C19	(S)-mephenytoin	>100	51	>2
2D6	Bufuralol	No inhibition	No inhibition	n.a.
3A4	Midazolam	No inhibition	>100	n.a.
	Testosterone	>100	>100	n.a.

n.a. = not applicable

4. **Benznidazole Levels in Blood Vary with Age in Rats.** Bulffer RF, Castro JA, Fanelli SL, Mem Inst Oswaldo Cruz, Rio de Janeiro, 106: 374-377 (2011).

Methods

Outbred male Sprague Dawley rats of variable age ranges (14, 21, and 70 days old) were administered a single administration of 100 mg/kg benznidazole by oral gavage, then blood samples were collected at 1, 3, 6, and 24 hours after dosing and blood benznidazole concentrations were measured using a HPLC-UV technique.

Results

Benznidazole concentrations in rat blood were higher in young rats and decreased with the age of the rats (Table 17). The authors speculated that juvenile rats metabolized and degraded benznidazole slower than older adult rats.

Table 17: Benznidazole (Bz) Content in Rat Whole Blood Samples Obtained at Staggered Intervals after a Single Administration by Oral Gavage. (Table from the Results Section of the Manuscript)

Bz concentration (µg/mL blood)

Excretion

As described in Study Report # CDCO_DNDi_09_032_PK in rats, approximately 10% of benznidazole administered by intravenous or oral routes was excreted in urine. In C3H/Km mice, approximately 5% of the total dose of benznidazole, and approximately 3% of the benznidazole amine metabolite was excreted in urine within 24 hours of dosing (Workman *et al.*, 1984).

6 General Toxicology

6.1 Single-Dose Toxicity

Single-dose toxicology studies have been conducted in rats and reported in the literature as described below.

1. **Ultrastructural Alterations in Ovaries from Nifurtimox or Benznidazole-Treated Rats: Their Relation to Ovarian Nitroreductive Biotransformation of Both Drugs.** de Castro CR, de Toranzo EGD, Bernacchi AS, Carbone M, and Castro JA, Exp Mol Pathol, 50:385-397 (1989).

Methods

Nifurtimox or benznidazole (gift from Hoffman LaRoche) were administered in single-oral doses of 100 mg/kg (in 1% carboxymethylcellulose) to female Sprague-Dawley rats during the diestrus or estrus stage of the estrus cycle. Control animals received the vehicle in the same volume. Animals were euthanized 24 hours after dosing, and their ovaries were removed and prepared for examination with electron microscopy.

Results

Ovaries obtained during the diestrus or estrus stages did not differ in their findings. In all the different cellular components of the rat ovary, nifurtimox and benznidazole were shown to increase mitochondrial swelling leading to disorganization of ovarian ultrastructure.

2. **Benznidazole-induced Ultrastructural Alterations in Rat Adrenal Cortex. Mechanistic Studies.** Rodriguez de Castro C, Diaz de Toranzo EG, and Castro JA, Toxicology, 74:223-232 (1992).

Methods
Male Sprague Dawley Rats were administered a single dose of 100 mg/kg benznidazole (obtained from Hoffman La Roche) in 1% carboxymethlycellulose or vehicle. Adrenal glands were removed and either processed for isolation of subcellular fractions and enzymatic and chemical determinations or electron microscopy

Results
Adrenal histopathology in rats included ultrastructural alterations of organelles within epithelial cells in the zonae fasciculata and reticularis within the adrenal glands. Changes included nuclei with large marginated chromatin clumps, rarefaction or loss of cristae within mitochondria, and lipid accumulation in the epithelial cell cytoplasm. Benznidazole nitroreductase activity was measured in adrenal microsomal and mitochondrial factions suggesting the ultrastructural changes may have resulted from reactive metabolites resulting from adrenal nitroreductase activity.

3. **Benznidazole-induced Ultrastructural and Biochemical Alterations in Rat Esophagus.** Rodriguez de Castro C, Montalto de Mecca M, Fanelli SL, de Ferreyra EC, Diaz, EG, Castro JA, Toxicology, 191:189-198 (2003).

Methods
Sprague-Dawley rats were administered a single dose of 100 mg/kg benznidazole (gift from Hoffman La Roche) or vehicle (1% carboxymethylcellulose) by intragastric administration, then animals were euthanized at different time points following dosing and esophageal samples were rapidly excised and processed. Benznidazole content and benznidazole nitroreductase activity was determined in esophageal tissue samples. Other esophageal samples were fixed and examined with transmission electron microscopy.

Results
Benznidazole was shown to be present in esophageal samples in nmol/L concentrations after intragastric administration. Like in the adrenal epithelial cells, esophageal epithelial cells were shown to metabolize benznidazole to active metabolites and measurable benznidazole nitroreaductase activity was detected. The polyhedral cells of the esophagus from benznidazole-teated rats at 24 hours post treatment contained accumulations of polyribosomes as conglomerates and as rosette-like formations, and the number of ribosomes per polysomes were increased compared to controls.

4. **Benznidazole-induced Ultrastructural and Biochemical Alterations in Rat Colon.** Diaz EG, Rodriguez de Castro C, Montalto de Mecca M, and Castro JA: Acta Pharmacol Sin, 21:961-966 (2000).

Methods
Male Sprague-Dawley rats were administered single intragastric doses of 100 mg/kg [^{14}C]benznidazole (gift from Hoffman La Roche). Animals were euthanized at several timepoints after administration and whole colon samples were excised for determination of benznidazole content in colonic tissue, metabolism studies, measurement of covalent binding of benznidazole metabolites to colonic cell constituents, and transmission electron microscopy.

Results
As in other tissues, benznidazole reductase was active, and reactive metabolites were formed. In epithelial cells of colonic mucosa in rats 24 hours after administration of 100 mg/kg oral benznidazole, microvilli were shortened and irregularly distributed. Epithelial nuclei were convoluted with margination of chromatin, enlargement of nucleoli, and contained numerous vacuoles. In the cytoplasm, endoplasmic reticulum and Golgi were dilated. Goblet cells were hypertrophic and characterized by an increase in mucus-secreting globules occupying a larger portion of the cytoplasm.

5. **Benznidazole Biotransformation in Rat Heart Microsomal Fraction Without Observable Ultrastructural Alterations: Comparison to Nifurtimox-induced Cardiac Effects.** Montalto de Mecca M, Bartel LC, Rodriguez de Castro C, Castro JA, Mem Inst Oswaldo Cruz, Rio de Janeiro, 103:549-553 (2008).

Methods
Male Sprague-Dawley rats were administered a single intragastric dose of 100 mg/kg benznidazole or 1% carboxymethylcellulose. At different times (1, 3, and 6 hours) after administration, heart samples were collected and examined for benznidazole content, benznidazole nitroreductase activity, and using transmission electron microscopy.

Results
In heart tissue from rats administered single doses of 100 mg/kg benznidazole, measureable concentrations of benznidazole, and nitroreductive biotransformation in the cytosol of heart cells was detected. However, unlike in other tissued, ultrastructural changes in heart tissue were not observed .

6.2 Repeat-Dose Toxicity

Repeated-dose toxicology studies include a 26-week toxicology study in rats that is reviewed below and several literature reports which are summarized below. In three studies in dogs (Scharer, 1972; Flores-Vieira *et al.*, 1997a and 1997b) benzindazole was shown to produce dose-dependent neurological toxicity. The benznidazole-induced neurological toxicity was observed in dogs but similar findings were not reported in studies with rabbits, guinea pigs, rats, or mice at systemically toxic doses. In humans,

treatment with benznidazole has been associated with paresthesia and peripheral neuritis.

1. **Selective Purkinje Cell Damage in Dogs After Oral Administration of High Doses of Nitroimidazole Derivatives.** Scharer K: Verhandlungen der Deutschen Gesellschaft fur Pathologie, 56:407-10 (1972)

Methods
Dogs and other species were administered multiple different doses and repeated-dose regimens of benznidazole (R0 7-1051), then observed for clinical signs. Subsequently, brain samples were obtained and fixed for histopathology analysis.

Results
Four dogs receiving 50 mg/kg/day benznidazole for 8-20 days and 3 dogs receiving 100 mg/kg for 7 days exhibited strong contractions in back and leg muscles, opisthotonos with nystagmus proceeding to death. Decreased Purkinje cells and degenerative Purkinje cells (cell swelling, vacuolization, and aggregation of protoplasm) were observed in the cerebellem of afflicted dogs. However, neurological toxicity was not observed in four dogs receiving 25 mg/kg/day benznidazole for 30 days. In separate repeated-dose studies with mice (16 days of dosing with 400 mg/kg/day), rats (10 days with 1000 mg/kg/day), guinea pig (14 days of dosing with 400 mg/kg/day), rabbit (14 days of dosing with 400 mg/kg/day), benznidazole did not produce clinical or histological findings indicative of neurological toxicity.

Reviewer Comment: *This five page published paper did not include detailed methods even omitting the strain of dog that was used.*

2. **Experimental Benznidazole Encephalopathy: I Clinical-Neurological Alterations.** Flores-Vieira CLL and Barreira AA, J Neurol Sci, 150:3-11 (1997a).

Methods
Benznidazole was administered orally BID to male mongrel dogs in doses ranging from 5 to 40 mg/kg/day for multiple dosing durations ranging from 7 days to over a year. During dosing, dogs were evaluated neurologically for mental status, locomotion, postural reflexes, and cranial nerve dysfunction according to a standardized method.

Results
Neurological signs associated with 15-days treatment with 30 mg/kg/day benznidazole or 7-days treatment with 40 mg/kg/day benznidazole included apathy, hypertonia, hyperreflexia, ataxia, loss of balance, oscillatory movements of the trunk and head, and asymmetrical gait persisting to necropsy or death. A 20 mg/kg/day dose of benznidazole administered for longer periods produced similar neurological signs later in the course of therapy. Lower doses produced reduced toxicity indicating dose dependency. After discontinuation of dosing, neurological signs were significantly reduced, but a complete reversal did not occur.

3. **Experimental Benznidazole Encephalopathy: II Electroencephalographic and Morphological Alterations.** Flores-Vieira CLL, Chimelli, L, Fernandez RMF, and Barreira AA, J Neurol Sci, 150:13-25 (1997b).

Methods
Benznidazole was administered orally BID to male mongrel dogs in doses of 30 mg/kg/day for 15 days followed by doses of 10 mg/kg/day for an additional 15 days (Group I), or in Group 2, doses of 40 mg/kg/day for 7 days followed by chronic treatment at lower doses of 20 and 5 mg/kg/day for over a year. At different times during the treatment, dogs received EEG evaluations. Following euthanasia, sections of brain, spinal cord, and peripheral nerves (tibial, fibular, dorsal interdigital nerves of the 2nd and 3rd digits) were obtained for histological analysis.

Results
Dose-dependent changes in EEG measurements ranging from moderate to severe occurred in dogs administered benznidazole. Brain histopathology associated with the neurological signs included: lymphocyte infiltrates, focal neuronal degeneration, necrosis, demyelination, satellitosis, early cavitation, microglial proliferation, and vascular proliferation. Focal neuronal degeneration and satellitosis of the spinal cord was observed. Axonal degeneration, hemorrhagic foci, spongy degeneration, and Purkinge cell degeneration were also observed, but no changes were seen in peripheral nerves.

Study title: Benznidazole - 26-week oral (twice daily gavage, 6 hours apart) toxicity study in the Wistar rat followed by a 4-week treatment-free period.

Study no.:	AB20563
Study report location:	Electronic transmission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	July 16, 2014
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Benznidazole, Lot # 00015, purity of 99.7%. The benznidazole was supplied by Chemo France and manufactured by (b) (4).

Key Study Findings
- Benznidazole produced a number of tissue-specific toxicities at doses of 30 and 100 mg/kg/day. The NOAEL value for this study was considered to be 10 mg/kg/day for both male and female rats.
- Testes and epididymides were target organs for benznidazole with toxicity including dose-dependent tubular atrophy, peritubular interstitial edema and accumulation of syncytial/degenerate sperm in seminiferous tubules in testes and decreased tubular sperm or aspermia in epididymides. After a 1-month recovery

period, only minor reversal of the testicular and epididymal changes was observed.

- Other organs were also affected in a dose-dependent manner including liver (minimal centrilobular hepatocellular hypertrophy), kidney (eosinophilic droplets and karyomegaly in tubular epithelial cells), pituitary gland (vacuolation in the pars distalis), spleen (increased incidence of extramedullary hematopoiesis and hemosiderin deposits), and thyroid gland (minimal follicular hypertrophy). After a 1-month recovery period, only the liver changes were completely reversed.

Methods

Doses:	0, 10, 30, and 100 mg/kg/day
Frequency of dosing:	BID
Route of administration:	Oral gavage
Dose volume:	5 ml/dose
Formulation/Vehicle:	1% carboxymethylcellulose
Species/Strain:	Wistar rat: Crl:WI (Han)
Number/Sex/Group:	20/sex/group for the Main Study
Age:	At initiation of treatment: 6 weeks
Weight:	At initiation of treatment: 137 to 157 grams for males and 132 to 156 grams body weight for females.
Satellite groups:	1. Toxicokinetic animals: 3/sex/group for the vehicle control group and 6/sex/group for all other groups. 2. Recovery animals: 10/sex/group for Group 1 (vehicle control) and Group 4 (high-dose).
Unique study design:	Wistar rats (20/sex/group for the Main Study) were administered benznidazole by oral gavage twice per day for 26 weeks. Main Study animals were euthanized after 26 weeks of dosing and Recovery animals were euthanized 4 weeks later.
Deviation from study protocol:	Multiple protocol deviations were noted, but none was considered to have altered the results or compromised the integrity of the study.

Observations and Results

Table 18: Schedule of Observations for the 26-Week, Oral-Dose Study in Rats.

Observations	Schedule
Mortality/morbidity	All animals were observed at least twice daily. Main Study and Recovery Study animals found dead, judged to be in moribund condition or sacrificed for ethical reasons were necropsied.
Clinical signs	Animals were observed daily. During the Main Study, the animals were observed before and at least once after each dose. Animals were observed once daily during the Recovery Study. A full

<table>
<tr><td></td><td>clinical examination was performed at least once weekly during the Main and Recovery studies.</td></tr>
<tr><td>Ophthalmology</td><td>Ophthalmology measurements were performed in Main Study control and high-dose animals during Weeks 13 and 26.</td></tr>
<tr><td>Body Weight</td><td>Body weights were obtained at the time of randomization, prior to dosing, then once weekly during the Main and Recovery studies.</td></tr>
<tr><td>Food Consumption</td><td>Food consumption was measured weekly for each cage of animals during the Main and Recovery studies and reported as grams/animal/day.</td></tr>
<tr><td>Clinical Pathology</td><td>Blood samples were obtained for hematology, coagulation, and serum chemistry measurements in Main Study animals during Weeks 13 and 26 and in Recovery animals on Day 210.

Urine was collected in individual metabolism cages for 12 hours from fasted animals in Main Study animals during Weeks 13 and 26.</td></tr>
<tr><td>Toxicokinetics</td><td>Blood was collected on Days 0, 90, and 181 at the timepoints shown below.
<table>
<tr><td>Blood sampling times (hours)</td><td colspan="3">After the first daily dosing</td><td colspan="3">After the second daily dosing</td></tr>
<tr><td></td><td>1</td><td>2</td><td>6[a]</td><td>2</td><td>8</td><td>18</td></tr>
<tr><td>Tube labelling/occasion (corresponding to “x” hours after the first daily dosing)</td><td>1h</td><td>2h</td><td>6h</td><td>8h</td><td>14h</td><td>24h</td></tr>
<tr><td>First 3 control males + females</td><td>+</td><td></td><td>+</td><td></td><td>+</td><td></td></tr>
<tr><td>First 3 treated males + females</td><td>+</td><td></td><td>+</td><td></td><td>+</td><td></td></tr>
<tr><td>Last 3 treated males + females</td><td></td><td>+</td><td></td><td>+</td><td></td><td>+</td></tr>
</table>
[a]: corresponding to within 5 minutes before the second daily dosing.</td></tr>
<tr><td>Necropsy</td><td>Main Study animals were necropsied on Day 185 and Recovery Study animals were necropsied four weeks later on Day 210.</td></tr>
</table>

Mortality

Three benznidazole-treated animals were found dead (one high-dose female on Day 148) or euthanized due to morbidity (one mid-dose female on Day 10 and on high-dose female on Day 150). However, the causes of death were either not clear and not clearly related to benznidazole administration or due to a cause (head trauma) that was not related to benznidazole administration.

Clinical Signs

In the Main Study, all high-dose animals were observed to hypersalivate after dosing with incidences ranging from once to ten times for individual animals. Also fewer high-dose animals demonstrated lower incidences of abnormal foraging and/or pedaling compared to control animals.

Body Weights

Body weight gain was significantly reduced in high-dose females from Day 113 to Day 181. On Day 181 the mean difference in body weight gain between the high dose females and control females was -13.61 grams. Weight gain in the high-dose females was similar to control values during the Recovery period.

Feed Consumption
Reportedly food consumption was not altered by benznidazole administration at any dose.

Ophthalmoscopy
Examination of the adnexa, optic media and fundus was performed by indirect ophthalmoscopy.

No benznidazole-related ophthalmological findings were observed.

ECG: Not performed

Hematology and Coagulation Parameters
The hematology and coagulation parameters that were assessed are listed in Table 65 in the Appendix.

Hematology Parameters: A persistent trend in both males and females on Days 94 and 185 was reduced RBCs, hemoglobin and packed cell volume and increased reticulocytes (percent and absolute values) and platelets. The effects were dose-dependent and generally significant for high-dose animals compared to control values (Table 19 and Table 20). However, although statistically significant, the magnitude of the changes for most of the parameters was low suggesting little biological relevance. The benznidazole-related hematology changes that achieved a meaningful ≥ 10% difference in high-dose animals compared to control values on Day 94 were increases in absolute reticulocytes in males, and relative reticulocytes in males and females, and decreases in absolute monocytes in males and females and relative monocytes in males. On Day 185 the hematology parameters that were changed by 10% or more in high-dose animals included: increased absolute and relative reticulocytes in males and females and decreased absolute monocytes in males and females and relative monocytes in males. The pattern of increased reticulocytes coupled with low magnitude decreases in red blood cells suggests red blood cell regeneration following a reduction in red blood cells caused by high-dose benznidazole. The results suggest a similar benznidazole-mediated reduction of monocytes. For both RBCs and monocytes, the severity of the benznidzole effect was not sufficient to have overwhelmed the regenerative mechanisms. At the end of the Recovery Period, absolute and relative reticulocytes were decreased in high-dose females by more than 10% relative to control values and monocyte numbers were similar to control values (Table 21). These results suggest a homeostatic response upon cessation of benznidazole dosing to the earlier increase in reticulocyte numbers.

Blood platelets were also increased 9.9% on Day 94 and 9.2% on Day 185 in high-dose males but not females compared to control values (Table 20). The changes in platelets

were consistent with similar increases in prothrombin time, suggesting a potential for benznidazole to stimulate blood clotting. However, given no increase in platelets in high-dose females and decreases in activated partial thromboplastin time (APTT), the relevance of the platelet and prothrombin findings is not clear.

Table 19: Mean Group Values for the Hematology Parameters that were Significantly Changed by Benznidazole Administration Compared to Control Values on Day 94.

Parameter	Sex	Mean Group Values [a]			
		Group 1	Group 2	Group 3	Group 4
Red Blood Cells (T/L)	Male	9.059	9.098	9.000	8.699* (-4.0%)
	Female	8.216	8.064	8.089	7.962* (-3.1%)
Hemoglobin (T/L)	Male	161.0	159.8	159.8	154.2** (-4.2%)
	Female	152.6	150.9	151.8	149.3* (-2.2%)
Packed Cell Volume (%)	Male	49.49	48.84	48.68	47.26** (-4.5%)
	Female	46.18	46.01	46.13	45.45
Absolute Reticulocytes (Giga/L)	Male	204.31	214.64	217.21	247.97** (+21.4%)
	Female	228.88	223.60	250.16	248.49
Relative Reticulocytes (%)	Male	2.26	2.35	2.43	2.86** (+26.5%)
	Female	2.80	2.78	3.09* (+10.4%)	3.14* (+12.1%)
Platelets (Giga/L)	Male	822.5	819.8	893.8* (+8.7%)	904.2** (+9.9%)
	Female	823.7	824.4	848.5	859.8
Absolute Monocytes (Giga/L)	Male	0.188	0.166	0.153	0.142** (-24.5%)
	Female	0.115	0.086	0.115	0.088*(-23.5%)
Relative Monocytes (%)	Male	2.73	2.48	2.26* (-17.2%)	2.13* (-22.0%)
	Female	2.28	2.13	2.78	2.02

Group 1 = vehicle control; Group 2 = 10 mg/kg/day benznidazole; Group 3 = 30 mg/kg/day benznidazole; Group 4 = 100 mg/kg/day benznidazole.

[a] The percent change relative to vehicle-control values is shown in parentheses.

* $p \leq 0.05$

** $p \leq 0.01$

Table 20: Mean Group Values for the Hematology Parameters that were Significantly Changed by Benznidazole Administration Compared to Control Values on Day 185.

Parameter	Sex	Mean Group Values [a]			
		Group 1	Group 2	Group 3	Group 4
Red Blood Cells (T/L)	Male	8.868	9.053	8.778	8.638
	Female	8.216	8.010* (-2.5%)	8.084* (-1.6%)	7.815** (-4.9%)
Hemoglobin (T/L)	Male	159.2	161.6	159.7	155.7* (-2.6%)
	Female	157.4	155.0	155.4	149.8** (-4.8%)
Packed Cell Volume (%)	Male	48.17	49.08	47.47	46.87* (-2.7%)
	Female	46.39	45.62	45.96	44.41** (-4.3%)
Absolute Reticulocytes (Giga/L)	Male	183.70	190.50	189.85	219.44** (+19.5%)
	Female	191.80	180.80	195.84	256.41** (+33.7%)
Relative Reticulocytes (%)	Male	2.08	2.13	2.17	2.56** (+23.1%)
	Female	2.34	2.26	2.43	3.29** (+40.6%)
Platelets (Giga/L)	Male	878.9	856.0	924.2	959.5* (+9.2%)
	Female	844.3	850.2	844.0	851.2
Absolute Monocytes	Male	0.206	0.212	0.203	0.205

(Giga/L)	Female	0.188	0.166	0.153	0.142** (-24.5%)
Relative Monocytes (%)	Male	3.15	3.35	3.14	3.17
	Female	2.73	2.48	2.26* (-17.2%)	2.13* (-22.0%)

Group 1 = vehicle control; Group 2 = 10 mg/kg/day benznidazole; Group 3 = 30 mg/kg/day benznidazole; Group 4 = 100 mg/kg/day benznidazole.
[a] The percent change relative to vehicle-control values is shown in parentheses.
* $p \leq 0.05$
** $p \leq 0.01$

Table 21: Mean Group Values for the Hematology Parameters that were Significantly Changed by Benznidazole Administration Compared to Control Values on Day 210 (End of Recovery)

Parameter	Sex	Mean Group Values [a]	
		Group 1	Group 4
Absolute Reticulocytes (Giga/L)	Male	173.65	181.74
	Female	180.12	156.22* (-13.3%)
Absolute Reticulocytes (%)	Male	1.92	1.97
	Female	2.27	1.95* (-14.1%)

Group 1 = vehicle control; Group 4 = 100 mg/kg/day benznidazole.
[a] The percent change relative to vehicle-control values is shown in parentheses.
* $p \leq 0.05$
** $p \leq 0.01$

Coagulation Parameters: Plasma APTT was significantly decreased by 6.7% in high-dose males but not females at the interim measurement on Day 94. Other coagulation parameters, prothrombin, and fibrinogen were significantly increased by 8.1 and 12.3% respectively in high-dose males but not females on Day 94. At the end of dosing, on Day 185, APTT remained decreased by 8.0% and prothrombin remained elevated by 5.8% compared to control values in high-dose males but not females (Table 22). Generally APTT and prothrombin time, which both measure the time for blood to clot, change in the same direction. The results suggest a complicated effect where benznidazole increases the speed of clotting mediated by the extrinsic pathway (measured by prothrombin time), but not the speed of clotting mediated by the intrinsic pathway (measured by APTT). Given that the effects were of relatively modest magnitude and did not occur in female rats, the biological relevance of this finding is not clear. Coagulation parameters were not measured at the end of the recovery period (Day 210).

Table 22: Mean Group Values for the Coagulation Parameters that were Significantly Changed by Benznidazole Administration Compared to Control Values on Days 94 and 185.

Parameter	Sex	Mean Group Values [a]			
		Group 1	Group 2	Group 3	Group 4
Day 94					
APTT	Male	16.55	16.27	16.03	15.44** (-6.7%)
	Female	15.07	14.86	14.96	14.83
Prothrombin	Male	22.11	21.50	22.60	23.90** (+8.1%)
	Female	22.70	22.68	23.05	23.13

Fibrinogen	Male	3.0475	3.1730	3.2916* (+8.0%)	3.4212** (+12.3%)
	Female	2.4249	2.3820	2.4197	2.3142
Day 185					
APTT	Male	16.54	16.63	16.70	15.21** (-8.0%)
	Female	15.40	15.07	14.94	15.39
Prothrombin	Male	22.09	22.05	22.30	23.44* (+5.8%)
	Female	22.35	22.77	22.47	22.52

Group 1 = vehicle control; Group 2 = 10 mg/kg/day benznidazole; Group 3 = 30 mg/kg/day benznidazole; Group 4 = 100 mg/kg/day benznidazole.
[a] The percent change relative to vehicle-control values is shown in parentheses.
* $p \leq 0.05$
** $p \leq 0.01$

Clinical Chemistry
The clinical chemistry parameters that were assessed are listed in Table 66 in the Appendix.

Multiple clinical chemistry parameters were significantly altered compared to control values in a dose-related manner on Days 94 (Table 23) and 185 (Table 24) in male and female rats. The changes were often modest and did not present a clear toxicity pattern but some changes (increased serum urea and creatinine) were consistent with kidney toxicity. Liver enzyme activity was suppressed on both Day 94 and 185 compared to control values. This finding may indicate reduced liver activity, but it is not consistent with liver damage which usually is accompanied by increased liver enzyme activity. On Day 94 in high-dose males and females, serum calcium, phosphorus, creatinine (males only), and albumin (males only) were significantly increased and serum glucose (males only) and the liver enzymes, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), lactate dehydrogenase (LDH), and creatinine phosphokinase (CK) were significantly decreased in a benznidazole dose-dependent manner (Table 23).

Table 23: The Mean Group Values for the Serum Chemistry Parameters that were Significantly Changed by Benznidazole Administration Compared to Control Values on Days 94.

Parameter	Sex	Mean Group Values [a]			
		Group 1	Group 2	Group 3	Group 4
Calcium (mmol/L)	Male	2.535	2.553	2.590* (+2.2%)	2.614** (+3.1%)
	Female	2.552	2.619** (+2.6%)	2.632** (+3.1%)	2.624** (+2.8%)
Phosphorus (mmol/L)	Male	1.951	2.019	2.146** (+10.0%)	2.209** (+13.2%)
	Female	1.696	1.702	1.756	1.814* (+7.0%)
Glucose (mmol/L)	Male	7.672	7.740	7.945	6.416** (+16.4%)
	Female	5.611	6.218*	6.084	5.733
Creatinine (mcmol/L)	Male	43.4	43.7	45.6	47.7** (+9.9%)
	Female	46.6	46.5	46.5	47.5
Cholesterol (mmol/L)	Male	1.543	1.614	1.627	1.603
	Female	1.550	1.581	1.722	1.890** (+21.9%)
Protein (g/L)	Male	64.99	64.95	65.22	66.12
	Female	67.57	69.56	69.11	69.98* (+3.6%)
Albumin	Male	33.92	34.33	33.99	35.22** (+3.8%)

(g/L)	Female	37.84	39.11	38.36	39.28* (+3.8%)
ASAT (IU/L)	Male	97.1	92.5	84.6** (-12.9%)	76.3** (-21.4%)
	Female	95.0	73.6** (-22.5%)	72.0** (-24.2%)	67.1** (-29.4%)
ALAT (IU/L)	Male	28.9	25.9	23.1** (-20.1%)	19.6** (-32.2%)
	Female	21.3	17.9** (-16.0%)	17.4** (-18.3%)	15.8** (-25.8%)
LDH (IU/L)	Male	1887.0	1754.3	1536.7	970.7** (-48.6%)
	Female	1716.4	1243.2** (-27.6%)	1007.3** (-41.3%)	956.4** (-44.3%)
Creatinine Phosphokinase (IU/L)	Male	602.0	573.7	569.9	332.3* (-44.8%)
	Female	583.8	457.8** (-21.6%)	381.0** (-34.7%)	379.9** (-34.9%)

Group 1 = vehicle control; Group 2 = 10 mg/kg/day benznidazole; Group 3 = 30 mg/kg/day benznidazole; Group 4 = 100 mg/kg/day benznidazole.
[a] The percent change relative to vehicle-control values is shown in parentheses.
* $p \leq 0.05$
** $p \leq 0.01$

On Day 185, in high-dose males and females serum calcium, phosphorus, urea (males only), creatinine (males only), total bilirubin, protein and albumin were significantly increased and serum glucose and the liver enzymes, ASAT, ALAT, LDH, and CK were significantly decreased in a benznidazole dose-dependent manner. In addition in high-dose females, serum cholesterol was significantly increased and triglycerides were significantly decreased (Table 24).

Table 24: The Mean Group Values for the Serum Chemistry Parameters that were Significantly Changed by Benznidazole Administration Compared to Control Values on Days 185.

Parameter	Sex	Group Values [a]			
		Group 1	Group 2	Group 3	Group 4
Calcium (mmol/L)	Male	2.533	2.575	2.600** (+2.6%)	2.644** (+4.4%)
	Female	2.582	2.614	2.657** (+2.9%)	2.657** (+2.9%)
Phosphorus (mmol/L)	Male	1.782	1.785	1.894* (+6.3%)	2.051* (+15.1%)
	Female	1.545	1.605	1.736** (+12.4%)	1.767** (+14.4%)
Glucose (mmol/L)	Male	8.074	8.189	7.597	6.714** (-16.8%)
	Female	6.098	6.810*	6.191	5.921
Urea (mmol/L)	Male	4.424	4.397	4.618	5.073** (+14.7%)
	Female	6.156	6.370	6.560	6.662
Creatinine (mcmol/L)	Male	42.9	43.0	45.1* (+5.1%)	47.6** (+11.0%)
	Female	49.3	50.0	48.9	52.2
Cholesterol (mmol/L)	Male	1.805	1.879	1.878	1.912
	Female	1.789	1.806	2.003	2.362** (+32.0%)
Triglycerides (mmol/L)	Male	1.488	1.731	1.901	1.393
	Female	0.730	0.740	0.556* (-23.8%)	0.545* (-25.3%)
Total Bilirubin (mcmol/L)	Male	2.41	2.70* (+12.0%)	2.57* (+6.2%)	2.62* (+8.7%)
	Female	2.80	3.05	3.27* (+16.8%)	3.28* (+17.1%)
Protein (g/L)	Male	66.21	67.19	67.14	68.73** (+3.8%)
	Female	69.74	70.58	70.56	72.51** (+4.0%)
Albumin (g/L)	Male	34.06	34.99* (+2.7%)	34.55* (+1.4%)	35.96** (+5.6%)
	Female	38.63	39.61	39.21	40.79** (+5.6%)
ASAT (IU/L)	Male	104.3	100.8	87.4* (-16.2%)	88.9** (-14.8%)
	Female	99.9	83.6** (-16.3%)	75.4** (-24.5%)	77.3** (-22.6%)

ALAT (IU/L)	Male	33.3	34.5	23.9** (-28.2%)	24.4** (-26.7%)
	Female	25.6	22.3* (-12.9%)	19.4** (-24.2%)	18.6** (-27.3%)
LDH (IU/L)	Male	2194.7	1747.9* (-20.4%)	1621.7** (-26.1%)	1182.2** (-46.1%)
	Female	1822.7	1360.9* (-25.3%)	952.1** (-47.8%)	1026.7** (-43.7%)
Creatinine Phosphokinase (IU/L)	Male	674.2	619.2	681.6	409.7* (-39.2%)
	Female	573.0	418.9* (-26.9%)	332.2** (-42.0%)	346.0** (-39.6%)

Group 1 = vehicle control; Group 2 = 10 mg/kg/day benznidazole; Group 3 = 30 mg/kg/day benznidazole; Group 4 = 100 mg/kg/day benznidazole.
a The percent change relative to vehicle-control values is shown in parentheses.
* $p \leq 0.05$
** $p \leq 0.01$

After the recovery period, serum potassium, urea, and creatinine were significantly increased in high-dose males compared to vehicle-control males. In high-dose females, ASAT, LDH and CK remained significantly reduced compared to vehicle control females.

Table 25: Mean Group Values for the Serum Chemistry Parameters that were Significantly Changed by Benznidazole Administration Compared to Control Values on Day 210 (End of Recovery).

Parameter	Sex	Group Values [a]	
		Group 1	Group 4
Potassium (mmol/L)	Male	4.43	4.68* (+5.6%)
	Female	3.97	4.12
Urea (mmol/L)	Male	4.787	5.607* (+17.1%)
	Female	6.157	6.426
Creatinine (mcmol/L)	Male	42.5	47.8** (+12.5%)
	Female	49.8	51.2
ASAT (IU/L)	Male	96.4	100.4
	Female	82.9	72.7* (-12.3%)
LDH (IU/L)	Male	2159.7	1695.4
	Female	1157.5	794.0** (-31.4%)
Creatinine phosphokinase (IU/L)	Male	844.3	981.2
	Female	434.4	308.3* (-29.0%)

Group 1 = vehicle control; Group 4 = 100 mg/kg/day benznidazole.
a The percent change relative to vehicle-control values is shown in parentheses.
* $p \leq 0.05$
** $p \leq 0.01$

Urinalysis
The following urinalysis parameters were examined: pH, protein, glucose, ketones, urobilinogen, bilirubin, blood, and leucocytes.

Urine volume significantly increased (+ 19.5%) compared to the vehicle control mean value and specific gravity and urine pH significantly decreased 0.18% and -3.9%

respectively in a benznidazole dose-dependent manner. However, these changes were not considered to be toxicologically important.

Gross Pathology
Gross pathology findings attributed to benznidazole administration included bilaterally small testes in all high-dose males and 3/20 males receiving 30 mg/kg/day benznidazole. In the same animals, epididymides were bilaterally small. After the 1-month recovery period, testes and epididymides were still small in 9/10 high-dose males. The small testes correlated with lower absolute and relative testes weights and benznidazole-related atrophic changes in seminiferous tubules, peritubular edema, and syncytial/degenerate spermatozoa. The small epididymides correlated with lower absolute and relative epididymides weights and decrease sperm or aspermia in the epididymides.

Also alopecia foci were observed in several body surface areas in all groups for males and females receiving benznidazole with a dose-dependent increase in incidence. After the recovery period, alopecia was still observed in 2 low-dose and 4 high-dose females.

Organ Weights
The organs that were weighed are listed in Table 67 in the Appendix.

Multiple organ weights were changed in association with benznidazole administration (Table 26). At the end of dosing, significant benznidazole-related organ weight loss was observed in male reproductive organs including testes (-68% absolute weight, -67% relative weight), epididymides (-41% absolute weight, -40% relative weight), and to a lesser extent, prostate (-12 absolute weight and -11 relative weight). The effects were dose related and significant. During the recovery period the organ weight losses for all three organs only partially reversed. The decreased organ weights correlated with histopathology findings of atrophy of the seminiferous tubules, peritubular edema, syncytial/degenerate sperm, and in the epididymides, the lower weight correlated with decreased sperm content or aspermia.

Mean values for the absolute and relative liver weights were significantly higher for high dose males and females compared to control values (11-24% increase) during dosing. The higher liver weights occurred in a dose-dependent manner and correlated with minimal hepatocellular hypertrophy. After the recovery period, the liver weight increases were smaller but still elevated by 5-12%.

Mean absolute and relative spleen weights were also significantly increased in all the male benznidazole-dose groups and in high-dose females. The increased spleen weights correlated with an increased incidence and/or severity of extramedullary hematopoiesis and hemosiderin deposits. Spleen weight increases were smaller relative to vehicle control values after the recovery period, but still elevated indicating incomplete reversal.

Kidney absolute and relative weights were significantly increased in all the male benznidazole dose groups. In contrast female kidney weights were not significantly

different than control values. The increased kidney weights in males correlated with a histological finding of cytoplasmic accumulation of eosinophilic droplets in renal epithelium. After the recovery period, male kidney weights were no longer higher than control values.

Absolute and relative weights for adrenal glands were increased in high-dose males at the end of dosing (mean absolute increase of 14%), and the weights increased further after the recovery period (mean absolute increase of 34%). Females were not similarly affected after dosing or after recovery.

Absolute and relative thyroid glands were generally higher than controls in both sexes, but the differences were not significant. However, the slight increases correlated to a slightly higher incidence of follicular cell hypertrophy in the thyroid of both sexes.

In high-dose males, absolute and relative pituitary weights were higher than control values, but pituitary weights were similar to control values for males in the other dose groups and for females in all dose groups. Pituitary weight elevations for high-dose males after the recovery period were further increased relative to the weight increases during dosing.

Table 26: Organ Weight Differences from Control Values as a Percentage.
(Sponsor's Table)

	Males				Females			
	Day 184			Day 210	Day 184			Day 210
Dosage (mg/kg/day):	2x5	2x15	2x50	2x50	2x5	2x15	2x50	2x50
Necropsy body weight (g)	=	+5	-2	-3	=	-2	-7	-2
Testes								
Mean absolute weight (g)	+3	-8	-68	-55	na	na	na	na
Mean relative weight	+3	-13	-67	-54	na	na	na	na
Epididymides								
Mean absolute weight (g)	+5	-8	-41	-42	na	na	na	na
Mean relative weight	+6	-13	-40	-41	na	na	na	na
Prostate gland								
Mean absolute weight (g)	=	+1	-12	-12	na	na	na	na
Mean relative weight	=	-3	-11	-10	na	na	na	na
Liver								
Mean absolute weight (g)	+1	+10	+22	+10	-3	+2	+11	+5
Mean relative weight	+2	+5	+24	+12	-3	+4	+19	+7
Spleen								
Mean absolute weight (g)	+7	+10	+18	+17	=	+4	+9	+5
Mean relative weight	+7	+5	+20	+21	+1	+6	+17	+7
Adrenal gland								
Mean absolute weight (g)	+1	-3	+14	+34	-13	-9	-6	+4
Mean relative weight	+1	-9	+17	+36	-13	-7	+1	+5

Table 26 continued

Kidneys								
Mean absolute weight (g)	+9	+16	+18	+5	-7	-11	-8	+5
Mean relative weight	+9	+11	+19	+7	-6	-8	=	+7
Thyroid gland								
Mean absolute weight (g)	-5	+6	+6	-6	+2	+7	+9	+15
Mean relative weight	-4	+1	+8	-3	+1	+8	+15	+18
Pituitary gland								
Mean absolute weight (g)	-8	-2	+8	+15	-4	-4	-7	-16
Mean relative weight	-8	-7	+9	+17	-4	-2	=	-14

na: not applicable; =: difference lower than 0.5

Histopathology

Adequate Battery: Yes as listed in Table 67 in the Appendix.

Peer Review: Yes

Histological Findings

Benznidazole-related histopathology findings were noted in multiple organs including the testes, epididymides, liver, kidney, spleen, pituitary gland, thyroid gland, and ovaries as summarized below.

Testes: In the testes, treatment-related histopathological changes included dose-related tubular atrophy, peritubular interstitial edema, and accumulation of syncytial/degenerate sperm in seminiferous tubules (Table 27). All high-dose males were affected along with 7/20 mid-dose males and 2/20 low-dose males. After the recovery period, the severity of testes histopathology was only slightly reduced consistent with minimal recovery.

Epididymides: in the epididymides at the end of treatment, aspermia was observed in all high-dose males. Also minimal to markedly decreased tubular sperm content was observe in 7 mid-dose males. At both doses, the decreased sperm was accompanied by an increased amount of degenerate/syncytial sperm in epididymides. No benznidazole-related effects were observed in epididymides in low-dose males. Only minor reversal was observed after the recovery period.

Liver: Minimal centrilobular hepatocellular hypertrophy was observe in 7/20 and 4/18 high-dose males and females respectively. At the end of the recovery period, none of the high-dose animals exhibited hepatocellular hypertrophy consistent with complete recovery.

Table 27: Incidence and Severity of Benznidazole-related Histopathology Changes in Testes. (Sponsor's Table)

Text Table 2. Testes - Incidence of Treatment-related Histopathologic Findings					
	Males				
	Day 184				Day 210
Dosage (mg/kg/day):	0	2x5	2x15	2x50	2x50
Testes [a]	20	20	20	20	10
Tubular atrophy	0	2	7	20	10
Minimal	-	2	3	-	3
Slight	-	-	4	-	3
Moderate	-	-	-	-	4
Marked	-	-	-	12	-
Severe	-	-	-	8	-
Oedema, peritubular	0	0	2	19	7
Minimal	-	-	2	11	5
Slight	-	-	-	8	2
Syncytial/degenerate sperms	0	0	4	15	6
Minimal	-	-	4	9	6
Slight	-	-	-	5	-
Moderate	-	-	-	1	-

[a] = Number of tissues examined from each group. -: finding not recorded in the group

Kidney: At the end of treatment, dose-dependent cytoplasmic accumulation of eosinophilic droplets and karyomegaly was observed in epithelial cells lining the proximal convoluted tubules. Eosinophilic droplets were more pronounced in males which were affected at all doses with dose-dependent severity. High-dose males displayed slight or moderate severity. In females only mid- and high-dose animals were affected and at a lower incidence and severity than males. Droplet accumulation was observed in the cortical region of the kidneys, and in both sexes in mid- and high-dose animals, droplets were accompanied by minimal or slight karyomegaly in epithelial cells lining the proximal convoluted tubules with the highest severity in males. At the end of the recovery period, tubular accumulation of eosinophilic droplets was no longer observed, but karyomegaly in cortical tubules was still observed in all 10 high-dose males and in 3/10 high-dose females.

Pituitary Gland: Benznidazole-related vacuolation in the pars distalis of the pituitary gland was observed at a minimal severity in 7/20 high-dose males. Pituitary vacuolation was still observed in 2/10 high-dose males at the end of recovery.

Spleen: An increased incidence in extramedullary hematopoiesis and hemosiderin deposits in high-dose males and mid- and high-dose females was observed in at the end of treatment compared to vehicle control values (Table 28). At the end of the recovery period, hemosiderin deposits were still observed in 4/10 and 5/10 high-dose males and females respectively.

Table 28: Incidence and Severity of Benznidazole-related Histopathology Changes in Spleen at the End of Dosing. (Sponsor's Table)

	Males				Females			
Dosage (mg/kg/day):	0	2x5	2x15	2x50	0	2x5	2x15	2x50
Spleen [a]	20	20	20	20	20	20	20	20
Extramedullary hematopoiesis	4	4	5	9	2	4	8	10
Minimal	2	4	5	6	2	4	6	8
Slight	2	-	-	2	-	-	2	2
Moderate	-	-	-	1	-	-	-	-
Hemosiderin-type deposits, increased	0	2	3	5	1	0	5	7
Minimal	-	2	3	4	1	-	5	6
Slight	-	-	-	1	-	-	-	1

[a] = Number of tissues examined from each group.

Thyroid gland: At the end of treatment, minimal follicular hypertrophy was observed at a higher incidence in high-dose males compared to control animals (Table 29). In females the pattern was less clear. At the end of recovery, only one high-dose male was affected.

Table 29: Incidence and Severity of Benznidazole-related Histopathology Changes in Thyroid at the End of Dosing. (Sponsor's Table)

	Males				Females			
Dosage (mg/kg/day):	0	2x5	2x15	2x50	0	2x5	2x15	2x50
Thyroid gland [a]	20	20	20	20	20	20	20	20
Follicular cell hypertrophy	3	3	4	9	-	3	-	4
Minimal	3	3	4	9	-	3	-	4

[a] = Number of tissues examined from each group.

Ovaries: At the end of the treatment period, 5/20 high-dose females and 1/20 control female demonstrated a marked decrease in the size and number of luteal bodies which was generally accompanied by unilateral or bilateral follicular cysts. Ovaries were not examined in recovery animals.

Special Evaluation: No special evaluations were performed.

Toxicokinetics

Plasma AUC and C_{max} values increased a roughly dose-dependent manner and there were no apparent sex-related differences in any of the toxicokinetic parameters. The results indicate that benznidazole did not substantially accumulate with repeated dosing (Table 30).

Table 30: Mean Toxicokinetic Parameters for the 26-Week Rat Study (Sponsor's Table)

Gender		Male			Female		
Day		0	91	181	0	91	181
Dose (mg/kg/day)	TK Parameters	Mean					
10	AUC_{last} (µg*h/mL)	37.3	61.1	66.5	44.4	56.6	61.5
	C_{max1} (µg/mL)	3.61	5.52	5.27	4.57	7.31	7.32
	T_{max1} (h)	1	2	1	1	1	1
	C_{max2} (µg/mL)	5.45	6.67	6.92	5.94	6.26	6.98
	T_{max2} (h)	2	2	2	2	2	2
30	AUC_{last} (µg*h/mL)	133	163	159	139	188	195
	C_{max1} (µg/mL)	15.9	15.6	15.4	14.3	18.0	15.9
	T_{max1} (h)	1	2	1	1	1	2
	C_{max2} (µg/mL)	17.1	17.7	16.1	17.3	18.7	20.4
	T_{max2} (h)	2	2	2	2	2	2
100	AUC_{last} (µg*h/mL)	528	592	706	615	575	585
	C_{max1} (µg/mL)	34.1	44.5	54.4	42.1	47.8	49.5
	T_{max1} (h)	2	1	2	2	2	2
	C_{max2} (µg/mL)	42.5	52.2	48.1	43.6	48.9	44.2
	T_{max2} (h)	2	2	2	2	2	2

C_{max1} and C_{max2} are following the first and second daily doses, respectively.
T_{max1} and T_{max2} are following the first and second daily doses, respectively.

Dosing Solution Analysis

Dosing solution preparations were analyzed for achieved concentrations and homogeneity on the first day of treatment and during Weeks 6, 10, 18, and 24. Assessments of homogeneity utilized samples obtained from the top, middle and bottom level from each formulation preparation.

All the actual concentrations for the benznidazole dosing solutions were within ± 15% compared of the nominal concentrations. Generally the preparations were also within the acceptance criteria for homogeneity.

7 Genetic Toxicology

No study reports for any genetic toxicity studies were included in the meeting package. However, several studies have been published in the literature with results indicating benznidazole was mutagenic in an Ames test (Ferreira and Ferreira, 1986; Nagel, 1987), but produced mixed results in a chromosome aberration and sister chromatid exchange assays in human lymphocytes and in a sister chromatid exchange and micronucleus assay in a human hepatoma cell line (Santos *et al.*, 1994), and in micronucleus assays using bone marrow from rats and mice (Sousa *et al.*, 1991). In these assays, benznidazole increased sister chromatid exchange in both cell systems and increased the frequency of micronuclei in hepatoma cells. However, benznidazole did not consistently increase the frequency of chromosome aberrations in peripheral blood cells or micronuclei in bone marrow cells from healthy rats or healthy and chagasic mice treated acutely (rats and mice) or chronically (mice) with benznidazole. In an assessment of cytogenetic damage in chagasic children before and after treatment with benznidazole, the incidence of micronucleated lymphocytes and chromosome aberrations both significantly increased approximately two fold after benznidazole treatment (Gorla *et al.*, 1988). Limitations for these studies include a lack of GLP compliance, lack of clear validation criteria, and use of dose ranges that did not always include a maximum tolerated or maximum feasible dose or concentration.

1. **Mutagenicity of Nifurtimox and Benznidazole in the Salmonella/Microsome Assay.** Ferreira RCC and Ferreira LCS: Braz. J. Med. Biol. Res., 1986, 19:19-25.

Methods
The *Salmonella typhimurium* strain TA100 was tested in an Ames test with multiple benznidazole concentrations ranging from 0 to 400 mcg with and without S9 activation. Incubations were carried out for 48 hours at 37°C.

Results
The concentration of benznidazole associated with 50% or greater cytotoxicity was 30 mcg/plate. Benznidazole produced a dose-dependent increase in revertant TA100 colonies (Figure 2). At a concentration not associated with ≥ 50% cytotoxicity, 5 mg/plate, benznidazole produced an average 177% increase in revertants without S9 activation and 171% with S9 activation compared to control values (Table 31). A positive control agent, 6 mcg/plate aflatoxin B1 produced an average 531% increase in revertants with S9 activation compared to vehicle control values.

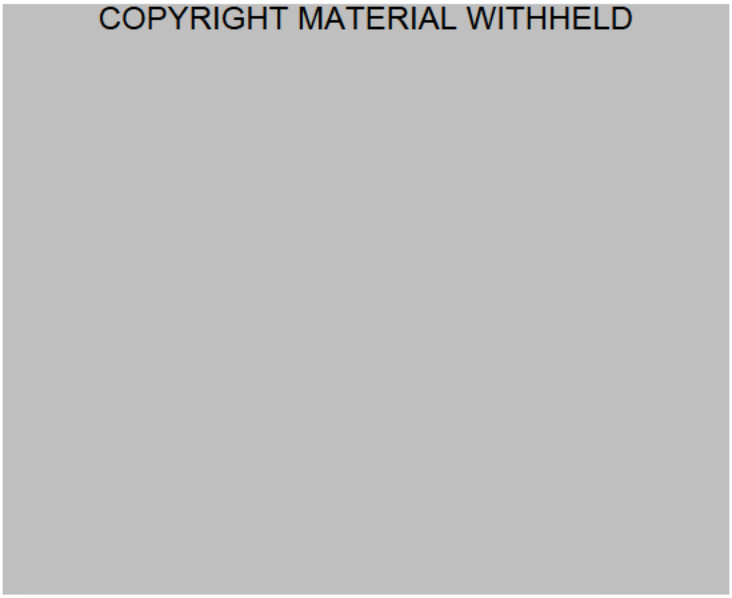

Figure 2: Mutagenicity Activity of Benznidazole with the Salmonella Strain TA100. Average values from three separate experiments are shown. (Manuscript Figure)

Table 31: Mutagenicity (number of revertants/plate) for Subcytotoxic Concentrations of Benznidazole. (Manuscript Table)

Drug	Concentration	S9 mixture	Number of revertants
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2. **Mutagenicity of 2 Anti-Chagasic Drugs and Their Metabolic Deactivation.** Nagel R and Nepomnaschy I: Mutation Research, 117:237-242 (1983).

Methods

Ames assays were performed with benznidazole and nifurtimox in *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98, and TA100. For the TA100 strain, incubations with benznidazole were conducted with and without S9 activation. Incubation mixtures of bacteria, different concentrations of benznidazole or nifurtimox plus (TA100 only) or minus S9 activation mixture were incubated for 25 minutes at 37°C before plating. Positive-control agents were 2-aminoantracene (2AA) and methylmethansulfonate (MMS). Benznidazole was tested in concentrations of 0, 50, 100, 250, 500, and 1000 mcM.

Results

Benznidazole produced a dose-dependent increase in revertant numbers in all five *S. typhimurium* strains with the greatest increases occurring in TA100 (Table 32). The addition of S9 to the TA100 incubations reduced the number of benznidazole-related revertants compared to incubations without S9 activation, but revertant values with S9 activation were still increased by several-hundred fold compared to control values.

Table 32: Mean Number of his+ Revertants per Plate Induced by Benznidazole. (Manuscript Table)

Concentration Strain

3. **Genotoxicity Studies with Two Antichagasic Drugs.** Nagel R: Mut Res. 1987; 191, 17- 20.

Methods: Benznidazole was assessed in an Ames assay using *Salmonella typhimurium* strains TA100 uvrB and UTH8414 rfa. Cells were incubated for approximately 46 hours with benznidazole concentrations of 0, 25, 50, 100, 500, 1000, and 2500 mcg/plate with and without S9 metabolic activation. Concurrent untreated and solvent controls were performed, but a positive control was not included.

Results: Benznidazole was found to be mutagenic in both the TA100 and UTH8414 strains with a dose-dependent increase in the number of his+ revertants. The mean number of revertants produced by a 500 mcM concentration of benznidazole in TA100 was reduced with the addition of mammalian S9 (2420 revertants) compared to incubations minus S9 (3168 revertants) by approximately 20%. However the number of revertants for both conditions was many-fold higher than mean vehicle-control values (approximately 150 revertants).

4. **Screening the Mutagenic Activities of Commonly Used Antiparsite Drugs by Simultest, A Simplified Salmonaell/Microsome Plate Incorporation Assay.** Melo MEB and Ferreira LCS: Rev Inst Med Trop Sao Paulo, 32: 269-274 (1990)

Methods
Using an Ames test format (Simultest) allowing simultaneous qualitative (spot) testing with multiple bacterial strains, benznidazole and other drugs were tested for mutagenicity. Simultest testing was performed with *Salmonella tiphimurium* strains, TA97, TA98, TA100, and TA102 incubated on agar plates with addition of different concentrations of benznidazole and other drugs for 48 hours at 37°C with and without S9 activation. In a follow-up experiment, benznidazole was incubated with TA100 and a mixture of all four strains in a traditional Ames test to provide quantitative measurements of revertant colonies.

Results
Benznidazole produced qualitatively positive results in the pool of *S. tiphimurium* strains in the Simultest assay with and without the addition of S9. In the follow-up Ames assay, benznidazole produced a dose-dependent increase in the number of TA100 revertants and strain pool revertants at concentrations ranging from 0 to 75 mcg/plate (Figure 3). The assay was somewhat more sensitive with TA100 alone versus the pool of four *S. tiphimurium* strains.

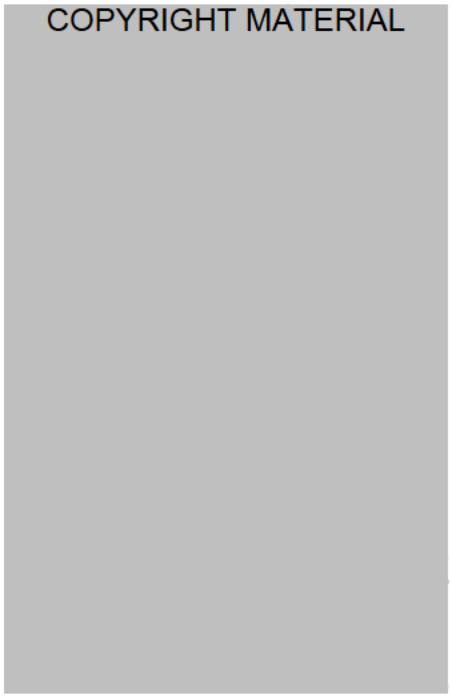


Figure 3: Dose response curve of benznidazole in plate incorporation assays. The mutagenic activities were based on the number of His+ colonies per plate determined by the TA100 strain (o) and the strain pool (Δ) without addition of S9 mix. (Manuscript Figure)

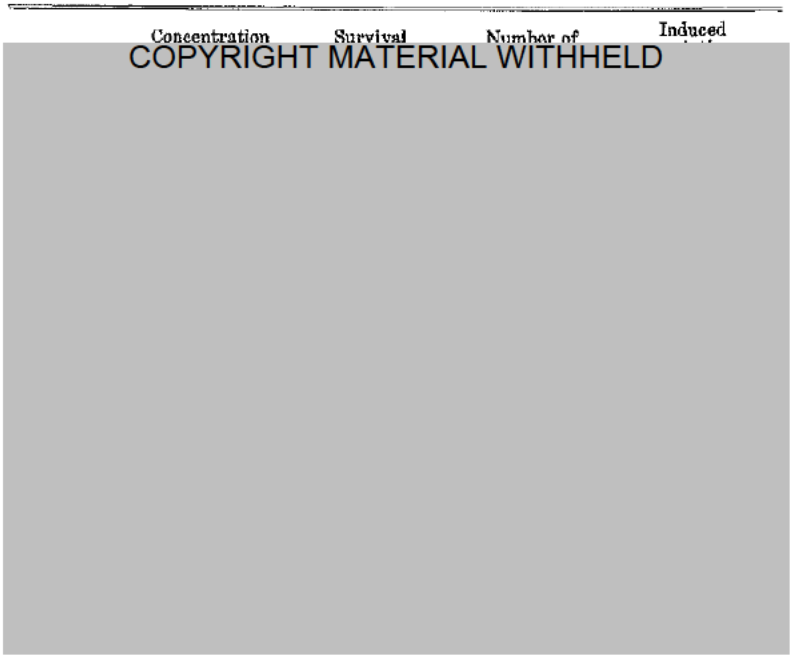
5. **Mutagenicity of Anti-trypanosomal Drug, Ro 7-1051 in Escherichia coli.** Ohnishi T, Ohashi Y, Nozu K, and Inoki S: Jpn J Genet, 58:505-509 (1983).

Methods

Benznidazole (Ro 7-1051, Radanil®) was tested in an Ames test using three strains of *Escherichia coli* (H/r30R, Hs30R, and NG30) at concentrations ranging from 0 to 50 mcg/ml.

Results
Benznidazole increased the number of revertants (arginine independence) in two of the three *E. coli* strains approximately 5-fold above control values (Table 33).

Table 33: Mutation Results with Benznidazole in Three Strains of *E. coli*.
(Manuscript Table)



6. **The Mutagenic Action of Nitroimidazoles, II. Effects of 2-Nitroimidazoles.** Voogd CE, Van Der Stel JJ, and Jacobs JJJAA, Mutation Research, 31:149-152 (1975).

Methods
Benznidazole (Ro-7-1051) was tested for mutagenesis in a modified Ames test using a Klebsiella pneumoniae mutant requiring uracil and proline for growth. Test plates were incubated for 3 days at 37°C using a benznidazole concentration range of 0 (vehicle control) to 1 mM.

Results
Benznidazole produced a dose-dependent over 7-fold increase in mutation frequency.

7. **Cytogenetic Effects of the Antichagasic Benznidazole on Human Cells in vitro.** Santos SS, Takahashi, CS, Natarajan AT, Mutation Research, 320: 305-314 (1994).

Methods

Benznidazole was tested in human lymphocytes and a human hepatoma cell line (Hep G2) for its ability to induce chromosome aberrations (lymphocytes), micronucleus formation (HepG2 cells) and sister chromatid exchange (both cell types). Human peripheral blood lymphocytes were obtained from 5 healthy donors and lymphocyutes were cultured with benznidazole concentrations of 0, 20, 50, 100 mcg/ml for 48 hours at 37°C for chromosome aberration analysis and for 72 hours at 37°C for analysis of sister chromosome exchange. Mitotic arrest was achieved by adding 0.08 mcg/ml colchicine 2 hours before harvest and 5-bromo-2-deoxyuridine (BrdU, 10 mcg/ml) was added to the cultures intended for analysis of sister chromatid exchange. Hep G2 cells were incubated with benznidazole concentrations of 0 (1% DMSO), 20, 50, 100, and 200 mcg/ml or 150 and 300 mcM cyclophosphamide (positive control) for 1 hour, washed twice, then grown in complete media until harvest after 24 hours for micronucleus analysis and at 48 hours for analysis of sister chromatid exchange. For micronucleus analysis, 3 mg/ml cytochalasin B was added to the 24 hour cultures 4 hours after the start of the incubation. At the end of the incubation, cells were fixed on slides and 1000 binucleate cells were scored for each treatment for micronuclei. The 48 hour treatments were treated and processed in a manner similar to the lymphocyte cultures for analysis of sister chromatid exchange.

Results
In human lymphocytes, benznidazole did not induce chromosome aberrations at any concentration, but sister chromosome exchange was significantly induced at benznidazole concentrations of 50 and 100 mcg/ml although values plateaued above 50 mcg/ml.

In Hep G2 cells, benznidazole significantly increased micronucleus formation and sister chromatid exchange in a dose-dependent manner.

8. **Micronucleus Formation in Bone Marrow of Mice Treated with Nifurtimox or Benznidazole.** Gorla NB and Castro JA, Toxicology Letters, 25:259-263 (1985).

Methods
Male Swiss mice, 9-11 weeks old were treated with oral or ip nifurtimox (600, 1200, and 2000 mg/kg) and oral or ip benznidazole (850 and 2000 mg/kg) administered in two doses 24 hours apart. Each treatment group included 2-9 mice. From each animal, 1000 polychromatic bone-marrow cells were obtained and processed for micronucleus analysis.

Results
Benznidazole administered in two oral doses of 850 and 2000 mg/kg or two intraperitoneal doses of 200 mg/kg did not significantly increase micronucleus formation in bone marrow cells. Treatment with oral nifurtimox at all tested concentrations (600, 1200, and 2000 mg/kg) significantly increased bone marrow micronucleus formation by at least 2 fold. However nifurtimox administered by the intraperitoneal route in two doses of 1200 mg/kg did not increased micronucleus formation. These results suggest that oral doses of nifurtimox are more effective in producing clastogenic changes

compared to benznidazole, and that intraperitoneal dosing does not result in claustogenic changes in mouse bone marrow cells for either compound. In this study, two doses of benznidazole did not stimulate micronucleus formation in rat bone-marrow cells when administered by either the oral or intraperitoneal routes.

9. **Clastogenic Activity of Two Antichagasic Drugs**. Navarro ML, Dain L, Migliorini AM, and Nagel R, Comunicaciones Biologicas, 3: 25-28 (1984).

Methods
Female hybrid mice (C57BL x C3H; F1), 10 weeks of age, were administered five consecutive daily intraperitoneal doses of 0, 125, 250, and 500 mg/kg benznidazole or 0, 62.5, 125, and 250 mg/kg nifurtimox (n = 4/group) in a vehicle of 1:10 DMSO: corn oil. Bone marrow cells were obtained from mouse femurs 4 hours after the last doses and processed for micronucleus analysis. Approximately 2000 polychromatic erythrocytes were analyzed per animal.

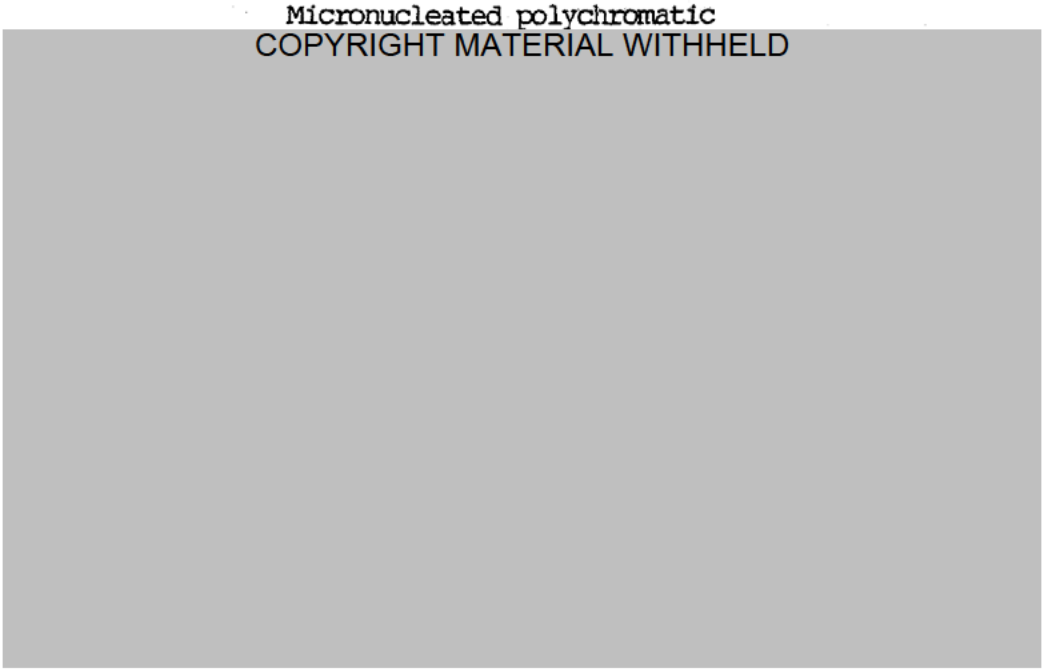
In another chromosome aberration study, human lymphocytes isolated from a normal donor and activated with phytohemagglutinin were incubated with 50 mcM nifurtimox or benznidazole concentrations dissolved in DMSO for 24 hours. After further incubation for 48 hours, cultures were arrested by the addition of colchicine at a final concentration of 500 ng/ml 75 minutes before the completion of the incubation. Lymphocytes were subsequently collected, lysed, fixed, and stained before metaphase analysis for chromosome aberrations.

Results
Both benznidazole and nifurtimox induced significant increases in micronucleus formation in bone marrow cells in a dose-dependent manner (Table 34).

In the chromosome aberration assay, the percentages of chromosome breaks and gaps were respectively 14 and 9 in benznidazole cultures and 16 and 16 in nifurtimox cultures compared to 3 and 9 in control cultures indicating a significant claustogenic effect for both benznidazole and nifurtimox.

Table 34: Percentage of Micronucleated Polychromatic Erythrocytes induced by Nifurtimox and Benznidazole in Mice. (Manuscript Figure)

Micronucleated polychromatic



10. **Evaluation of the Mutagenic Potential of the Antichagasic Drug Rochagan in Healthy and Chagasic Rodents.** Souza SC, Takahashi CS, and da Silva, JS: Mutation Research, 259: 139-45. (1991)

Methods

Rat Single-dose Experiment: Benznidazole (Rochagan) was orally administered to male and female Wistar rats in a single dose of vehicle (water) or 2000 mg/kg benznidazole. Rats (6/timepoint) were euthanized 6, 12, and 24 hours later and bone marrow metaphase cells were examined. Rats were administered ip colchicine 1.5 hours before sacrifice and 50 metaphase cells per animal were examined.

Rat Repeated-dose Experiment: Male and female Wistar rats received oral gavage doses of 150, 300, 1500, and 3000 mg/kg benznidazole TID for one day. After the last dose animals were euthanized 8, 12, and 18 hours later and bone marrow metaphase cells were analyzed (100 metaphases per animal). Rats were administered ip colchicine 1.5 hours before sacrifice.

Mouse Repeated-dose Experiment: Male and female chagasic Balb/c mice (5 per group) were orally administered 100 mg/kg/day benznidazole for 25 days. Mice were treated with ip colchicine 2 hours before sacrifice and bone marrow metaphase cells (100 per animal) were analyzed.

Micronucleus Assay in Mice: Healthy male and female Balb/c mice were orally administered 100 mg/kg/day benznidazole for 10 days. Negative control mice received oral water, and positive control mice receive a single ip dose of 50 mg/kg

cyclophosphamide administered 24 hours before sacrifice. Peripheral blood was obtained from each animal and 1000 normochromatic erythrocytes were analyzed for polychromatic erythrocytes and micronucleated cells.

Results
Rat Single-dose Experiment: Treatment with a single oral dose of 2000 mg/kg benznidazole increased the number of chromosome aberrations in bone marrow cellsin a time-dependent fashion with aberrations increasing by 300, 167, and 133% at 6, 12, and 24 hours after dosing respectively (Table 35).

Table 35: Frequency of Chromosome Aberrations in Bone Marrow Cells of Rats Treated with a Single Oral Gavage Dose of Benznidazole. (Manuscript Table)

Dose (bz)	Time of	Types of aberrations [a]	Total number of cells

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Rat Repeated-dose Experiment: Rats administered oral benznidazole in doses ranging from 150 to 3000 mg/kg TID for one day did not demonstrate significantly increased chromosome aberrations compared to vehicle control rats (Table 36)

Mouse Repeated-dose Experiment: Mice administered oral 100 mg/kg/day benznidazole for 25 days did not demonstrate increased chromosome aberrations compared to vehicle control mice (Table 37).

Table 36: Frequency of Chromosome Aberrations in the Bone Marrow Cells of Rats Treated with Oral Gavage Benznidazole Three Times at 8-h Intervals for 24 hours and Euthanized at 8, 12, and 18 hours after the Last Administration.
(Manuscript Table)

Dose (bz)	Number of	Time of	Number of	Types of aberrations [a]	Total number of

Table 37: Frequency of Chromosome Aberrations in the Bone Marrow of Normal and Chagasic Mice Treated with Oral Gavage Benznidazole Once per Day for 25 Days and Euthanized at 8, 12, and 18 hours after the Last Administration.
(Manuscript Table)

Group	Treatment	Mitotic	Types of aberrations	Total number of cells

Mouse Micronucleus Assay: Mice administered 100 mg/kg/day benznidazole for 10 days did not demonstrate an increased frequency on micronuclei in peripheral polychromatic erythrocytes compared to vehicle treated animals. In contrast, the positive control agent, cyclophosphamide produced a significant increase in micronucleus cells (Table 38).

Table 38: Micronuclei (MN) in Polychromatic (PCE) and Normochromatic (NCE) Peripheral Blood Erythrocytes From Balb/c Mice Treated by Oral Gavage with 100 mg/kg/day Benznidazole for 10 days. (Manuscript Table)

Treatment	Dose	PCE	MN	MN

11. **Assessment of Cytogenetic Damage in Chagasic Children Treated with Benznidazole.** Gorla NB *et al.*: Mutat Res, 206:217-228 (1988)

Methods

Blood samples from chagasic children ages 2 to 12 years of age in Argentina were obtained before beginning treatment with benznidazole and after being treated with 5 mg/kg benznidazole for 30 days. Chromosomal aberrations in 10 patients and micronuclear cell counts in 20 patients in peripheral lymphocytes were used to evaluate cytogenetic damage. Peripheral blood lymphocytes were incubated in complete media with 3 mcg/ml bromodeoxyuridine (BrdU) for the chromosome analysis or without BrdU for the micronucleus analysis for 72 hours at 37°C with colchicine added for the final 1.5 hours. Structural chromosome damage was analyzed in first-division metaphases identified by uniform staining of sister chromatids. Scored aberrations included: acentric fragments, deletions, dicentrics, translocations, gaps and breaks. For both the chromosomal aberration analysis using 100 cells per subject, and micronucleus analysis using 1000 interphase cells per subject, slides were randomized, coded, and scored blind. Statistical analysis was performed with non-parametric, Wilcoxon 2-sample test.

Results

Chromosomal aberrations before treatment with benznidazole ranged from 0 to 8 per 100 cells with a median value of 3. Cells collected after benznidazole treatment contained 2-9 chromosomal aberrations/100 cells with a median of 6. The difference was significantly different. The micronucleus results were variable before treatment ranging from 1-15 micronuclei per 1000 interphase cells with a median value of 5 compared to a median value of 11.5 micronuclei per 1000 interphase cells (range of 4-24) after benznidazole treatment. The results after benznidazole treatment were significantly greater than before treatment for both chromosome aberrations and the frequency of micronuclei (Table 39). These results suggest benznidazole may be genotoxic in humans. Because the subjects were suffering from Chagas disease and received benznidazole later in the disease progression compared to control measurements, it is not absolutely clear if the increases in chromosome aberrations and micronuclei resulted from benznidazole treatment or were due to other factors associated with the disease progression.

Table 39: Chromosome Aberrations and Micronuclei Frequency in Blood Lymphocytes in 2 Groups of Chagasic Children After and Before Benznidazole (Bz) Treatment. (Manuscript Table)

Patients	Chromosomal aberrations/100 cells [b]	Frequency of micronuclei/1000 cells

8 Carcinogenicity

Nonclinical carcinogenicity studies are not recommended for benznidazole, because benznidazole is intended for a relatively short 60-day course of treatment, and carcinogenicity studies are normally recommended only for drugs that are intended for ≥ 6-months of dosing. However, an understanding of the carcinogenic potential for benznidazole is of interest because benznidazole has been shown to be genotoxic in some nonclinical assays. The carcinogenic potential for benznidazole has not been examined in nonclinical carcinogenicity studies conducted by the Sponsor or in controlled clinical trials, but some nonclinical carcinogenicity data has been published in the literature.

In published animal studies in mice and rabbits, benznidazole has been linked to non-Hodgkin's lymphoma. In a mouse study, benznidazole in daily doses of 8 mg/kg/day for 15, 30 or 60 days increased the incidence of lymphomas proportionally to the duration of dosing (Teixeira *et al., 1994)*. In another study, mice infected with *trypanosoma cruzi and* treated with 100 mg/kg/day benznidazole administered by oral gavage for 5 days a week for 90 days, malignant non-Hodgkin's lymphoma developed in 1/31 treated mice compared to no incidence in control animals (Andrade *et al.*, 2003). In a rabbit study, in animals infected with Chagas and treated with 8 mg/kg/day intraperitoneal benznidazole for 60 days beginning 5 days before infection or 6-months after infection, more than 30% of animals presented with lymphoma compared to no animals with lymphoma in control groups (Teixeira *et al.*, 1990a). In another rabbit study, more than 40% of rabbits infected with *trypanosoma cruzi* and treated with intraperitoneal benznidazole presented with lymphoma growths (Teixeira *et al.*, 1990b).

These results suggest a carcinogenicity potential for benznidazole, but the studies are limited by a lack of GLP compliance and positive carcinogenicity results only in association with intraperitoneal dosing as opposed to the oral route of dosing for clinical administration. Due to these limitations, the results of these studies will not be included on the product label. However, because another approved drug in the same nitroimidazole class, metronidazole, has been shown to be carcinogenic in mice and rats, a carcinogenic potential for benznidazole based its classification in the nitroimidazole class of drugs will be included on the product label.

1. **Chagas Disease. Carcinogenic Activity of the Antitrypanosomal Nitroarenes in Mice.** Teixeira *et al.*: Mutat Res, 1994, 305:189-196.

Methods

Three nitroarenes (MK-346, nifurtimox and benznidazole) were administered intraperitoneally to female Swiss mice at clinical doses for different durations. For benznidazole, the daily dose was 8 mg/kg/day for 15, 30 or 60 days. Control mice received daily injections of 0.15 M saline for 15, 30, and 60 days. The number of mice in each group was 25-30 mice. At the end of treatment, each mouse underwent a complete necropsy and all organs and tissues were examined for gross lesions.

Results

Mice receiving benznidazole for 30 and 60 days demonstrated significantly reduced survival compared to control mice (Table 40) and a significantly increased incidence of lymphomas (Table 40, Figure 4). Survival decreased and the incidence of lymphomas increased with the duration of benznidazole dosing.

Table 40: Carcinogenic Effects of Benznidazole and Two other Nitroarenes in Mice. (Manuscript Table)

Drugs Days Proportions of growths

Figure 4: Proportions of Lymphomas in Groups of Mice Receiving Daily Injections With Antitypanosomal Nitroarenes including Benznidazole. Significant differences at the 5% level between groups are shown by proportion bars that do not intersect. (Figure from the Manuscript)

2. **Treatment with Benznidazole in Association with Immunosuppressive Drugs in Mice Chronically Infected with Trypanosoma cruzi: Investigation into the Possible Development of Neoplasms.** Andrade *et al.*: Rec Soc Bras Med Trop, 2003, 36:441-447.

Methods: Swiss mice were treated in 8 groups as shown below (Table 41). The mice that were treated with benznidazole received oral gavage doses of 100 mg/kg/day for 5 days a week for 90 days. Immunosuppressive drugs consisted of: azathioprine, betamethazone, and cyclosporin. When administered concomitantly with immunosuppressive drugs, benznidazole treatment began 4 weeks after the immunosuppressive treatment. Measured endpoints included white blood counts and histopathology.

Table 41: Study Design for Andrade *et al.*, 2003

		Number of

Results: In Group 2, infected mice treated with oral benznidazole, 1/31 mice presented with neoplastic proliferation of lymphoid cells of the spleen, histologically characterized as a malignant non-Hodgkins's lymphoma. None of the animals in the other groups, including Group 6 (uninfected mice treated with benznidazole) presented with neoplasms. These results suggest a low potential for oral benznidazole to stimulate lymphoma in mice in the absence of immunosuppression.

3. **Malignant Non-Hodgkins Lymphoma in Trypanosoma cruzi-infected Rabbits Treated with Nitoarenes.** Teixeira *et al.*, J Comp Path, 1990a, 103:37-48.

Methods
New Zealand white male and female rabbits (outbred, 2 months old, n = 8/group) were infected with *Typanosoma cruzi*, and then treated with intraperitoneal injections of nitroarenes including benznidazole which was administered at a dose of 8 mg/kg/day for 60 days. Other non-infected rabbits also received the same course of treatment with benznidazole.

Results
A higher incidence of lymphoma and other neoplasms occurred in chagasic rabbits treated with benznidazole (3/7 rabbits with lymphoma) and non-infected rabbits treated with benznidazole (2/6 rabbits with lymphoma and 1/6 rabbits with adenocarcinoma of the colon) compared to untreated control rabbits (no neoplasms) (Table 42). These results suggest that treatment with intraperitoneal benznidazole at a dose similar to the clinical dose range (5-10 mg/kg/day) stimulates the occurrence of lymphomas in rabbits. Also both groups of rabbits treated with benznidazole had significantly reduced days of survival after treatment compared to control rabbits.

Table 42: Effect of Benznidazole Administration to Chagasic and Un-Infected Rabbits. (Manuscript Table)

Rabbit	*Sex*	*T. cruzi**	*Histopathological findings*	*Survival† (days*

Reviewer Comment: *A deficiency in the study design for this study is that control rabbits did not receive sham intraperitoneal injections or injections with vehicle.*

4. **Chagas Disease Lymphoma Growth in Rabbits Treated with Benznidazole.** Teixeira *et al.*, Am J Trop Med Hyg, 1990b, 43:146-158.

Methods: New Zealand White rabbits were treated as shown in the table below. The infected rabbits received subcutaneous administrations of 10^6 trypomastigotes (*trypanosoma cruzi*)/kg body weight. Benznidazole was administered by the intraperitoneal route.

Table 43: Study Design for the Teixeira *et al.*, 1990b paper. (Manuscript Table)

		Number of

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Results: In Group B (infected animals treated with benznidazole-acute phase) 3/7 animals presented with lymphoma. In Group D (infected animals treated with benznidazole-chronic phase), 3/8 animals presented with lymphoma, and in Group F (immunized animals treated with benznidazole), 2/6 animals presented with lymphoma and 1/6 animals presented with adenocarcinoma of the bowel with lung metastasis. No lymphomas were reported for control animals in Groups A, C, E, and G.

9 Reproductive and Developmental Toxicology

Benznidazole-related toxicity resulting in testicular atrophy and/or impair spermatogenesis has been reported in the literature in mice and rats (Bernacchi *et al*., 1986; Vieira *et al.*, 1989; and Navarro and Nagel, 1990) and in the 26-week rat study submitted in support of this NDA application. Because of this available data, it was recommended that the applicant conduct a male fertility study in rats as a postmarketing requirement.

The literature reports describing testicular toxicity in rodents are summarized below. Additional studies examining the effect of benznidazole on female fertility in rats, embryo-fetal toxicity in rats and rabbits, and pre-postnatal toxicity in rats were conducted by the Sponsor in support of this NDA application, and the study reports for these studies are reviewed below.

9.1 Fertility and Early Embryonic Development

1. **Effects of Nifurtimox or Benznidazole Administration on Rat Testes: Ultrastructural Observations and Biochemical Studies.** Bernacchi AS, de Castro CR, de Toranzo EG, Castro JA, Exp Mol Pathol 45:245-256 (1986).

Methods
Male Sprague Dawley rats were orally administered a single dose of 100 mg/kg nifurtimox or benznidazole in 1% carboxymethlycellulose. Animals were euthanized at different times following administration and testes were obtained for different histological assessments including ultrastructural changes in testes using electron microscopy.

Results

The following ultrastructural changes in testicular Sertoli cells were noted following nifurtimox or benznidazole treatment: alternations in the shape of nuclei and mitochondria. Dilated and some disruption of mitochondria cristae, increased numbers of lysosomes, more sparse and electron lucent cytoplasm, and severely disrupted endoplasmic reticulum. In spermatids, the formation of the acrosomal cap appeared disrupted, and spermatozoa were altered exhibiting unusual shapes and configurations. Leydig cells were not affected, and reportedly nifurtimox produced more pronounced effects than benznidazole.

2. **Testes Alterations in Pubertal Benznidazole-treated Rats.** Vieira CL, Lamano-Carvalho TL, Favaretto AL, Valenca MM, Antunes-Rodridgues J, Barreira AA, Braz J Med Biol Res, 22:695-698 (1989).

Methods
Male Wistar rats (n = 5/group) were treated with 80 mg/kg/day benznidazole or vehicle (carboxypolymethylene-carbopol gel) for 30 days On the last day of treatment, animals were euthanized and blood was collected for measurement of plasma testosterone. Also testes, seminal vesicles and prostate glands were removed and weighed, then fixed and processed for histological examination. The degree of germinal epithelial maturity was determined from 100 cross sections of seminiferous tubules per animal, randomly analyzed and scored.

Results
Treatment with benznidazole did not affect plasma testosterone levels, or seminal vesicle or prostate weights. In contrast the relative weights of testes were significantly reduced in benidazole-treated rats (0.25 ± 0.02 g/100g body weight) compared to control rats (0.44 ± 0.02 g/100g body weight). Associated histopathology included the presence of numerous atrophic tubules and depletion of germinal epithelium. Also the degree of maturity of germinal epithelium was reduced in benznidazole-treated rats with approximately 25% of tubular sections exhibiting spermatogenic arrest, and approximately 33% of tubular sections with only a small amount of mature spermatids. The results indicate that benznidazole did not affect the andogenic activity of rat testes, but did separately interfere with spermatogenesis.

3. **Abnormal Sperm Induced in Mice by Oral Administration of Antichagasic Drugs.** Navarro ML and Nagel R, Comunicaciones Biologicas, 8:251-258 (1990).

Methods
Male C57BL x C3H F1 mice age 11 weeks old (n = 8/group) were administered 8 consecutive daily doses of benznidazole (doses of 0, 125, 250, 500, and 1000 mg/kg/day in 0.5% carboxymethylcellulose) by oral gavage. Four mice from each dose group were euthanized in Weeks 4 and 11 after administration. Sperm collected from the cauda epididima from each two mice were pooled and examined for sperm abnormalities.

Results

Treatment with the high-dose (1000 mg/kg/day) of benznidazole produced an approximately 48% decrease in total sperm counts on both euthanasia days. The percent of abnormally shaped sperm also increased by as much as five-fold and in a dose-dependent manner on both euthanasia days. The effects were more pronounced 4 weeks after the end of dosing compared to 11 weeks after the end of dosing indicating a trend toward reversibility.

4. **Pituitary-Testicular Axis in Benznidazole-treated Rats.** Favaretto AL, Antunes-Rodriques J, Vieira CL, Lamano-Carvalho TL, Braz J Med Biol Res, 23:719-722 (1990).

Methods
Pubertal male Wistar rats were orally administered benznidazole in doses of 10 (n =5), 40 (n = 5), and 80 (n = 17) mg/kg/day or vehicle (carboxypolymethylenecarbopol gel, n = 17) for 30 days. On Day 31, all the animals in the lower dose groups and 12 animals each in the vehicle-control and high-dose groups were euthanized. The remaining 5 animals in the vehicle control and high-dose groups were allowed to recover for an additional 90 days. Following euthanization, blood samples were collected and plasma testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin levels were measured.

Results
Benznidazole treatment at all of the dose levels did not significantly change plasma testosterone, LH, or prolactin levels at the primary euthanasia timepoint or after the recovery period compared to vehicle control values. In contrast, plasma FSH levels were significantly increased in rats receiving 40 (+ 25%) and 80 (+ 84%) mg/kg/day benznidazole. In high-dose animals, FSH levels remained significantly elevated (+ 43%) after the recovery period.

Study title: Benznidazole – Fertility Toxicity Study by the Oral Route (Twice Daily Gavage) in the Female Rat (Segment I) Followed by a 13-Week Treatment-Free Period.

Study no.:	AB21207
Study report location:	Electronic transmission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	March 31, 2016
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Benznidazole, lot # 00019 (102607/14009743), purity of 99.6%

Key Study Findings
- Benznidazole at doses as high as 150 mg/kg/day did not alter the estrous cycle or pre-coital interval, or impair pregnancy rates in female rats.

- High-dose benznidazole was associated with significant increases in post-implantation loss, and the mean number of live embryos was significantly decreased in high-dose females.
- The NOAEL was considered to be the mid-dose, 50 mg/kg/day based on the significantly increased post-implantation loss and decreased mean number of live embryos for high-dose females.

Methods

Doses:	0, 15, 50, and 150 mg/kg/day
Frequency of dosing:	BID dosing of half daily doses (2x0, 2x7.5, 2x25, and 2x75 mg/kg/day). 6 hour interval between doses.
Dose volume:	10/ml/kg/day (2x5 mg/kg/day)
Route of administration:	Oral gavage
Formulation/Vehicle:	1% [w/v] Carboxymethylcellulose 400-800 centipoises in water for injection
Species/Strain:	Wistar rat: Crl: WI (Han)
Number/Sex/Group:	20/sex/group
Satellite groups:	10/sex/group
Study design:	Main study females were treated for 14 days prior to mating, throughout mating, and until Gestation Day 7 (GD 7). Male and female animals were paired for a maximum of 14 days. Vaginal smears were obtained daily and used to determine the stage of estrus in each female from one week before the start of dosing until confirmation of mating or separation from the male. The day of mating was confirmed by the presence of sperm in the vaginal smear and that day was recorded as GD 0. Mated females were separated for males once mating had been confirmed and smearing ceased. Any females with no evidence of mating after 7 days were re-paired with a proven male from the same group.
Deviation from study protocol:	Several protocol deviations were noted, but the deviations were not considered to have affected the outcome or integrity of the study.

Observations and Results

Mortality

All animals were observed twice daily for clinical mortality, morbidity.

No unscheduled deaths occurred.

Clinical Signs

Females were observed daily during the study for clinical signs. Also during the treatment period, females were observed once before and once after each treatment to detect abnormalities in appearance and behavior. A full clinical examination was performed weekly.

No benznidazole-related clinical signs were reported.

Body Weight
Individual male body weights were recorded weekly. Individual female body weights were recorded twice weekly during the pre-mating and mating periods and on GDs 0, 4, 8, and 13.

Mid and high-dose females demonstrated a slightly lower (nonsignificant) mean body weight gain (-39% and -30% respectively compared to control values) during the first week of treatment (Days 1-8). However, mean body weight gain up to GD 13 for both groups was comparable to control values.

Feed Consumption
Female food consumption was measured twice weekly during the premating period and on GDs 0-4, 4-8, and 8-13.

A slight but statistically significant reduction in food consumption was noted in mid- and high-dose females compared to controls from Days 1-4 (-7 and -12% for the mid- and high-dose groups respectively) during the pre-mating period. Reduced food consumption persisted for high-dose females from Days 4-8, but thereafter food consumption was similar for all groups.

Toxicokinetics: Not performed.

Dosing Solution Analysis
Samples of the dosing formulations prepared on the first day of treatment and during the last week of the mating period were tested for benznidazole concentration.

The actual concentrations of all the dosing formulations including the vehicle control group except for the Group 3 (5 mg/ml) solution used on the first day of dosing were with the acceptance criteria (± 15%) of the nominal concentrations. For the Group 3 sample in question, the actual concentrations deviated from the nominal concentration in a range of 19.2% to 26.6%. Frozen duplicate samples of the same Group 3 dosing solution were subsequently tested and the actual concentrations in the duplicate sample were within ± 15% of the nominal concentration.

Necropsy
Female rats were euthanized then necropsied for caesarean section on GD 13 for pregnant females or one week after completion of the mating period for females that were considered to be unmated. The body weights of all females were recorded before necropsy. All females were examined macroscopically for structural or pathological changes. The ovaries and uterus of each female was removed and examined along with

the placenta and the pregnancy status, number of corpora lutea, number of intrauterine implantations, and number of intrauterine deaths (resorption sites). The ovaries were weighed paired, and the vagina, uterus, and ovaries of all females were retained along with gross lesions.

Ovary weights were unchanged by benznidazole treatment, and no gross pathology findings in the vagina, uterus, or ovaries or gross lesions were reported.

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

Benznidazole administration did not affect the estrous cycle in any group compared to the control group values. Also, benznidazole had no effect on mating performance. All animals except for two control females mated successfully. The mean pre-coital interval was normal, less than 4 days, in all groups. No effect on fertility was noted; only two low-dose females did not become pregnant.

The caesarian section data (Table 44) indicated an absence of benznidazole-related effects on the number of corpora lutea, the number of implantations, and the percent pre-implantation loss. However, high-dose benznidazole was associated with significant increases in post-implantation loss (mean and mean percent), and the mean number of live embryos was significantly decreased in high-dose females.

Table 44: Mean Caesarian Section Data for the Female Fertility Study. (Sponsor's Table)

Sex: Female Day(s) Relative to Mating (Litter: A)		2x0 mg/kg/day	2x7.5 mg/kg/day	2x25 mg/kg/day	2x75 mg/kg/day
Females Pregnant	N	18	20	18	20
Dams with Viable Embryos		18	20	18	20
No. of Corpora Lutea [GEN AN]	Mean	13.8 R[1]	14.3	14.2	13.5
	SD	1.9	1.7	2.3	3.0
	Sum	248 R[1]	285	255	269
No. of Implantations [GEN AN]	Mean	13.1 R[1]	13.4	12.6	12.8
	SD	2.4	2.3	3.2	3.4
	Sum	236 R[1]	268	227	256
Pre-Implant Loss (%) [KWLWCX]	Mean	5.16	6.14	11.50	5.01
	SD	11.09	12.43	14.19	11.57
	N	18	20	18	20
Post-Implantation Loss [GEN AN]	Mean	0.6 R[1]	0.6	1.0	7.7 SSS[2]
	SD	0.8	1.2	1.0	3.9
	Sum	11 R[1]	11	18	153 SSS[2]
Post-Implantation Loss (%) [KWLWCX]	Mean	4.46 kkk[3]	3.61	7.88	56.47 ddd[4]
	SD	6.32	7.86	8.36	22.69
No. of Live Embryos [GEN AN]	Mean	12.5 I[5]	12.9	11.6	5.2 www[6]
	SD	2.4	2.2	3.1	2.5
	N	18	20	18	20

[GEN AN] - Generalised Anova/Ancova Test
[KWLWCX] - Kruskal Wallis & Wilcoxon
1 [R - Automatic Transformation: Rank]
2 [SSS - Test: Shirley 2 Sided p < 0.001]
3 [kkk - (All Groups) Test: Kruskal-Wallis p < 0.001]
4 [ddd - Test: Dunnett Non-Parametric 2 Sided p < 0.001]
5 [I - Automatic Transformation: Identity (No Transformation)]
6 [www - Test: Williams 2 Sided p < 0.001]

THE NOAEL for female conception was considered to be the high-dose, 150 mg/kg/day. The NOAEL for cesarean section data was considered to be the mid-dose, 50 mg/kg/day based on the significantly increased post-implantation loss and decreased mean number of live embryos for high-dose females.

9.2 Embryonic Fetal Development

Study title: Benznidazole – Embryo-Foetal Toxicity Study by the Oral Route (Gavage) in the rat (Segment II).

Study no.:	AB20567
Study report location:	Electronic transmission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	September 22, 2014
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Benznidazole, Batch No.: 00015, purity of 99.7%

Key Study Findings

- High-dose dams had significantly lower weight gain (-39%) and food consumption (-19%) compared to control females.
- Gravid uterine weight was significantly lower (-25%) in high-dose dams compared to control dams.
- High-dose dams experienced significantly increased early resorptions and mean post-implantation loss.
- Mean fetal weights and live litter size were significantly reduced for high-dose dams compared to control values.
- High-dose fetuses had a higher incidence of total malformations, as well as external, visceral, and skeletal malformations compared to the control group indicating benznidazole was teratogenic in pregnant rats. Also anasarca in one mid-dose fetus is considered to be possibly related to benznidazole administration.

- The NOAEL for maternal toxicity was considered to be the mid-dose of 50 mg/kg/day. The NOAEL dose was associated with maternal plasma C_{max} and AUC values of 42.9 mcg/ml and 357 mcg•hr/ml respectively.
- The NOAEL for fetal toxicity and benznidazole-related malformations was considered to be the low dose of 15 mg/kg/day which was associated with plasma C_{max} and AUC values of 14.7 mcg/ml and 73.6 mcg•hr/ml.

Methods

Doses:	0, 15, 50, and 150 mg/kg/day
Frequency of dosing:	Once daily
Dose volume:	10 ml/kg/day
Route of administration:	Oral
Formulation/Vehicle:	1% carboxymethylcellulose in water
Species/Strain:	Wistar rats: Crl: WI(Han)
Number/Sex/Group:	22 females/group
Satellite groups:	3 females in the vehicle control group and 6 females/group for all the other groups.
Study design:	Vehicle or benznidazole was administered by daily oral gavage at doses of 0, 15, 50, and 150 mg/kg/day to female Wistar rats from gestation days (GD) 6 to 17 inclusive. Clinical condition, body weight, food consumption, Four satellite groups were sampled at specific time-points for toxicokinetics on GDs 6 and 17. Females were euthanized and underwent caesarean section on GD 20 and litter parameters were recorded. At necropsy, the females were examined macroscopically and live fetuses were weighed, sexed, and examined for external abnormalities. Half of the fetuses were examined internally prior to processing for skeletal assessment. The remaining fetuses were fixed for visceral examination of the head for serial sectioning.
Deviation from study protocol:	Multiple deviations from the study protocol were noted but none was considered to have altered the results of the study or compromised the study integrity.

Observations and Results

Mortality

All animals were observed at least twice daily for morbidity and mortality.

No unscheduled deaths occurred.

Clinical Signs

During the treatment period, the animals were observed at least once before and once after dosing for clinical signs. A full clinical examination was performed on each weighing day.

No benznidazole-related clinical signs were reported. Incidental clinical observations included scabs and localized hair loss.

Body Weight
Each female was weighed on Days 0, 6, 9, 12, 15, 18, and 20 of gestation.

High-dose females demonstrated a significant reduction in mean weight gain (-39%) during the dosing period (GD 6 – GD 17) compared to control values, and the effect persisted after dosing until GD 20. The finding was more pronounced for net mean body weight gain following adjustment for the gravid uterus weight (-59% compared with control values). Consequently, absolute mean body weights for high-dose females were significantly lower than in the control group from GD 15 through termination on GD 20.

Feed Consumption
Individual food consumption was measured on GDs 0-6, 6-9, 9-12, 12-15, 15-18, and 18-20 during gestation.

Food consumption was significantly decreased averaging -19% in high-dose females throughout the treatment period from GD 6-18.

Toxicokinetics
Blood was collected for toxicokinetic measurements of plasma benznidazole according to the schedule shown below (Table 45). Plasma benznidazole was measured using a validated HPLC-MSMS method.

Table 45: Toxicokinetic Sampling Schedule. (Sponsor's Table)

Time after dosing (hours)	1	2	6	10	24
3 control females	+			+	
First 3 treated females/treated group	+		+		+
Last 3 treated females/treated group		+		+	

Benznidazole concentrations in all the vehicle control plasma samples were below the limit of quantification. In the benznidazole-treatment groups, plasma AUC exposure increased in a roughly dose-proportional manner while plasma C_{max} values increased in a less than dose-proportional manner. Plasma benznidazole AUC exposures were roughly the same for GDs 6 and 17 indicating that benznidazole did not accumulate in plasma with repeated administration (Table 46). The plasma $t_{1/2}$ values (not shown in the table) reportedly ranged from approximately 2-4 hours with no apparent relationship to dose.

Table 46: The Toxicokinetic Parameters for Plasma Benznidazole in Pregnant Rats. (Sponsor's Table)

Gestation Day	6			17		
Dose (mg/kg/day)	15	50	150	15	50	150
AUC_{last} (µg*h/mL)	69.8	352	894	73.6	357	934
C_{max} (µg/mL)	15.4	41.8	78.9	14.7	42.9	73.2
T_{max} (h)	1	1	2	1	2	2

Dosing Solution Analysis
The actual concentration of benznidazole in all the dosing formulations except for Group 3 (5 mg/ml) was within the acceptance criteria (± 15 %) relative to the nominal concentrations. The original Group 3 sample for dosing Day 1 tested outside the acceptance criteria, but the actual concentration of a frozen duplicate was shown to fall within the acceptance criteria.

Reviewer Comment: *The original test sample for the Group 3 dosing formulation for the first day of dosing was also outside the acceptance criteria for the female fertility study. The two studies (embryo-fetal study in rats and female fertility study) were not conducted at the same time. This appears to be an odd coincidence, but for all of the studies including the 6-month repeated-dose toxicology study and the embryo-fetal study in rabbits, at least one sample did not meet acceptance criteria when first tested. The study reports indicate that sonication was used to mix the sample solutions and that increased sonication time was used in some cases to make sure duplicate test samples were mixed more thoroughly. This practice suggests the dosing solutions were prone to stratification unless mixed thoroughly, and this characteristic may have adversely influenced the concentration measurements for some samples when first tested.*

Necropsy
On GD 20 all females were euthanized, and examined for gross pathology. Abnormal organs or tissues were sampled and preserved. In addition, the ovaries and uterus of each female as well as placentae were removed and examined.

No gross pathology related to benznidazole administration was noted. Incidental gross pathology included: mass on the left uterine horn, pale liver, sore/crust and/or alopecia in individual females.

Cesarean Section Data (Implantation Sites, Pre- and Post-Implantation Loss, etc.)
The following parameters were recorded: pregnancy status, gravid uterus weight, number of corpora lutea, number of implantations, number and distribution of live fetuses. Also the number and distribution of embryonic/fetal deaths were classified as follows: early with only the placenta visible at termination, late with both the placenta and embryonic tissue visible at termination, dead fetus. Fetuses were weighed and their sexes determined. Toxicokinetic animals, upon completion of blood collection, were euthanized and discarded without necropsy following verification of pregnancy status.

Gravid uterine weight was significantly lower (approximately -25%) in high-dose females compared to control values. Caesarian section data is shown in Table 47. Changes in several caesarian-section parameters were attributed to benznidazole and occurred in dose-related manner. High-dose females demonstrated significantly increased mean early resorptions (number and percent) and mean post-implantation loss (number and percent). The mean fetal weights for total fetuses and for the individual sexes were significantly lower compared to control values (Table 47). Also the mean live-litter size for high-dose females (8.2 fetuses/litter) was lower than for the concurrent control group (10.3 fetuses/litter).

Table 47: Mean Caesarean Section Data for the Rat Embryo-fetal Study. (Sponsor's Table)

Sex: Female Day(s) Relative to Mating (1)		0 mg/kg/day	15 mg/kg/day	50 mg/kg/day	150 mg/kg/day
No. of Pregnant Females	N	20	19	19	20
Dams with Viable Foetuses		20	19	18	19
No. of Corpora Lutea	Mean	11.8 R[1]	13.2	11.8	12.1
	SD	2.5	2.0	2.8	2.1
	Sum	235	250	224	241
No. of Implantations	Mean	11.2 R[1]	12.1	11.0	11.4
	SD	3.0	2.7	2.9	2.7
	Sum	223	229	209	228
Pre-Implantation Loss	Mean	0.60 R[1]	1.11	0.79	0.65
	SD	1.31	1.41	1.62	1.09
	Sum	12.0	21.0	15.0	13.0
Pre-Implantation Loss (%)	Mean	6.21	9.00	6.11	6.46
	SD	16.45	11.95	12.46	13.40
No. of Early Resorptions	Mean	0.8 R[1]	0.7	0.9	3.0 SS[2]
	SD	0.9	1.0	0.9	3.0
	Sum	16	13	18	59
Early Resorptions (%)	Mean	6.35 kk[3]	5.62	12.97	26.71 dd[4]
	SD	7.15	8.51	22.62	26.38
No. of Late Resorptions	Mean	0.1 R[1]	0.1	0.0	0.3
	SD	0.3	0.2	0.0	0.4
	Sum	2	1	0	5
Late Resorptions (%)	Mean	0.97	0.35	0.00	2.23
	SD	3.03	1.53	0.00	4.08
No. of Scars	Mean	0.0 R[1]	0.0	0.0	0.0
	SD	0.0	0.0	0.0	0.0
	Sum	0	0	0	0
Scars (%)	Mean	0.00	0.00	0.00	0.00
	SD	0.00	0.00	0.00	0.00

1 [R - Automatic Transformation Selected: Rank]
2 [SS - Test: Shirley 2 Sided p < 0.01]
3 [kk - Group Factor Test: Kruskal-Wallis p < 0.01]
4 [dd - Test: Dunnett Non-Parametric 2 Sided p < 0.01]

Table 47 continued.

Sex: Female Day(s) Relative to Mating (1)		0 mg/kg/day	15 mg/kg/day	50 mg/kg/day	150 mg/kg/day
No. of Dead Foetuses	Mean	0.0 R[1]	0.0	0.0	0.0
	SD	0.0	0.0	0.0	0.0
	Sum	0	0	0	0
Post-Implantation Loss	Mean	0.90 R[1]	0.74	0.95	3.20 SS[2]
	SD	1.07	1.05	0.91	2.91
	Sum	18.0	14.0	18.0	64.0
Post-Implantation Loss (%)	Mean	7.32 kkk[3]	5.97	12.97	28.94 dd[4]
	SD	9.07	8.69	22.62	25.72
No. of Live Foetuses	Mean	10.3 R[1]	11.3	10.1	8.2
	SD	2.8	2.7	3.3	3.7
No. of Male Foetuses	Mean	5.1 I[5]	5.5	5.2	4.7
	SD	2.4	2.1	1.5	1.9
	Sum	102	105	93	90
No. of Female Foetuses	Mean	5.2 I[5]	5.8	5.4	3.9
	SD	1.9	2.3	2.3	2.5
	Sum	103	110	98	74
Male Foetuses (%)	Mean	49.12	49.25	50.40	57.81
	SD	14.65	15.86	15.71	20.19
Total Litter Weight (g)	Mean	35.710	40.215	35.854	25.456
	SD	10.519	9.726	7.258	9.529
	N	20	19	18	19
	%Diff	.	12.616	0.403	-28.714
Mean Foetal Weight (both) (g)	Mean	3.49 I[5]	3.57	3.39	2.98 www[6]
	SD	0.30	0.21	0.18	0.24
	N	20	19	18	19
	%Diff	.	2.27	-2.89	-14.49
Mean Foetal Weight (M) (g)	Mean	3.60 I[5]	3.66	3.46 w[7]	3.04 www[6]
	SD	0.31	0.27	0.19	0.21
Mean Foetal Weight (F) (g)	Mean	3.38 I[5]	3.45	3.32	2.91 www[6]
	SD	0.29	0.21	0.19	0.31

1 [R - Automatic Transformation Selected: Rank]
2 [SS - Test: Shirley 2 Sided p < 0.01]
3 [kkk - Group Factor Test: Kruskal-Wallis p < 0.001]
4 [dd - Test: Dunnett Non-Parametric 2 Sided p < 0.01]
5 [I - Automatic Transformation Selected: Identity (No Transformation)]
6 [www - Test: Williams 2 Sided p < 0.001]
7 [w - Test: Williams 2 Sided p < 0.05]

Offspring (Malformations, Variations, etc.)
Each fetus was examined for external defects and live fetuses were subsequently euthanized. Approximately one half of each litter was submitted to fresh visceral examination of the body (abdominal and thoracic cavities) and then processed for skeletal examination. The remaining half of the fetuses in each litter was fixed in Harrison's fluid for subsequent examination of the head only by serial sectioning. The number of fetuses submitted for external, visceral, and skeletal examination is summarized in Table 48.

Table 48: The Number of Fetuses (Litters) Submitted for External, Visceral, and Skeletal Examination. (Sponsor's Table)

Group	1	2	3	4
External examination	205 (20)	215 (19)	191 (18)	164 (19)
Visceral examination				
– Body	108 (20)	111 (19)	100 (18)	88 (19)
– Head	97 (20)	104 (19)	91 (18)	76 (19)
Skeletal examination				
– Body	108 (20)	111 (19)	100 (18)	88 (19)
– Head	108 (20)	111 (19)	100 (18)	88 (19)

External Findings: in the mid- and high-dose groups there were 1(1) and 2(2) fetuses with the same external malformation, hyper-extension of the right hindpaw (
Table 49). No external malformations were observed in the vehicle control and low-dose benznidazole groups.

Visceral (Soft Tissue) Findings: High-dose fetuses exhibited visceral malformations in 5 fetuses in a total of 4 litters. Four of the fetuses had eye malformations (bilateral or unilateral anophthalmia or microphthalmia) and one fetus had a three chambered heart with multiple large blood vessel malformations (
Table 49). The control group had no visceral malformations and the low- (anophthalmia) and mid-dose (multiple malformations including anasarca) groups each had malformations in one fetus in one litter. Three of the high-dose fetuses with eye malformations (dams No.: 74, 87, and 81) had dilated lateral-brain ventricles. This is an unusual finding that was not reported in the vehicle control or the lower-dose fetuses or in any of the historical control findings.

Skeletal Findings: There were 2 high-dose fetuses in 2 litters with skeletal malformations (multiple skeletal abnormalities and multiple abnormalities of the lumbar vertebra) compared to no fetuses with skeletal malformations in any of the other groups (
Table 49).

Historical Control Data: In the historical control data for the same colony of Wistar rats that were used in the current study a low incidence of fetal malformations were recorded (Table 50). Bilateral anophthalmia which occurred in one low-dose fetus and two high-dose fetuses was observed in one historical control study conducted in 2013 with a litter incidence of 4% (1/25 litters) and a fetal incidence of 0.36% (1/2586 fetuses).

Table 49: Summary of External, Visceral, and Skeletal Malformations in the Rat Embryo-fetal Study. (Sponsor's Table)

Dose level (mg/kg/day)	Female number	Foetus number	Malformation(s)#
0	-	-	-
15	26	10	Anophthalmia (left)
50	64	2	Anasarca Hyperextension (right hindpaw) Situs inversus (abdominal and thoracic) Abnormal lobation of the lung (right lobes not separated and azygos absent)
150	67	4	Anophthalmia (left)
	74	6	Anasarca Gross disruption of head with proboscis Malrotation (hindpaws) Three chambered heart and multiple abnormalities of the great blood vessels Multiple skeletal abnormalities (gross disruption of the head, vertebrae, ribs, pectoral girdle and pelvic girdle)
		8	Anophthalmia (bilateral)
	76	1	Multiple abnormalities of lumbar vertebra
	81	10	Hyperextension (right hindpaw)
	82	2	Microphthalmia (right)
	87	11	Microphthalmia (right)

-: No abnormality detected.

#: Including external, visceral and skeletal examinations.

Table 50: The Latest (2011-2013) Historical Control Data for the Test Colony of Wistar Rats for External, Internal and Skeletal Malformations. (Sponsor's Table)

Study	Year	Number of litters examined	Number of fetuses examined	Number of litters with malformed fetuses	Litter incidence %	Number of malformed fetuses	Fetal Incidence %	Type of malformation by fetus
B13-2	2013	25	291	0	-	0	-	
C13	2013	25	274	1	4,00	1	0,36	1: Anophthalmia (bilateral), malformed great blood vessels and malpositioned testis (bilateral, cranially)
D13	2013	5	63	0	-	0	-	
E13	2013	6	70	0	-	0	-	
F13	2013	6	69	0	-	0	-	
G13	2013	22	234	0	-	0	-	
H13	2013	23	234	1	4,35	1	0,43	1: Malformed maxilla (short maxilla and premaxilla, and misshapen zygomatic arches)
I13	2013	20	231	0	-	0	-	
A12	2012	25	269	1	4,00	1	0,37	1: Agnathia, cleft palate, malpositioned annulus, malformed thoracic and lumbar vertebrae
B12	2012	5	52	0	-	0	-	
C12	2012	8	86	0	-	0	-	
D12	2012	24	255	1	4,17	1	0,39	1: Malformed major blood vessels
E12	2012	6	63	0	-	0	-	
F12	2012	6	72	1	16,67	1	1,39	1: Sternoschisis
D11	2011	23	246	0	-	0	-	
E11	2011	6	77	0	-	0	-	
Total 2011 to 2013		*235*	*2586*	*5*	**2,13%**	*5*	**0,19%**	
Total 2008 to 2010		*264*	*2918*	*7*	**2,65%**	*10*	**0,34%**	

Study nos. E11, F12, E12, B12, B13-2, D13, E13, F13 and I13: Dose Range Finding studies with external examination only

Skeletal Variations: High-dose fetuses exhibited a higher incidence of skeletal variations associated with unossified or incompletely ossified bones occurring predominantly in the skull, vertebral column, sternum and digits compared to the vehicle control and the lower-dose benznidazole fetuses (Table 51). A higher incidence of one variation, unossified 5th metacarpal also occurred in the mid-dose group. Given the substantial reduction in maternal weight gain and food consumption that occurred with benznidazole treatment, it is considered likely that the increased incidence of unossified bones in the high-dose group was related to maternal toxicity.

Table 51: Skeletal Variations in the Rat Embryo-Fetal Study. (Sponsor's Table)

Finding with % of affected foetuses		Historical control data (2008 to 2010 - 2011 to 2013)	Group 1 Control	Group 2 15 mg/kg/day	Group 3 50 mg/kg/day	Group 4 150 mg/kg/day
Unossified bones						
Sternebra	5th	2.7-5.2	7	5	7	**13**
	6th	2.3-3.1	4	2	2	**8**
Thoracic vertebra	1-9th centrum	0-0.1	0	0	1	**3**
Metacarpal	5th digit	0-3.8	12	13	**31**	**77**
Caudal vertebra		0.5-0.7	2	0	0	**11**
Caudal vertebra	1/2nd arches	18.9 –24.6	31	32	37	**64**
Incomplete ossification of bones						
Supraoccipital		22.5-17.5	9	5	6	**46**
Parietal		21.3-14.9	17	10	14	**25**
Squamosal		8.4-7.3	3	5	2	**16**
Zygomatic arch		0-0.1	3	0	1	**7**
Sternebra	1/3rd	3.4-3.3	2	0	0	**11**
	2/4th	7.8-10.7	4	2	0	**17**
	6th	29.2-24.8	25	14	20	**53**
Thoracic vertebra	1/9th	NA*	1	1	1	**6**
Metacarpal	2-4th digit	0-0.2	1	0	2	**9**
Metatarsal		1-0.3	1	0	0	**13**
Sacral vertebra	arch	1.9-3.7	4	2	1	**24**

NA*: Not applicable. Change in manner of examination does not allow comparison with previous historical

Conclusions

Pregnant female rats receiving the high dose of benznidazole (150 mg/kg/day) demonstrated maternal toxicity in the form of significant weight loss. In addition, caesarian section data in the high-dose group including increased early resorptions (number and percent), and increased post-implantation loss (number and percent) may have been influenced by maternal toxicity. Also significantly reduced fetal weights and increased numbers of fetal skeletal variations (unossified and incompletely ossified bones) may have occurred as a consequence of maternal toxicity.

Fetal malformations increased in a benznidazole dose-dependent manner and are not considered related to maternal toxicity. Of the observed fetal malformations, anasarca occurred in single mid-dose and high-dose fetuses and anophthalmia occurred in two fetuses in two litters in the high-dose group. The litter incidence for anophthamia in the high-dose group (2/19; 10.5%) is above the highest litter incidence in the historical control data (4.0%). A single low-dose fetus also had anophthalmia, but the litter incidence (1/19; 5.2%) is close to the highest incidence in the historical control data (4.0%). In addition, another eye malformation, microphthalmia occurred in 2 fetuses in 2 litters in the high-dose group with no incidence in the concurrent vehicle control group or in historical control data. The Sponsor also reported that 3 of the high-dose fetuses with eye malformations had an associated rare malformation, dilated lateral-brain ventricles.

The increased number and types of fetal malformations in the high-dose group indicate that the malformations are associated with benznidazole administration. The single fetal incidence of anasarca in the mid-dose group is consistent with a single incidence of anasarca in the high-dose group suggesting that this malformation which did not occur in historical control data is possibly related to benznidazole administration. However, the single incidence of anophthalmia in the low dose group has a similar incidence in the historical control data, and thus it is not clearly related to benznidazole administration.

The NOAEL for maternal toxicity is considered to be the mid-dose of 50 mg/kg/day which was associated with plasma C_{max} and AUC values of 42.9 mcg/ml and 357 mcg•hr/ml respectively on GD 17. The NOAEL for fetal toxicity and benznidazole-related malformations is considered to be the low dose of 15 mg/kg/day which was associated with plasma C_{max} and AUC values of 14.7 mcg/ml and 73.6 mcg•hr/ml respectively on GD 17.

Study title: Benznidazole–Embryo-fetal Toxicity Study by the Oral Route (Gavage) in the Rabbit (Segment II)

Study no.:	AB20565
Study report location:	Electronic transmission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	October 20, 2014
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Benznidazole, lot #00015, purity of 99.7%

Key Study Findings

- Two high-dose females receiving 25 mg/kg/day from GD 6 to GD 19 aborted their fetuses
- High-dose dams experienced substantial weight loss, approximately 40 grams in the first 4 days of dosing compared to 40 grams weight gain for the vehicle control group. Subsequently high-dose females gained weight, but in much lower (-30-100%) amounts than control dams.

- Food consumption was reduced 28% in high-dose dams compared to control dams during dosing.
- The number of late resorptions was significantly increased in high-dose dams compared to controls. Fetal weights were not significantly reduced in any benznidazole-treatment groups.
- A serious visceral malformation, ventricular septal defect, occurred in mid- and high-dose fetuses and is considered related to benznidazole administration.
- Benznidazole did not increase the number of most skeletal variations, but did increase a few variations in the high-dose group mostly related to decreased ossification.
- The NOAEL for maternal toxicity was considered to be 7.5 mg/kg/day which was associated with plasma C_{max} and AUC values of 2.29 mcg/ml and 7.36 mcg•hr/ml respectively.
- The NOAEL for fetal toxicity was considered to be the low-dose of 2.5 mg/kg/day which was associated with plasma C_{max} and AUC values of 0.569 mcg/ml and 0.723 mcg•hr/ml respectively.

Methods

Doses:	0, 2.5, 7.5, and 25 mg/kg/day
Frequency of dosing:	Once per day
Dose volume:	4 ml/kg/day
Route of administration:	Oral gavage
Formulation/Vehicle:	1% carboxymethylcellulose in water for injection
Species/Strain:	New Zealand White Rabbit
Number/Sex/Group:	22 females/group
Satellite groups:	none
Study design:	Benznidazole or vehicle (1% carboxymethylcellulose in water) was administered by daily oral gavage in doses of 2.5, 7.5, and 25 mg/kg/day to four groups (22/group) of mated female New Zealand White rabbits from Gestation Days (GD) 6-19 inclusive. Dams were monitored for clinical condition, body weight, and food consumption throughout the study. Three females per group were sampled at specific time-points for toxicokinetics on GDs 6 and 19. On GD 29, the dams underwent caesarean section followed by necropsy and litter parameters were evaluated. Dams were examined for gross pathology, having a gravid uterus, and live fetuses were weighed. All of the fetuses were examined for external, visceral, and skeletal malformations, and the heads of half of the fetuses were fixed, and sectioned

	serially.
Deviation from study protocol:	Multiple deviations from the study protocol were noted. However, none of the deviations was considered to have altered the results or compromised the integrity of the study.

Observations and Results

Mortality
All animals were observed at least twice daily for mortality and morbidity.

Two high-dose females were euthanized after aborting on either GD 22 or GD 24. Previous findings in the two females included reduced food consumption and body weight loss. At necropsy, one female had dark fluid in the abdominal cavity and several ovarian cysts, and the other female had a pale liver with an irregular surface.

Clinical Signs
All animals were observed daily for clinical signs. During the treatment period, all animals were observed once before and at least once after treatment for signs of abnormal behavior or reaction to treatment.

In all the benznidazole-dose groups reduced food intake and increased body weight loss occurred in a dose-dependent manner compared to control females. In association with the pattern of reduced food intake, reduced fecal output occurred in a dose-dependent manner. Red traces, thought to be associated with fetal resorption, were observed in one mid-dose female. Also 3/22 high-dose dams exhibited localized hair loss as did 1/20 mid-dose dams.

Body Weight
All animals were weighed on GDs 0, 6, 9, 13, 16, 20, 24, and 29 of gestation.

During the first three days of dosing on GD 6-9, high-dose females lost approximately 40 grams of body weight compared to a 40 gram body-weight gain in control females. For the rest of the dosing period (until GD 20), high-dose females gained weight, but in mean amounts 30-100% lower than control values. After the cessation of dosing (GDs 21-29), high-dose females gained equal or greater amounts of body weight compared to control females. The low- and mid-dose benzindazole groups also lost body weight during dosing, but the weight changes were not significantly different than control values.

Feed Consumption
Individual food consumption was measured daily from the day of arrival to GD 29. The mean food consumption (g/animal/day) was calculated for GDs 0-6, 6-9, 9-13, 13-16, 16-20, 20-24, and 24-29.

During the dosing period (GD 6-20), mean food consumption was significantly reduced 28% in high-dose females and 7 and 5% in low- and mid-dose females respectively

compared to vehicle control females. After the end of dosing, food consumption in benznidazole-treatment groups recovered to levels equal to or greater than food consumption in control females.

Toxicokinetics
Blood samples for toxicokinetic measurements of benznidazole were collected on GDs 6 and 19 at 1, 2, 6, 10 and 24 hours after dosing.

Benznidazole was not detected in plasma samples from vehicle control animals. Plasma C_{max} and AUC values increased in a greater than dose-dependent manner (Table 52). Benznidazole accumulated approximately 1.5 fold in plasma after the full term of dosing compared to values on the first day of dosing. The plasma $t_{1/2}$ value for the high-dose group was 2.6 hours.

Table 52: Toxicokinetic Parameters for Benznidazole in Pregnant Rabbits
(Sponsor's Table)

Gestation Day	6			19		
Dose (mg/kg/day)	2.5	7.5	25	2.5	7.5	25
AUClast (µg*h/mL)	0.497	5.58	52.3	0.723	7.36	75.2
Cmax (µg/mL)	0.362	1.35	9.86	0.569	2.29	12.1
Tmax (h)	1	2	1	1	1	1

Dosing Solution Analysis
The actual concentrations of the test formulations prepared on the first and last days of dosing were within the acceptance criteria of ± 15% of the nominal concentrations. One first dose sample for the 2.5 mg/kg dose did not meet the acceptance criteria, but a frozen duplicate sample was shown to meet the acceptance criteria after a longer sonication period. Benznidazole was not measureable in the vehicle samples.

Necropsy
All surviving females were euthanized on GD 29, and examined for gross pathology. Abnormal organs were sampled and preserved. The ovaries, uterus, and placenta from each female was removed and examined.

No gross pathology findings were noted other than those (pale liver with an irregular surface, dark fluid in the abdominal cavity, pale heart, ovarian cyst) observed in the two high-dose dams that were euthanized after aborting.

Cesarean Section Data (Implantation Sites, Pre- and Post-Implantation Loss, etc.)
Caesarian section data included: pregnancy status, gravid uterus weight, number of corpora lutea, number of implantations, number and distribution of live fetuses, individual fetal weights, fetal sex and the number and distribution of embryonic/fetal deaths classified as early, late, or dead fetuses.

Pregnancy occurred in nearly all of the females in each group. Also mean gravid uterus weights were comparable for all groups. The mean number of late resorptions/litter was significantly increased 300% in high-dose females compared to control females (Table 53). Other benznidazole-related trends were apparent including increased post-implantation loss and reduced fetal weights, but none of the trend parameters were significantly different compared to concurrent control or the historical control values.

Table 53: Mean Caesarian Section Data. (Sponsor's Table)

Sex: Female Day(s) Relative to Mating (1)		0 mg/kg/day	2.5 mg/kg/day	7.5 mg/kg/day	25 mg/kg/day
No. of Pregnant Females	N	20	21	20	18
Dams with Viable Foetuses		20	20	20	18
No. of Corpora Lutea	Mean	9.9 I[1]	9.7	10.5	10.8
	SD	1.7	2.2	2.1	2.3
	Sum	197	204	209	195
No. of Implantations	Mean	8.6 I[1]	8.1	9.3	9.5
	SD	2.3	3.2	2.5	2.4
	Sum	172	171	185	171
Pre-Implantation Loss	Mean	1.25 R[2]	1.57	1.20	1.33
	SD	1.55	1.96	1.74	1.28
	Sum	25.0	33.0	24.0	24.0
Pre-Implantation Loss (%)	Mean	13.13	18.31	11.49	12.29
	SD	16.45	24.48	18.43	11.80
No. of Early Resorptions	Mean	0.3 R[2]	0.1	0.4	0.5
	SD	0.4	0.4	0.6	0.9
	Sum	5	3	7	9
Early Resorptions (%)	Mean	2.71	5.82	3.38	6.04
	SD	4.86	21.84	5.62	12.80
No. of Late Resorptions	Mean	0.1 R[2]	0.1	0.3	0.4 S[3]
	SD	0.4	0.3	0.6	0.6
	Sum	2	2	5	7
Late Resorptions (%)	Mean	0.77	0.87	2.58	4.21
	SD	3.44	2.82	5.55	6.52
No. of Dead Foetuses	Mean	0.0 R[2]	0.0	0.0	0.1
	SD	0.0	0.0	0.0	0.2
	Sum	0	0	0	1
Post-Implantation Loss	Mean	0.35 R[2]	0.24	0.60	0.94
	SD	0.59	0.54	0.68	1.21
	Sum	7.0	5.0	12.0	17.0

Table 53 continued

Post-Implantation Loss (%)	Mean	3.48	6.69	5.96	10.65
	SD	5.58	22.06	6.64	14.47
No. of Live Foetuses	Mean	8.3 R[1]	7.9	8.7	8.6
	SD	2.1	3.3	2.3	2.5
No. of Male Foetuses	Mean	4.2 I[2]	4.4	4.2	4.2
	SD	1.8	2.1	1.9	1.7
	Sum	84	87	84	75
No. of Female Foetuses	Mean	4.1 I[2]	4.0	4.5	4.4
	SD	1.9	1.7	1.4	2.0
	Sum	81	79	89	79
Male Foetuses (%)	Mean	51.76	49.98	47.44	49.58
	SD	18.62	20.32	12.69	19.69
Total Litter Weight (g)	Mean	327.240	333.003	338.248	322.377
	SD	61.308	80.581	74.305	86.754
	N	20	20	20	18
	%Diff	.	1.761	3.364	-1.486
Mean Foetal Weight (both) (g)	Mean	40.56 I[2]	41.83	39.86	38.58
	SD	4.57	6.31	4.16	6.41
	N	20	20	20	18
	%Diff	.	3.11	-1.74	-4.88
Mean Foetal Weight (M) (g)	Mean	40.74 I[2]	40.99	40.40	38.94
	SD	5.27	6.01	4.43	6.98
Mean Foetal Weight (F) (g)	Mean	40.31 I[2]	41.93	39.60	38.67
	SD	4.55	6.64	4.69	6.53

[I - Automatic Transformation Selected: Identity (No Transformation)]

[R - Automatic Transformation Selected: Rank]

[S - Test: Shirley 2 Sided p < 0.05]

Offspring (Malformations, Variations, etc.)

All fetuses were examined for external defects then euthanized. All fetuses were examined viscerally and sexed at the time of caesarean section. Subsequently, the head of approximately half of the fetuses in each litter was removed, placed in Harrisson's fluid for later sectioning and examination. The eviscerated carcasses of these fetuses were processed for skeletal examination. The ossified skeleton was stained with alizarin red and skeletal and fixed-visceral examinations were performed under low power magnification using a binocular microscope. The number of fetuses submitted for external, visceral, and skeletal malformations is shown in Table 54.

Table 54: The Number of Fetuses (Litters) Submitted for External, Visceral, and Skeletal Examinations. (Sponsor's Table)

Group	1	2	3	4
External examination	165 (20)	166 (20)	173 (20)	155[1] (18)
Internal examination				
– Body	165 (20)	166 (20)	173 (20)	154 (18)
– Head	77 (20)	78 (20)	81 (20)	72 (18)
Skeletal examination				
– Body	165 (20)	166 (20)	173 (20)	154 (18)
– Head	88 (20)	88 (20)	92 (20)	82 (18)

[1]: Includes one dead foetus.

A summary of the external, visceral, and skeletal malformations is shown in Table 55.

External Malformations: No external malformations were noted in the low- and mid-dose benznidazole groups. In the control group, 2 fetuses in 2 litters exhibited external malformations (umbilical hernia and hyperflexed paw). In the high-dose benznidazole group, 3 fetuses in 3 litters demonstrated external malformations including 2 fetuses with umbilical hernia and one with a malrotated paw.

Visceral Malformations: Visceral malformations were observed in every group including in 3 fetuses in 3 litters in the control group (multiple abnormalities of the great blood vessels, small right heart ventricle and great vessel abnormalities and dilated renal pelvis), 1 fetus in the low-dose benznidazole group (absent gall bladder), 4 fetuses in 4 litters in the mid-dose benznidazole group (2 fetuses with ventricular septal defects, 1 fetus with narrowed pulmonary trunk and dilated aortic arch, 3 fetus with multiple abnormalities of the heart and/or great vessels), and 1 fetus in the high-dose benznidazole group (ventricular septal defect and multiple abnormalities of the great vessels)(Table 55). In the historical control data that was provided with the study report, in 1750 control rabbit fetuses in 203 litters used in studies conducted from 2010-2012, neither multiple malformations of the great vessels nor ventricular septal defects were reported to have occurred in any fetuses (Table 56). In the current study, ventricular septal defect occurred in 2 mid-dose fetuses and 1 high-dose fetus with no occurrence in vehicle control fetuses suggesting that this malformation may be related to benznidazole administration. Because multiple malformations of the great vessels occurred in 1 vehicle control fetus as well as 2 mid-dose fetuses and 1 high-dose fetus, the relationship of this malformation to benznidazole administration is less clear.

Skeletal Malformations: Skeletal malformations were observed in 1 control fetus (fused frontal bones), 3 fetuses in 2 litters in the low-dose benznidazole group (fused sternebrae), and 2 litters in 2 fetuses in the mid-dose benznidazole group (thoracic scoliosis and fused sternebrae). No skeletal malformations were reported for the high-dose benznidazole group (Table 55).

Table 55: Summary of the Fetal Malformations in the Rabbit Embryo-Fetal Study. (Sponsor's Table)

Dose level (mg/kg/day)	Dam no.	Foetus no.	Malformation(s) (including external, visceral and skeletal examinations)
0	7	4	Hyperflexed paw and malpositioned testis and multiple abnormalities of the great blood vessels (retroeosophageal aortic arch, absent innominate artery and absent common trunk)
	9	5	Umbilical hernia
	11	6	Small right heart ventricle (thick wall of the left and right ventricles) and multiple abnormalities of the great blood vessels (narrowed pulmonary trunk and dilated aortic arch)
	15	9	Dilated renal pelvis
	17	5	Fused frontal bones
2.5	26	2	Fused sternebrae
		7	Fused sternebrae
	32	3	Fused sternebrae
	36	2	Absent gall bladder
7.5	47	10	Narrowed pulmonary trunk and dilated aortic arch
	50	14	Multiple abnormalities of thoracic vertebrae (scoliosis)
	54	7	Multiple abnormalities of the heart (dilated right ventricle with thin wall, dilated left ventricle with thick wall and thick atrioventricular, pulmonary and aortic valves) and great blood vessels (dilated aortic arch and pulmonary artery)
	58	3	Ventricular septal defect and multiple abnormalities of the great blood vessels (dilated ductus arteriosus and pulmonary trunk)
	61	1	Multiple abnormalities of the heart (ventricular septal defect with large right atrium, small right ventricle and left ventricle with thick wall), dilated aortic arch, multiple uro-genital abnormalities, absent spleen and fused sternebrae
25	71	2	Umbilical hernia
	73	5	Umbilical hernia
	76	9	Malrotated paw
	77	13	Ventricular septal defect and multiple abnormalities of the great blood vessels (narrowed pulmonary trunk and dilated aortic arch)

Table 56: The Latest (2010-2012) Historical Control Data for the Test Colony of New Zealand White Rabbits for External, Internal and Skeletal Malformations.
(Sponsor's Table)

Study	Number of litters examined	Number of fetuses examined	Number of litters with malformed fetuses	Litter incidence %	Number of malformed fetuses	Fetal incidence %	Type of malformation by fetus
A12	7	56	0	0.00	0	0.00	
B12	23	185	1	4.35	2	1.08	1: Malpositioned kidney 2: Malformed thoracic vertebra
D12	23	194	4	17.39	6	3.09	1: Cleft palate, micrognathia and major fusion of the sternebra 2: Umbilical hernia and malformed sternebra 3: Malformed limbs, short forelimb, marked malrotation paws, malformed forepaw, ectrodactyly, malpositioned kidney, absence of a gonad, displaced scapula, malformed sternebrae and malformed vertebral column (thoracic and cervical) 4: Marked malrotation forepaws, absent gallbladder, asplenia and ectrodactyly 5: Malformed thoracic vertebra (scoliosis) 6: Haemorrhagic eye
A11	18	160	2	11.11	2	1.25	1: Malformed thoracic vertebra 2: Malformed hindpaw (brachydactyly), bilateral
B11	18	165	2	11.11	2	1.21	1: Malformed vertebral column 2: Ectrodactyly, forepaws
C11	6	51	0	-	0	-	
D11	20	189	1	5.00	1	0.53	1: Malformed thoracic vertebra
A10	20	180	0	-	0	-	
C10	22	164	1	4.55	1	0.61	1: Malformed thoracic vertebra
E10	23	208	1	4.35	1	0.48	1: Malformed thoracic vertebra, scoliosis
G10	23	198	1	4.35	1	0.51	1: Spina bifida, malformed lumbar vertebra
2010-2012	*203*	*1750*	*13*	**6.40%**	*16*	**0.91%**	
2007-2009	*523*	*4669*	*33*	**6.31%**	*36*	**0.77%**	

Study nos. C11 and A12: Dose Range Finding studies with external examination only.

Skeletal Variations: The majority of the many observed skeletal variations occurred with similar incidences in all the groups including the vehicle-control group. For some skeletal variations, the highest incidence occurred in vehicle-control fetuses. However, specific skeletal variations (Table 57) occurred with a higher incidence in the high-dose fetuses including small fontonelle, unossifed nasal line, unossified middle phalanx, incomplete ossification of the pubis, and 8 lumbar vertebrae instead of the more common 7 lumbar vertebrae. Given the small changes in litter incidence for these specific skeletal variations and the probable influence of maternal weight loss during gestation, the increases are considered to be probably related to maternal toxicity.

Table 57: Skeletal Variations Occurring with a Higher Incidence in Benznidazole Groups.

Skeletal Variation	Total Samples	0 mg/kg/day	2.5 mg/kg/day	7.5 mg/kg/day	25 mg/kg/day
	Total No. of Fetuses	165	166	173	154
	Total No. of Litters	20	20	20	18
Small fontanelle	Fetuses N(%)	0 (0.0)	2(2.3)	3(3.3)	4(4.9)
	Litters N(%)	0 (0.0)	2(10.0)	2(10.0)	2(11.1)
Nasal unossified line	Fetuses N(%)	0 (0.0)	0 (0.0)	0 (0.0)	4(4.9)
	Litters N(%)	0 (0.0)	0 (0.0)	0 (0.0)	4(22.2)
Unossified middle phalanx	Fetuses N(%)	5(3.0)	1(0.6)	5(2.9)	13(8.4)
	Litters N(%)	3(15.0)	1(5.0)	2(10.0)	6(33.3)
Incomplete ossification	Fetuses N(%)	3(1.8)	4(2.4)	1(0.6)	8(5.2)

of the pubis	Litters N(%)	3(15.0)	4(20.0)	1(5.0)	4(22.2)
8 Lumbar vertebrae	Fetuses N(%)	2(1.2)	2(1.2)	3(1.7)	8(5.2)
	Litters N(%)	1(5.0)	2(10.0)	3(15.0)	2(11.1)

Conclusions

Pregnant rabbits receiving the high dose of benznidazole (25 mg/kg/day) demonstrated maternal toxicity in the form of significant weight loss. In addition, the two abortions in high-dose dams, increased mean numbers of late resorptions/litter, and increased numbers of specific skeletal variations in high-dose fetuses may have been influenced by maternal toxicity.

The fetal malformations that occurred in this study are not considered to be related to maternal toxicity. A serious malformation, ventricular septal defect, occurred in 2 mid-dose fetuses in 2 litters and 1 high-dose fetus. This malformation did not occur in the historical control data or in the concurrent control group and is considered related to benznidazole administration. Another malformation that is not present in the historical control data, multiple abnormalities of the great blood vessels, occurred in 2 mid-dose fetuses in 2 litters, 1 high-dose fetus and 1 control fetus. Because this abnormality occurred in the vehicle control group, despite its absence in historical control data, it is not considered related to benznidazole administration. Multiple external and skeletal malformations occurred in all groups without a clear relationship to benznidazole administration.

The NOAEL for maternal toxicity is considered to be the mid-dose of 7.5 mg/kg/day which was associated with plasma C_{max} and AUC values of 2.29 mcg/ml and 7.36 mcg•hr/ml respectively on GD 19. The NOAEL for fetal toxicity and benznidazole-related malformations is considered to be the low dose of 2.5 mg/kg/day which was associated with plasma C_{max} and AUC values of 0.569 mcg/ml and 0.723 mcg•hr/ml respectively on GD 19.

9.3 Prenatal and Postnatal Development

Study title: Benznidazole – Pre- and Post-natal Development Study by the Oral Route (Gavage) in the Wistar Rat (Segment III)

Study no.:	AB20568
Study report location:	Electronic transmission
Conducting laboratory and location:	(b) (4)
Date of study initiation:	October 20, 2014
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Benznidazole, Batch No.: 00015, purity of 99.7%

Key Study Findings

1. High-dose F_0 dams administered 75 mg/kg/day from GD 6 to GD 20 demonstrated significantly reduced weight gain (-8%) and food consumption (-11%) during the gestation period compared to the control dams.
2. In high-dose dams, the mean duration of gestation was significantly longer and the mean number of implantations and the number of F_1 pups delivered was significantly lower compared to control values.
3. The absolute mean body weight for F_1 males was significantly reduced compared to control F_1 males on PND 21. Male body weights were similar for all groups on PND 91 and females in benznidazole-treatment groups did not demonstrate weight loss on PND 21 or after impregnation and during gestation (until PND 56).
4. In general, maternal benznidazole treatment did not alter the pre-weaning physical or functional development or neurological findings of F_1 pups.
5. Reproductive parameters for F_1 females were not significantly altered by benznidazole treatment.
6. Caesarean section data for F_1 females included significantly lower mean corpora lutea counts, mean number of implantations, and mean number of live embryos and significantly higher pre-implantation loss for the high-dose (75 mg/kg/day) group compared to the vehicle control group.
7. Maternal treatment with benznidazole did not alter the mean values for F_1 males for testes weight, testicular and epididymal sperm counts, and epididymal sperm motility and progression. However, in 1/20 mid-dose males and 2/20 high-dose males, small testes and/or epididymides were observed, and two of the affected animals (1 mid-dose and 1 high-dose) failed to mate or induce pregnancy.
8. Plasma benznidazole concentration increased in a dose-dependent manner in F_0 dams on Lactation Days (LDs) 4 and 19, and plasma benznidazole concentrations were measureable in all of the low- mid- and high-dose F_1 litters on PND 4 (LD 4) at 2 and 4 hours after maternal dosing.
9. The NOAEL was considered to be the mid-dose of 50 mg/kg/day based on the reduced mean corpora lutea counts, mean number of implantations, and mean number of live embryos in pregnant F_1 females in the high-dose group.

Methods

Doses:	0, 15, 50, and 75 mg/kg/day
Frequency of dosing:	Once per day
Dose volume:	10 ml/kg/day
Route of administration:	Oral gavage
Formulation/Vehicle:	1% carboxymethyl cellulose in water for injection
Species/Strain:	Wistar rats: Crl: WI (Han)
Number/Sex/Group:	24 females per group
Satellite groups:	None.
Study design:	Four groups of 24 mated female Wistar rats were administered either vehicle (Group 1) or 25, 50, and

75 mg/kg/day benznidazole (Groups 2-4 respectively) by daily oral gavage beginning on Gestation Day (GD) 6 until Lactation Day (LD) 20 or, when no litter delivered, up to Day 26 post-coitum. Clinical condition, body weight, and food consumption of the females was monitored throughout the study. The females were allowed to give birth.

Litter parameters including the number of pups born, pup survival, and pup weights were recorded up to postnatal day (PND) 21.

At least 10 dams /group were sampled for blood on LDs 4 and 19 in order to measure benznidazole concentrations in maternal plasma. Also F_1 pup blood was collected from 10 litters/group on PND 4.
One male and one female pup per litter were selected for a total of 20/sex/group for F_1 generation testing. The dams and the rest of the F_1 pups were necropsied on PND 21.

The F_1 pups selected for testing were maintained untreated and assessed for post-weaning development, behavioral tests, and mating. Body weights of the F_1 females were monitored during the pre-mating period and during gestation (until PND 56). Body weights of the F_1 males were monitored from selection up to necropsy (PND 91). F_1 males and females (20 each), with every litter represented, were mated on PND 21. The F_1 reproduction study was terminated with the necropsy of the F_1 males after the caesarean examinations of the F_1 females on Day 13 after mating.

All necropsied F_1 animals were examined for gross pathology. The testes and epididymides of males were weighed and used for sperm analysis. Female F_1 animals that were mated were assessed for pregnancy status, number of corpora lutea and numbers and types of uterine implantations.

Deviation from study protocol: Multiple deviations from the study protocol were noted. However, none of the deviations was considered to have altered the results or compromised the integrity of the study.

Observations and Results

F_0 Dams

Survival: No unscheduled deaths occurred in the study

Clinical signs: No clinical signs during gestation or lactation were

related to benznidazole treatment.

Body weight: High-dose dams experienced significantly reduced, dose-dependent, body weight gain during GD 6 to 20 in comparison to control values. At the end of the gestation period, absolute body weight was 8% lower in high-dose dams than in the control group. However mean body weight gain during the lactation period was similar for all groups.

Feed consumption: Food consumption was significantly lower in high-dose females by approximately 11% compared to control values in conjunction with treatment during the gestation period. In the lactation period, food consumption was similar for all groups.

Uterine content: The number of high-dose F_1 pups delivered was significantly lower than for control F_1 pups. This finding correlated with a significantly lower mean number of implantations (Table 59). No other uterine content parameters were significantly altered by administration of benznidazole.

Necropsy observation: No gross pathology findings in F_0 dams related to benznidazole were observed.

Toxicokinetics: Maternal plasma exposure was demonstrated and increased in a dose-dependent manner in dams on Lactation Days 4 and 19 (see Table 58 below).

Dosing Solution Analysis The actual concentrations of all the analyzed samples were within ± 15% of the nominal concentrations. The formulations were considered homogeneous with a precision of < 4.5%.

Other: The mean duration of gestation was significantly longer in high-dose dams compared to control dams and also outside the historical control range (Table 59). However, no clinical changes were reported that appeared indicative of delivery difficulties.

Table 58: Maternal Plasma Concentrations of Benznidazole. (Sponsor's Table)

Occasion	Dose (mg/kg/day)	Sex	Time (h)	Mean concentration (µg/mL)	SD	CV (%)	n
L4	0	Female	4	BLQ	0	NA	10
	15	Female	4	8.37	1.42	17.0	10
	50	Female	4	25.4	6.30	24.8	10
	75	Female	4	41.0	6.40	15.6	10
L19	0	Female	4	BLQ	0	NA	10
	15	Female	4	4.68	1.24	26.6	10
	50	Female	4	20.8	3.71	17.8	10
	75	Female	4	35.0	8.37	23.9	10

Table 59: Summary of F_0 Delivery and Litter Data. (Sponsor's Table)

Sex: Female Day(s) Relative to Littering (Litter: A)		0 mg/kg/day	15 mg/kg/day	50 mg/kg/day	75 mg/kg/day
Females on Study		24	24	24	24
Females Mated [CHSQFS]	N+ve	24	24	24	24
Mating Index (%)		100.0	100.0	100.0	100.0
Females Pregnant [CHSQFS]	N+ve	22	24	23	24
Female Fertility Index	%	91.7	100.0	95.8	100.0
Females with Liveborn [CHSQFS]	N+ve	22	24	23	24
Gestation Index (%)		100.0	100.0	100.0	100.0
Females Completing Delivery [CHSQFS]	N+ve	22	24	23	24
Females Completing Delivery(%)		100.0	100.0	100.0	100.0
with Stillborn Pups		2	1	1	1
with Stillborn Pups (%)		9.1	4.2	4.3	4.2
with All Stillborn Pups [CHSQFS]	N+ve	0	0	0	0
	%	0.0	0.0	0.0	0.0
With all dead PND 21 [CHSQFS]	N+ve	0	0	0	0
	%	0.0	0.0	0.0	0.0
Gestation Length (Days) [GEN AN]	Mean	22.2 R[1]	22.2	22.3	22.6 SS[2]
	SD	0.4	0.4	0.5	0.5
	N	22	24	23	24

Table 59 continued

Sex: Female Day(s) Relative to Littering (Litter: A)		0 mg/kg/day	15 mg/kg/day	50 mg/kg/day	75 mg/kg/day
Number of Implantation Sites [GEN AN]	Mean	12.8 R[1]	11.8	12.7	11.2 S[2]
	SD	1.5	2.7	1.7	2.0
	Sum	281 R[1]	283	291	269 S[2]
Pups Delivered/Litter [GEN AN]	Mean	11.5 R[1]	10.8	11.5	10.0 S[2]
	SD	1.3	2.5	1.7	2.1
	Sum	252 R[1]	259	264	241 S[2]
Pre-Birth Loss (%) [GEN AN]	Mean	10.06 R[1]	8.17	8.91	10.63
	SD	7.65	6.34	10.18	9.39
	N	22	24	23	24
Litters with Liveborn Pups [CHSQFS]	N+ve	22	24	23	24
Live Pups/Litter PND 0 [CHSQFS]	Mean	11.3	10.8	11.4	10.0
	SD	1.6	2.5	1.6	2.1
	Sum	248	258	263	240
Live Birth Index (%)		98.4	99.6	99.6	99.6
Culled PND 4	Sum	72	72	78	54
Liveborn not culled PND 21	Sum	176	186	185	186
Dead, Miss., Cannibal. PND 0 [CHSQFS]	Sum	4	1	1	1
Dead, Miss., Cannib. PND 0 (%)		1.6	0.4	0.4	0.4
Dead, Miss., Cannib. PND 1-4 [CHSQFS]	Sum	1	.	2	.
Dead, Miss., Cannib. PND 1-4 (%)		0.4	0.0	0.8	0.0

Table 59 continued

Sex: Female Day(s) Relative to Littering (Litter: A)		0 mg/kg/day	15 mg/kg/day	50 mg/kg/day	75 mg/kg/day
Dead, Miss., Cannib. PND 5-21 [CHSQFS]	Sum	0	1	0	1
Dead, Miss., Cannib. PND 5-21 (%)		0.0	0.4	0.0	0.4
Dead, Miss., Cannib. PND 0-21 [CHSQFS]	Sum	5	2	3	2
Dead, Miss., Cannib. PND 0-21 (%)		2.0	0.8	1.1	0.8
Cmb - Live Pups on Day 1 [GEN AN]	Mean	11.3 R[1]	10.8	11.3	10.0
	SD	1.6	2.5	1.8	2.1
	Sum	248 R[1]	258	261	240
No live Pups Preculling d4 [GEN AN]	Mean	11.2 R[1]	10.8	11.3	10.0
	SD	1.5	2.5	1.8	2.1
	Sum	247 R[1]	258	261	240
No live Pups Postculling d4 [GEN AN]	Mean	8.0 R[1]	7.8	8.0	7.8
	SD	0.2	1.0	0.2	0.7
	Sum	175 R[1]	186	183	186
No Live Pups PND 7 [GEN AN]	Mean	8.0 R[1]	7.8	8.0	7.7
	SD	0.2	1.0	0.2	0.9
	N	22	24	23	24
	Sum	175 R[1]	186	183	185

Table 59 continued

Sex: Female Day(s) Relative to Littering (Litter: A)		0 mg/kg/day	15 mg/kg/day	50 mg/kg/day	75 mg/kg/day
No Live Pups PND 14 [GEN AN]	Mean	8.0 R[1]	7.7	8.0	7.7
	SD	0.2	1.0	0.2	0.9
	N	22	24	23	24
	Sum	175 R[1]	185	183	185
No Live Pups PND 21 [GEN AN]	Mean	8.0 R[1]	7.7	8.0	7.7
	SD	0.2	1.0	0.2	0.9
	N	22	24	23	24
	Sum	175 R[1]	185	183	185
Viability Index (%)		99.6	100.0	99.2	100.0
Lactation Index (%)		100.0	99.5	100.0	99.5
Sex Ratio PND 1 - % Males [CHSQFS]	Mean	48.6	51.9	49.1	47.1
Sex Ratio PND 21 - % Males [CHSQFS]	Mean	50.7	53.1	49.7	48.6

[CHSQFS] - Chi-Squared & Fisher's Exact
[GEN AN] - Generalised Anova/Ancova Test
1 [R - Automatic Transformation: Rank]
2 [SS - Test: Shirley 2 Sided p < 0.01]
2 [S - Test: Shirley 2 Sided p < 0.05]

F_1 Generation

Survival: No unscheduled deaths occurred

Clinical signs: Incomplete hair growth was observed 1/24, 2/23, and 5/24 litters in the low- mid- and high-dose groups compared to no incidence in pups from control dams. No other clinical signs considered related to maternal benznidazole treatment were observed.

Body weight:	Male and female mean body weights were not altered in any benznidazole-treatment group compared to control values during the lactation period. Mean body weight gain for F_1 males and females were slightly (≈ 4-9%) lower for all benznidazole treatment groups for PNDs 4-7 compared to control values. However mean body weight gains were similar for all groups after PND 7 until weaning on PND 21. In weaned F_1 rats selected for reproduction and testing of postweaning development (beginning on PND 21, Postweaning Day 0), mean body weight gain and mean body weights for F_1 males were significantly reduced in all benznidazole-treatment groups by Postweaning Day (PWD) 21. After PWD 21, mean body weight gain for males was similar for all groups, and at terminal sacrifice on PWD 91 mean body weights for males was similar for all groups. Mean body weights for F_1 females were significantly reduced in the high-dose group on PWDs 7 and 14 but not on any other measurement days until PND 56. Mean body weight gains for F_1 females were not significantly reduced by benznidazole treatment during the pre-mating and gestation periods (until PND 56).
Feed consumption:	Not measured
Physical development:	In general, maternal benznidazole treatment did not alter the pre-weaning physical or functional development of F_1 pups. No changes in balano-preputial development of F_1 males or sexual maturation of F_1 females were noted for any of the benznidazole-treatment groups. However, one mid-dose pup and three high-dose pups did not exhibit a pupillary reflex on PND 21.
Neurological assessment:	In F_1 animals, no benznidazole-related changes in parameters measured in the Watermaze (learning capacity, memory, and motor activity) and Open Field (activity and exploratory behavior) tests were observed.
Reproduction:	F_1 males (n = 20) and females (n =20) were selected for mating on PND 21. Benznidazole-maternal treatment did not significantly affect the F_1 mating performance for males and females. The number of females that were inseminated and became pregnant was similar for all groups, as were the pre-coital interval, copulation index, and fertility index. Caesarean section data for the pregnant F_1 females

revealed significantly lower mean corpora lutea counts, mean number of implantations, and number of live embryos and higher pre-implantation loss for the 75 mg/kg/day benznidazole group compared to the vehicle control group (Table 60).

In F_1 males, a low incidence of small testes and/or epididymides were observed in the mid- (1/20 male) and high-dose (2/20 males) benznidazole groups. Of the afflicted males, the mid-dose male and one high-dose male failed to mate or induce pregnancy. However, mean values for testes weight, and testicular and epididymal sperm counts and epididymal sperm motility and progression were not significantly different in the high-dose group compared to the control group (Table 61 and Table 62). The mean weight of the R epididymides but not the L epididymides was significantly lower in all the benznidazole-treatment groups, but the effect was dose-independent for magnitude.

Other: Toxicokinetics: blood samples (processed to plasma) were obtained from culled F_1 pups in 10 litters/group from all groups including the vehicle control at 2 and 4 hours after maternal dosing (5 litters/group/timepoint) on PND 4. Benznidazole exposures were measurable in all litters in the low-, mid-, and high-dose groups with increasing plasma exposures at 4 hours compared to 2 hours after maternal dosing (Table 63).

Table 60: Mean Caesarean Section Data for F_1 Females. (Sponsor's Table)

Sex: Female Day(s) Relative to Mating (Litter: A)		0 mg/kg/day	15 mg/kg/day	50 mg/kg/day	75 mg/kg/day
Females Pregnant	N	19	19	19	18
Dams with Viable Embryos		19	19	19	18
No. of Corpora Lutea [GEN AN]	Mean	14.8 R[1]	13.9	13.9	12.7 S[2]
	SD	1.8	1.7	2.0	3.9
	Sum	282 R[1]	264	265	228 S[2]
No. of Implantations [GEN AN]	Mean	14.4 R[1]	13.3 S[2]	13.1 S[2]	11.5 S[2]
	SD	2.4	2.2	2.6	4.7
	Sum	273 R[1]	253 S[2]	249 S[2]	207 S[2]
Pre-Implantation Loss [GEN AN]	Mean	0.47 R[1]	0.58	0.84	1.17
	SD	1.17	1.35	1.26	2.41
	Sum	9.0 R[1]	11.0	16.0	21.0
Pre-Implantation Loss (%) [KWLWCX]	Mean	3.64	4.39	6.72	12.64
	SD	10.42	10.23	10.27	22.86
Post-Implantation Loss [GEN AN]	Mean	0.74 R[1]	0.89	0.79	0.50
	SD	1.24	0.99	1.40	0.79
	Sum	14.0 R[1]	17.0	15.0	9.0
Post-Implantation Loss (%) [KWLWCX]	Mean	4.89	7.36	5.94	4.37
	SD	8.01	8.60	10.11	7.61
No. of Live Embryos [GEN AN]	Mean	13.6	12.4	12.3	11.0 w,t[3]
	SD	2.5	2.6	2.8	4.7
	N	19	19	19	18

[GEN AN] - Generalised Anova/Ancova Test

[KWLWCX] - Kruskal Wallis & Wilcoxon

1 [R - Automatic Transformation: Rank]

2 [S - Test: Shirley 2 Sided p < 0.05]

3 [w,t - Test: Williams 2 Sided p < 0.05,Test: T-Test Parametric 2 Sided p < 0.05]

Table 61: Mean Testicular Sperm Counts. (Sponsor's Table)

Dose level		Testis weight (g)	Millions / gram testis
0 mg/kg/day	MEAN	1.975	123.2
	SD	0.122	22.5
	N	20	20
15 mg/kg/day	MEAN	1.901	122.8
	SD	0.127	18.3
	N	20	20
50 mg/kg/day	MEAN	1.844	126.4
	SD	0.367	35.6
	N	20	20
75 mg/kg/day	MEAN	1.906	117.3
	SD	0.289	35.8
	N	20	20

Table 62: Mean Epididymal Sperm Motility and Progression Data. (Sponsor's Table)

Dose level		Total number of sperm	Motile sperm		Progressive sperm	
			count	(%)	count	(%)
0 mg/kg/day	Mean	838	777	93	528	63
	SD	244	228	3	147	4
	N	20	20	20	20	20
15 mg/kg/day	MEAN	685	630	89	439	63
	SD	294	306	21	205	16
	N	20	20	20	20	20
50 mg/kg/day	MEAN	788	741	89	505	62
	SD	302	288	21	184	15
	N	20	20	20	20	20
75 mg/kg/day	MEAN	831	780	86	520	57
	SD	386	377	25	238	20
	N	20	20	20	20	20

Table 63: Plasma Benznidazole Concentrations in F_1 Pups on PND 4 at 2 and 4 Hours after Dosing. (Sponsor's Table)

Occasion	Dose (mg/kg/day)	Time (h)	Mean concentration (µg/mL)	SD	CV (%)	n
PND4	0	2	BLQ	0	NA	5
		4	BLQ	0	NA	5
	15	2	0.0436	0.0598	137	5
		4	0.155	0.106	68.4	5
	50	2	0.214	0.0973	45.5	5
		4	0.657	0.227	34.6	5
	75	2	0.352	0.124	35.3	5
		4	0.732	0.392	53.5	5

F_2 Generation

Survival: Not measured beyond tabulation of the number of live F_2 embryos which was reduced in the high-dose group.
Body weight: Not measured
External evaluation: Not measured
Male/Female ratio: Not measured
Other: None

10 Special Toxicology Studies

No special toxicology studies were conducted.

11 Integrated Summary and Safety Evaluation

Clinically relevant toxicity findings for benznidazole in nonclinical studies included teratogenicity in rats and rabbits, testicular atrophy and aspermia in male rats and mice, and *in vitro* and *in vivo* genotoxicity.

A significant finding in the rat embryo-fetal study was increased total, external, visceral, and skeletal malformations in pregnant females treated with the high-dose of 150 mg/kg/day benznidazole compared to vehicle-control dams. In addition, a visceral malformation, anasarca, was considered related to benznidazole administration in a mid-dose (50 mg/kg/day) fetus. In rabbits, a serious visceral malformation, ventricular septal defect occurred at a low incidence in mid- (two fetuses in two litters) and high-dose fetuses (1 fetus) and was considered related to benznidazole administration. In both rats and rabbits, pregnant females experienced pronounced weight reductions in a benznidazole dose-dependent manner indicating maternal toxicity. The product label will include restrictions for use during pregnancy based on the animal data. In order to better elucidate the potential fetal effects of benznidazole in human pregnancy, a postmarketing registry detailing pregnancy outcomes is recommended for Chagas patients treated with benznidazole during pregnancy.

Another clinically relevant toxicity associated with benznidazole treatment was dose-dependent testicular atrophy and inhibition or arrest of spermatogenesis in testes and epididymides in rats. The testicular and epididymal results were observed in the 26-week toxicology study conducted by the Sponsor in rats and also reported rats and mice in three literature reports (Bernacchi *et al*., 1986; Vieira *et al.*, 1989; and Navarro and Nagel, 1990). The toxicity was characterized by tubular atrophy, peritubular interstitial edema, and accumulation of syncytial/degenerate sperm in seminiferous tubules and epididymides. Testicular atrophy, and reduced spermatogenesis and fertility in rats have been reported for other drugs in the same nitroimidazole class as benznidazole including metronidazole and tinidazole. A male fertility study in rats is planned as a post-marketing requirement.

One question that has not been adequately addressed by the available study results for benznidazole is whether the testicular atrophy and inhibition of spermatogenesis is reversible. In the 26-week toxicology study in rats, the testicular toxicity did not substantially reverse during the 1-month recovery period. In the planned male fertility study in rats, the 13-week (b) (4) period (b) (4) (b) (4) Another question is whether the testicular and epididymal toxicity is limited to rodents or if benznidazole has the potential to produce similar toxicity in humans. Benznidazole-related testicular toxicity has not been reported in any test species other than rats and mice. However, it appears that most of the studies in species other than rodents did not assess testicular toxicity. Also, testicular toxicity has not been reported to occur in the hundreds of adult and juvenile patients that have been administered benznidazole in clinical trials. Additional clinical data including focused measurements designed to detect testicular toxicity and fertility outcomes in patients will help to clarify the issue. One route to obtaining this kind of data might be via a postmarketing registry reporting symptoms consistent with testicular toxicity (reduced testicular size, reduced ejaculate volume, aspermia and/or malformed sperm) and fertility outcomes in male patients treated with benznidazole. Until the issue is further clarified, the potential for benznidazole to impair male fertility and induce testicular toxicity based on study data in rodents will be reported in the product label.

Because of the questions remaining about the reversibility of benznidazole-related testicular toxicity and its potential to occur in humans, testicular toxicity remains a serious concern for infant and juvenile Chagas patients treated with benznidazole. In the pre-postnatal study in rats, 1/20 F_1 males in the mid-dose group (50 mg/kg/day) and 2/20 high-dose F_1 males in the high-dose group (75 mg/kg/day) exhibited small testes and/or epididymides. These results suggest that benznidazole received through breast milk from mothers administered benznidazole may have adversely affected a small percentage of F_1 offspring. A further implication of these results is that benznidazole

exposure in infant and juvenile males may adversely affect their reproductive capacity. Reportedly, in the several clinical studies that have included juvenile patients, benznidazole produced fewer adverse events compared to adult patients and no cases of testicular toxicity or later impairment of fertility have been reported for any patients administered benznidazole. The reproductive toxicity potential of benznidazole in infant and juvenile males is difficult to assess with juvenile animals studies using rats due to the rapid reproductive development in rats. Again, the best option for obtaining more information about possible effects in pediatric patients may be a postmarketing registry.

Another category of toxicity associated with the risk benefit assessment for clinical administration of benznidazole is its high potential for genotoxicity based on positive mutagenicity and clastogenicity results in nonclinical studies. Benznidazole has been assessed in a number of published genetic toxicology studies including a full battery of assays recommended in the ICH S2(R1) Guidance. Benznidazole was shown to be mutagenic in Ames assays, increase the frequency of sister chromatid exchange in human lymphocytes and increase micronuclei *in vitro* in Hep G2 cells and *in vivo* in mice. In contrast, in other published studies where benznidazole was orally administered to rats and mice, benznidazole did not increase the number of chromosome aberrations in rats and mice or the frequency of micronuclei in peripheral blood cells from mice (*Gorla et al.*, 1985; *Souza et al.*, 1991). However, in chagasic children, the incidence of micronucleated lymphocytes and chromosome aberrations both significantly increased approximately two fold after benznidazole treatment. The weight of evidence from the published studies suggests a genotoxic potential for benznidazole in humans. This is consistent with genotoxicity findings for other drugs in the same nitroimidazole class, including metronidazole and tinidazole.

Like another nitroimidazole drug, metronidazole, benznidazole has been reported to be carcinogenic in animals. In published studies, intraperitoneal benznidazole administration at doses of 8 mg/kg/day for 30 or 60 days was shown to increase the incidence of lymphomas in mice and rabbits. However in another study where benznidazole was administered in oral gavage doses of 100 mg/kg day for 5 days a week for 90 days to mice, only 1/31 mice developed lymphomas. These results and the genotoxicity results suggest a carcinogenicity potential for benznidazole, but due to limitations in the studies, the relevance to humans is not clear. The literature studies are limited by a lack of GLP compliance and positive carcinogenicity results only in association with intraperitoneal dosing as opposed to the oral route of dosing for clinical administration. Due to these limitations, the results of these studies will not be included on the product label. However, because another approved drug in the nitroimidazole class, metronidazole, has been shown to be carcinogenic in mice and rats, a

carcinogenic potential for benznidazole based its classification in the nitroimidazole class of drugs will be included on the product label.

Other dose-dependent toxicity findings reported in the 26-week toxicology study in rats included centrolobular hepatocellular hypertrophy, eosinophilic droplets and karyomegaly in kidney tubular epithelial cells accompanied by increased serum creatinine and urea in high-dose male rats, vacuolation in the pars distalis in the pituitary gland, increased incidence of extramedullary hematopoiesis and hemosiderin deposits in the spleen, and minimal follicular hypertrophy of the thyroid gland. After a 1-month recovery period, the liver changes were completely reversed, but the other organ toxicities did not completely reverse. Of these, the kidney karyomegaly may be the most important, but it is not clear that these toxicities will contribute to adverse events in patients treated for no more than 60 days. In safety data from 71 adult and 238 child Chagas patients treated with benznidazole, the primary reported adverse events were headache, pruritus, nausea, upper abdominal pain, hypersensitivity, neutropenia, and rash.

Another serious nonclinical toxicity of benznidazole associated with chronic-repeated dosing is severe neurotoxicity in dogs including seizure contractions, opisthotonos, and nystagmus proceeding to death. However similar findings were not observed in studies with other test species, including in rabbits, guinea pigs, rats, or mice where neurological signs were monitored. Safety findings from multiple clinical trials with benznidzole also indicate that the neurotoxicity findings in dogs do not appear to be clinically relevant. In clinical data for 71 adult patients, peripheral neuropathy was reported as a low incidence adverse event occurring in less than 2% of subjects. No neurological adverse events were reported in summary safety data for 238 children administered benznidazole in clinical trials. Because of the demonstrably low incidence of peripheral neuropathy, and its qualitative dissimilarity with the neurological toxicity in dogs which was specifically noted to not occur in peripheral nerves, it is unlikely that the findings in dogs indicate a potential for similar neuropathy in humans. However, other drugs in the same nitroimidazole class as benznidazole including metronidazole and tinidazole have been shown to produce central nervous system toxicity (encephalopathy for metronidazole and seizures for tinidazole) as well as peripheral neuropathy. Because these drugs have been used more commonly than benznidazole, it is possible that neurological toxicity more consistent with the CNS toxicity reported for benznidazole in dogs may become apparent in humans with wider use of benznidazole.

Benznidazole was evaluated for its ability to inhibit hERG potassium channels in stably transfected HEK-293 cells. Benznidazole produced a 17% inhibition of hERG potassium channels at the highest concentration of 100 mcM (26.0 mcg/ml). Based on this data, the potential for hERG inhibition at the lower plasma concentrations associated with

clinical dosing was expected to be minimal. No other safety pharmacology studies were conducted with benznidazole for this NDA.

The pharmacokinetics of benznidazole was evaluated in multiple studies. In an absorption study in rats, orally administered benznidazole was shown to be approximately 100% bioavailable with a plasma $t_{1/2}$ of 2-2.5 hours. Reportedly plasma $t_{1/2}$ values were shorter in mice (1.5 hours) but longer in sheep (4-5 hours) and dogs (9–11 hours). In the 6-month toxicology study, plasma AUC and C_{max} values increased in a roughly dose-dependent manner and benznidazole did not greatly accumulate in plasma with daily oral dosing.

Benznidazole was shown to distribute widely to tissues. In pregnant rats, the highest concentrations of radioactivity associated with a single oral dose of [^{14}C] benznidazole were shown to be covalently bound to tissues in the GI tract, liver, kidney, heart, lung, blood, brain and skeletal muscles. Radioactivity also distributed readily to placenta and fetal tissue at concentrations similar to the blood concentrations. Plasma-protein binding for benznidazole was reported to be 38-39% in mice, 52-58% in dogs, and 41-42% in sheep. The excretion pathways for benznidazole have not been fully elucidated, but 5-10% of oral doses in mice and rats were shown to be excreted in urine.

A primary metabolic pathway for benznidazole is thought to involve catalysis with nitroreductases, and nitroreductase enzyme activity was shown to correlate with histopathology in a variety of tissues including rat adrenal cortex, rat esophagus, and rat colon. These results suggest that benznidazole toxicity may to some extent be mediated by metabolites arising from nitroreduction in affected tissues. Plasma benznidazole concentrations were shown to be higher and remain elevated longer in juvenile rats compared to adult rats suggesting reduced metabolism in younger rats. Consistent with these results, clinical safety results indicate that pediatric Chagas patients receiving benznidazole exhibited fewer adverse events compared to adult patients, perhaps due to reduced nitroreduction of benznidazole to reactive intermediates in the pediatric patients.

The results of the nonclinical developmental and reproductive studies for benznidazole in females indicate little effect on female fertility, with more serious effects for pregnancy and first and second generation offspring. In the 6-month toxicology study in rats, five high-dose females (100 mg/kg/day) and 1 control female exhibited reduced ovary size and luteal bodies which was generally accompanied by unilateral or bilateral follicular cysts. However, in the female fertility, embryo-fetal, and pre-postnatal studies in rats, ovaries were not reduced in size or reported to be adversely effected by repeated oral dosing with benznidazole doses ranging from 75 to 150 mg/kg/day. Also no changes in

estrous cycle or pre-coital interval or pregnancy rates were observed in the female fertility study in rats where the high-dose of 150 mg/kg/day was administered for a total of 42 days. These results suggest the general form and function of ovaries is not expected to be adversely affected. However uterine findings associated with embryo patency did occur in the female fertility study with significant decreases in live embryos associated with increased postimplantation loss in pregnant females administered the high dose. This finding was consistent with similar findings in the rat embryo-fetal study with a high dose of 150 mg/kg/day and in the rat pre-postnatal study with a high dose of 75 mg/kg/day indicating benznidazole-related impairment of female reproduction at the level of embryo patency following implantation.

In the pre-postnatal study in rats, embryo implantation and patency was also affected in first generation dams born to mothers that were treated with 75 mg/kg/day benznidazole during gestation suggesting the potential for generational effects. Cesarean section findings in the F_1 dams in the high-dose group included: higher pre-implantation loss and reduced numbers of corpora lutea, implantations, and live embryos. Other developmental toxicity results in the pre-postnatal study included: reduced mean body weights for F_1 males in the high-dose group at PND 21, and as noted above, reduced testicular size and impaired spermatogenesis and mating in 5% of the mid- and high-dose F_1 males that were selected for mating. However, the testicular effects were not widespread and did not alter the mean fertility results or the mean results for testicular sperm counts in F_1 males or mean motility and progression data for epididymal sperm in F_1 males.

The NOAEL values for pertinent nonclinical studies, their associated human equivalent dose (HED) values, and calculated safety margins relative to the highest recommended human dose in adults are shown below (Table 64). The nonclinical data from the literature regarding benznidazole effects on testes and spermatogenesis in rats (Vieira *et al.*, 1989) and mice (Navarro *et al.*, 1990) and neurotoxicity in dogs (Scharer *et al.*, 1972) is included although the studies did not follow GLP guidelines and sometimes included only one dose of benznidazole. Similarly, the carcinogenicity studies in mice and rabbits that were positive for benznidazole-related lymphomas are limited by a lack of GLP-compliance and their use of intraperitoneal dosing as opposed to oral dosing in humans. The safety margins arising from comparison of the nonclinical NOAEL doses and their associated HED with clinical doses predominantly indicate that the nonclinical safety data does not strongly support the highest recommended clinical dose in adults.

Table 64: Safety and Associated Exposure Data for Nonclinical Studies and Comparison to the Expected Clinical Dose and its Associated Plasma Exposure.

Study Type/ Study Number or Citation	Subcategory	NOAEL	HED	Safety

		(mg/kg/day)[a]	(mg/kg/day)[b]	Margin[c]
6-month general toxicology study in rats/ Study No.: AB20563	Male	10	1.61	0.20
	Female	10	1.61	0.20
Repeated-dose studies in dogs/ Scharer *et al.*, 1972	Male (30 days of dosing)	25	13.9	1.7
Carcinogenesis in mice/Teixeira *et al.*, 1994	Female (30 and 60 days of dosing	Dose associated with toxicity – 8 mg/kg/day	0.65	0.08
Carcinogenesis in rabbits/ Teixeira *et al.*, 1990a,b	Male and Female (60 days of dosing)	Dose associated with toxicity – 8 mg/kg/day	2.6	0.32
Repeated-dose, sperm counts and shape in mice/ Navarro *et al.*, 1990	Male (8 days of dosing)	500	40.7	5.1
Repeat-dose, testes histopathology and spermatogenesis in rats/ Vieira *et al.*, 1989)	Male (30 days of dosing)	< 80	<12.9	< 1.6
Female Fertility Study in rats/ Study No.: AB21207	Females	50	8.1	1.0
Embryo-Fetal Study in Rats/ Study No.: AB20567	Maternal	50	8.1	1.0
	Fetal	15	2.5	0.31
Embryo-Fetal Study in Rabbits/ Study No.: AB20565	Maternal	7.5	2.4	0.30
	Fetal	2.5	0.81	0.13
Pre-postnatal study in rats/Study No.: AB20568	Male and female offspring	50	8.1	1.0

a NOAEL = No adverse effect level

b HED = human equivalent dose. The conversion factors for determining the HED values based comparison of human whole body surface area to surface area for rats, rabbits and dogs are the NOAEL dose divided by 6.2, 3.1, and 1.8 respectively.

c The highest recommended clinical dose in adults is 8 mg/kg/day.

Although most of the nonclinical toxicology data do not strongly support the safe use of benznidazole at the highest recommended clinical dose for the treatment of Chagas disease, the extensive history of use in Latin America with fairly extensive clinical data in pediatric and adult Chagas patients provide some clinical evidence of safety. Clinical data from recent well-controlled clinical trials is lacking, however, and some questions remain regarding the relevance and reversibility of specific toxicities including toxicity to male reproductive organs and possible inhibition of male fertility. Because of this

limitation, further clinical safety evaluation and monitoring is recommended postmarketing including registries recording effects on male fertility and female pregnancy outcomes.

12 Appendix/Attachments

Table 65: Hematology and Coagulation Parameter Table

Study No.	AB20563
Species	**Rat**
Hemoglobin concentration	X
Hemaglobin distribution width	
Hematocrit	X
Erythrocyte count	X
Platelet count	X
Plateletcrit / thrombocrit	
Mean platelet volume	
Mean corpuscular volume	X
Mean corpuscular hemoglobin	X
Mean corpuscular hemoglobin concentration	X
Red cell distribution width	
Total leukocyte count	
Reticulocyte count	X
Reticulocyte hemoglobin content	
Differential leukocyte count (Absolute and relative neutrophil, lymphocyte, monocyte, eosinophil, basophil counts)	X
Blood smear for cell morphology (if necessary for interpretation)	
Activated partial thromboplastin time (APTT)	X
Prothrombin time (PT)	X
Fibrinogen	X

Table 66: Clinical Chemistry Parameter Table

Study No.	AB20563
Species	**Rat**
Aspartate aminotransferase	X
Alanine aminotransferase	X
Alkaline phosphatase	X
Blood urea nitrogen	
Urea	X

Creatinine	X
Creatine kinase	X
Glucose	X
Cholesterol	X
Triglycerides	X
Total protein	X
Albumin	X
Total bilirubin	X
Sodium	X
Sorbitol dehydrogenase	
Potassium	X
Chloride	
Calcium	X
Inorganic phosphorus	X
Gamma-glutamyl transferase	X
Glutamate dehydrogenase	
Globulin	X
Albumin/globulin ratio	X

Table 67: Histopathology and Organ Weight Inventory Table

Study #	**AB20563**
Species	**Rat**
Adrenals	X, *
Aorta	X
Bone Marrow smear	X
Bone (sternum, and/or femur and/or rib)	X
Brain	X, *
Bronchi	X
Cecum	X
Cervix	X
Colon	X
Conjunctiva	
Duodenum	X
Epididymides	X, *
Esophagus	X
Eye	X
External ear	
Fallopian tube	
Gall bladder	
Gross lesions	
Harderian gland	X
Heart	X, *
Hypophysis	
Ileum	X

Infusion site	
Jejunum	X
Joint, tibiofemoral	
Kidneys	X, *
Lachrimal gland	
Larynx	
Liver	X, *
Lungs	X
Lymph nodes, inguinal	
Lymph nodes, mediastinal	
Lymph nodes mandibular	X
Lymph nodes, mesenteric,	X
Mammary Gland	X
Muscle (biceps, femoris)	
Nasal cavity	
Nasal turbinates	X
Optic nerves	X
Ovaries	X, *
Oviduct	X
Pancreas	X
Parathyroid	X, *
Peripheral nerve	
Peyer’s patches	X
Pharynx	
Pituitary	X, *
Prostate	X, *
Rectum	X
Salivary gland	X
Sciatic nerve	X
Seminal vesicles	X
Skeletal muscle	X
Skin	X
Spinal cord	X
Spleen	X, *
Sternum	
Stomach	X
Testes	X, *
Thymus	X, *
Thyroid	X, *
Tongue	X
Tonsils	
Trachea	X
Ureter	X
Urinary bladder	X

Uterus	X, *
Vagina	X
Vertebra, Lumbar	
Zymbal gland	X

X, histopathology performed

*, organ weight obtained

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