CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER:

204630Orig1s000

PHARMACOLOGY REVIEW(S)

MEMORANDUM

Date: March 1, 2016

To: File for NDA 204630

From: Brenda J Gehrke, PhD
Pharmacology-Toxicology Reviewer
Division of Hematology Oncology Toxicology (DHOT)
Office of Hematology and Oncology Products (OHOP)

Through: Christopher Sheth, PhD
Pharmacology-Toxicology Supervisor
Division of Hematology Oncology Toxicology (DHOT)
Office of Hematology and Oncology Products (OHOP)

Subject: NDA Resubmission
NDA: 204630
Drug: Provayblue (methylene blue 0.5%) injection
Indication: Acquired methemoglobinemia
Applicant: Provepharma SAS

Methylene blue is currently used to treat methemoglobinemia and marketed in the United States as an unapproved product without an approved NDA. Provepharma SAS is developing a methylene blue product for the treatment of acquired methemoglobinemia. NDA 204630 was initially submitted in August 2012. The Application was determined to be deficient for a substantial review and a Refuse to File letter was issued on October 11, 2012, mainly due to clinical deficiencies. NDA 204630 was resubmitted in September 2013 and the FDA issued a Complete Response letter to the Applicant in October 2014 with clinical, clinical pharmacology, and product quality deficiencies. The primary pharmacology/toxicology review of the NDA was completed and filed on May 28, 2014, and a separate pharmacology/toxicology review for the carcinogenicity studies was completed and filed on March 25, 2014. There were no pharmacology/toxicology concerns with the application and the recommended regulatory action from pharmacology/toxicology was approval.

The current submission, Supporting Document 48 for NDA 204630, is a Class 2 Resubmission. This resubmission contains no new pharmacology/toxicology information. Labeling was completed with this resubmission and changes were made to the pregnancy related sections of the prescribing information in order for the label to be in compliance with the Pregnancy and Lactation Labeling Rule (PLLR).

Recommendation:

Recommending approval. There are no pharmacology/toxicology issues for NDA 204630 to preclude approval of the drug for the proposed indication.

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/s/

BRENDA J GEHRKE
03/01/2016

CHRISTOPHER M SHETH
03/02/2016

MEMORANDUM

Date: May 28, 2014
From: Haleh Saber, Ph.D.
Pharmacology/Toxicology Supervisor
Division of Hematology Oncology Toxicology (DHOT)
Office of Hematology and Oncology Products (OHOP)
Re: Approvability for Pharmacology and Toxicology
NDA: 204630
Drug: (b) (4) Injection (methylene blue)
Indications: Treatment of acquired methemoglobinemia
Applicant: Provepharma SAS

Methylene blue is a small molecule that has been used for years in chemistry and biology fields. Due to its redox property, methylene blue can convert methemoglobin (MetHb) to hemoglobin in a non-enzymatic reaction. There are years of experience with methylene blue in treating methemoglobinemia as an unapproved product. Provepharma SAS is seeking approval of a methylene blue product for the treatment of acquired methemoglobinemia. Acquired methemoglobinemia can be induced by exposure to certain drugs and chemicals.

Methemoglobin results from the oxidation of the ferrous moiety (Fe2+) within the hemoglobin molecule to the ferric state (Fe3+). This may impair the oxygen delivery to tissues, causing tissue hypoxia and metabolic acidosis. In the body, methylene blue is reduced to leucomethylene blue. Leucomethylene blue can then reduce methemoglobin to hemoglobin. Methylene blue is an oxidizing agent and high doses can result in methemoglobinemia. Due to its redox properties, the recommended pharmacologic class for methylene is "oxidation-reduction agent".

This is a 505(b)(2) NDA. Proof-of-concept information provided by Provepharma SAS is based on published articles. The Applicant conducted a few toxicology studies with their product using the I.V. route of administration. They also submitted results of toxicology studies conducted by the National Toxicology Program (NTP). While the route of administration in animal studies conducted by NTP is oral, the studies are considered adequate mainly based on toxicities observed.

Drug-related toxicities in rats and /or dogs in general toxicology studies included the following: anemia as indicated by decreases in erythrocytes, hematocrit, and hemoglobin; methemoglobinemia as indicated by increases in methemoglobin, liver findings (increased weight, inflammatory cell foci, hemosiderin formation indicating hemolysis and hemoglobin degradation, increased bilirubin), enlarged and/or congested spleen and spleen capsule fibrosis, and injection site reactions (necrosis, hemorrhage, mixed inflammatory cell infiltrates and edema, and fibrosis).

Several genetic toxicology studies were conducted by NTP or the Applicant. Methylene blue was genotoxic in three assays (Ames test, *in vitro* sister chromatid exchange test and an *in vitro* chromosomal aberration test in CHO cells) and negative for micronucleus induction in animals. Based on these results, methylene blue has the potential to be carcinogenic. For acquired methemoglobinemia, carcinogenicity studies are not required due to the short term administration of the drug. However, as the studies were conducted by NTP and the reports were submitted to the NDA, they have been reviewed by Dr. Gehrke and the results discussed at the Executive Carcinogenicity Assessment Committee. Methylene blue caused pancreatic islet adenomas or carcinomas (combined) in male rats in the 2-year oral carcinogenicity study.

Methylene blue was teratogenic in rats and rabbits when administered during the period of organogenesis. Embryo-fetal toxicities may be due to methemoglobinemia developed in animals and the associated adverse effects (e.g. hypoxia and anemia). The clinical dose of methylene blue will be titrated to doses that will reduce methemoglobin in patients. Therefore, the findings in animals may not be applicable to patients receiving the therapeutic range of methylene blue; pregnancy category C is recommended. Embryo-fetal toxicities may occur if patients are dosed above the therapeutic range. In an *in vitro* study, methylene blue reduced the human sperm motility in a concentration-dependent manner, suggesting that the drug may reduce fertility in male subjects.

The nonclinical studies were reviewed by Drs. Brenda Gehrke and C.J. George Chang. The nonclinical findings are summarized in the "Executive Summary" of the NDA review and reflected in the product label.

Recommendation: I concur with Drs. Gehrke and Chang that from a nonclinical perspective, (b) (4) may be approved and that no additional nonclinical studies are needed to support approval of (b) (4) for the proposed indication.

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/s/

HALEH SABER
05/28/2014

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:	204630
Supporting document/s:	9
Applicant's letter date:	September 13, 2013
CDER stamp date:	September 13, 2013
Product:	(b) (4) Injection (methylene blue injection)
Indication:	Treatment of acquired methemoglobinemia
Applicant:	Provepharma SAS
Review Division:	Division of Hematology Oncology Toxicology
	(for Division of Hematology Products)
Reviewer:	Brenda J. Gehrke, Ph.D.
	C.J. George Chang, DVM, M.S., Ph.D., DABT
Supervisor/Team Leader:	Haleh Saber, Ph.D.
Division Director:	John Leighton, Ph.D., DABT
	Ann Farrell, M.D. (DHP)
Project Manager:	Kim J. Robertson

Disclaimer

Except as specifically identified, all data and information discussed below and necessary for approval of NDA 204630 are owned by Provepharma SAS or are data for which Provepharma SAS has obtained a written right of reference. Any information or data necessary for approval of NDA 204630 that Provepharma SAS does not own or have a written right to reference constitutes one of the following: (1) published literature, or (2) a prior FDA finding of safety or effectiveness for a listed drug, as reflected in the drug's approved labeling. Any data or information described or referenced below from reviews or publicly available summaries of a previously approved application is for descriptive purposes only and is not relied upon for approval of NDA 204630.

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1 Executive Summary

1.1 Introduction

Methemoglobinemia is a disease characterized by a reduced ability of the blood to carry oxygen due to a reduction in the levels of normal hemoglobin. Excessive levels of an altered form of hemoglobin (methemoglobin) can be caused by various genetic factors or substances (e.g. drugs, nitrates and nitrites, and pesticides). Methylene blue ((b) (4) is a thiazine dye that promotes a non-enzymatic redox conversion of methemoglobin (MetHb) to hemoglobin. Based on this mechanism of action, methylene blue is currently used to treat methemoglobinemia and marketed in the United States as an unapproved product without an approved NDA. Provepharma SAS is developing a methylene blue product (b) (4) Injection) for the treatment of acquired methemoglobinemia. The proposed clinical doses of 1 mg/kg (b) (4) in adults and children (b) (4) will be administered by intravenous injection over a period of 5 minutes as an initial dose (b) (4). To support the approval of methylene blue for the treatment of acquired methemoglobinemia, the Applicant submitted results of toxicology studies conducted with their product (b) (4) along with literature on the pharmacology, pharmacokinetics and toxicology of methylene blue, and studies of methylene blue trihydrate conducted by the National Toxicology Program (NTP).

1.2 Brief Discussion of Nonclinical Findings

General toxicology studies of methylene blue include a 3-month repeat-dose toxicology study conducted by NTP with oral administration of methylene blue trihydrate in rats and a 1-month repeat-dose toxicology study with intravenous administration of the Provepharma's methylene blue in dogs. In the 3-month repeat-dose toxicology study in rats, F344/N rats were administered methylene blue trihydrate (0, 25, 50, 100, or 200 mg/kg; 0, 150, 300, 600, and 1200 mg/m^2) by oral gavage at a dose volume of 5 mL/kg once daily 5 days per week for 14 weeks. In the 1-month repeat-dose toxicology study in dogs, Beagle dogs were administered Provepharma's methylene blue (0, 0.25, 0.50, or 1.0 mg/kg/day; 0, 5, 10, or 20 mg/m^2/day; concentration 5 mg/mL) or a comparator drug of methylene blue injection USP 1% w/v from Martindale (1.0 mg/kg/day; 20 mg/m^2) by intravenous infusion into the cephalic or saphenous vein at a flow rate of 0.5 mL/minute once daily for 4 weeks. Despite the difference in the routes of administration, similar toxicities were observed in rats and dogs. Hematological responses in both species included anemia as indicated by decreases in erythrocytes, hematocrit, and hemoglobin and increases in reticulocytes and methemoglobinemia as indicated by increases in methemoglobin and/or Heinz bodies (inclusions within red blood cells composed of denatured hemoglobin). Liver and spleen were organs of toxicity in both rats and dogs. Liver findings included increased liver weight, presence of inflammatory cell foci, and increased bilirubin. Increases in bilirubin may be secondary to hemolysis and degradation of hemoglobin. Increases in absolute and relative spleen weights, enlarged spleen, and congestion in the spleen were observed in both species. Additional microscopic findings in the spleen were hematopoietic cell proliferation,

lymphoid depletion of lymphoid follicles, and capsular fibrosis. Additional toxicities observed with intravenous administration of methylene blue in dogs were injection site toxicity (e.g. hemorrhage, edema, inflammatory cell infiltrates, fibrosis) and increased brown pigment in the kidney. Provepharma's methylene blue (5 mg/mL) had a similar toxicological and toxicokinetic profile in dogs as the comparator drug Methylene blue injection USP 1% w/v (Martindale) at a dose of 1 mg/kg/day (20 mg/m^2/day) when administered intravenously once daily for 4 weeks.

The genotoxicity of methylene blue has been evaluated in both in vitro and in vivo studies conducted by NTP with methylene blue trihydrate and in an in vitro bacterial reverse mutation assay (Ames test) conducted with Provepharma's methylene blue. Methylene blue trihydrate and Provepharma's methylene blue were mutagenic when tested in in vitro bacterial cell assays. Methylene blue trihydrate was also genotoxic in an in vitro sister chromatid exchange test and an in vitro chromosomal aberration test in Chinese hamster ovary (CHO) cells. Methylene blue trihydrate was negative for micronucleus induction in bone marrow or peripheral blood in male mice treated with single doses up to 150 mg/kg (450 mg/m^2) and in peripheral blood samples from male and female mice at the end of a 3-month repeat-dose toxicity study of doses up to 200 mg/kg (600 mg/m^2).

Two-year carcinogenicity studies in mice and rats were conducted by NTP with methylene blue trihydrate; the studies were done by oral gavage. Based on the FDA criteria for a positive carcinogenicity response, there are no statistically significant neoplastic findings in the 2-year mouse carcinogenicity study. The FDA concluded that methylene blue caused pancreatic islet adenomas or carcinomas (combined) in male rats in the 2-year carcinogenicity study based on the incidences exceeding the historical control incidence. In addition, there was a dose-related increase in pancreatic islet hyperplasia.

Embryo-fetal development studies in rats and rabbits were conducted by NTP; methylene blue trihydrate was administered by oral gavage. Methylene blue produced maternal toxicity as indicated by increases in maternal spleen weight at doses of ≥50 mg/kg/day (≥300 mg/m^2/day) in rats and maternal death at 100 mg/kg/day (1200 mg/m^2/day) in rabbits. Post-implantation loss, consisting primarily of resorptions, was increased compared to controls at doses of >200 mg/kg/day (≥1200 mg/m^2/day) in rats and ≥50 mg/kg/day (≥600 mg/m^2/day) in rabbits. Treatment with methylene blue caused spontaneous abortion at all doses (≥50 mg/kg/day; ≥600 mg/m^2/day) in rabbits. Fetal body weight was decreased with treatment of methylene blue compared to controls at doses of ≥200 mg/kg/day (≥1200 mg/m^2/day) in rats. Methylene blue produced teratogenicity including enlarged ventricles in rats and a malformation of umbilical hernia at doses of ≥100 mg/kg/day (≥1200 mg/m^2/day) in rabbits when administered during organogenesis. The embryo-fetal toxicities including teratogenicity were observed at maternally toxic doses. In addition, healthy animals given methylene blue develop drug-induced methemoglobinemia, causing hypoxia. The clinical dose of methylene blue will be titrated to doses that will reduce methemoglobin and hence hypoxia, and only in an overdose situation would adverse embryo-fetal effects be

anticipated. Since the above adverse fetal effects are not expected in patients at the therapeutic range of methylene blue, pregnancy category C is recommended.

Fertility studies with methylene blue have not been conducted. According to published literature (Coddington *et al.*, 1989), in vitro, methylene blue reduced motility of human sperm in a concentration-dependent manner.

1.3 Recommendations

1.3.1 Approvability

Recommended for approval. The nonclinical studies and literature submitted to this NDA provide sufficient information to support the use of methylene blue for the proposed indication.

1.3.2 Additional Non Clinical Recommendations

None

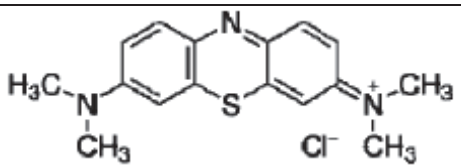
1.3.3 Labeling

The content for the labeling of methylene blue is contained in this review. Toxicokinetics were not conducted in the NTP embryo-fetal studies for methylene blue, therefore, dose to dose comparisons will be used for the animal-to-human conversions for labeling. Pregnancy category C is recommended. The embryo-fetal toxicities including teratogenicity were observed at maternally toxic doses. The clinical dose of methylene blue will be titrated to doses that will reduce methemoglobin and hypoxia, and only in an overdose situation would adverse embryo-fetal effects be anticipated. Since fetal toxicities are not expected at the therapeutic range, category C is appropriate for this drug.

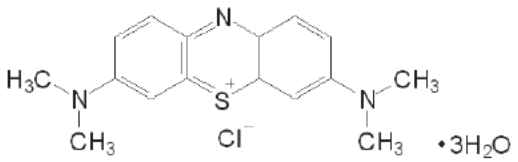
2 Drug Information

2.1 Drug

Provepharma's methylene blue

CAS Registry Number	61-73-4
Generic Name	Methylene blue; (b) (4)
Code Names	None
Chemical Name	3,7-bis(dimethylamino)-phenothiazin-5-ium chloride
Molecular Formula/ Molecular Weight	$C_{16}H_{18}N_3SCl$/ 319.86 g/mol
Structure or Biochemical Description	
Pharmacologic class	Oxidation-reduction agent

Methylene blue trihydrate

CAS Registry Number	7220-79-3
Generic Name	Methylene blue trihydrate; methylthioninium chloride
Chemical Name	3,7-bis(dimethylamino)-phenothiazin-5-ium chloride trihydrate
Molecular Formula/ Molecular Weight	$C_{16}H_{24}N_3O_3SCl$/ 373.9 g/mol
Structure or Biochemical Description	
Pharmacologic class	Oxidation-reduction agent

2.2 Relevant INDs, NDAs, BLAs and DMFs

Pre-IND 113942, Pre-IND 118156

2.3 Drug Formulation

The methylene blue injection (0.5%) is a sterile solution for intravenous administration supplied in 10 mL glass type 1 ampules. Each ampule contains 50 mg methylene blue and water for injection q.s., for a concentration of 5 mg/mL.

2.4 Comments on Novel Excipients

None

2.5 Comments on Impurities/Degradants of Concern

The proposed impurity specification for [redacted] in both the drug substance and drug product is not more than (NMT) [redacted]%, which is [redacted] the qualification thresholds in ICH Q3A (0.15% or 1.0 mg/day, whichever is lower) and ICH Q3B (1% or 50 µg whichever is lower). With a maximum cumulative dose for methylene blue of [redacted], the maximum dose of [redacted] is [redacted] mg/m^2. [redacted] Based on this information, the proposed specification of [redacted]% for [redacted] is qualified.

[redacted]

2.6 Proposed Clinical Population and Dosing Regimen

The proposed clinical population is patients with acquired methemoglobinemia. Methylene blue will be administered by intravenous injection over a period of 5 minutes as an initial proposed dose of 1 mg/kg (b) (4)

2.7 Regulatory Background

Methylene blue is currently used to treat methemoglobinemia and marketed in the United States as an unapproved product without an approved NDA. There is no reference listed product for methylene blue. Provepharma SAS is developing a methylene blue product for the treatment of acquired methemoglobinemia. A pre-NDA meeting was held with the FDA in January 2012, and NDA 204630 was initially submitted in August 2012. The Application was determined to be deficient for a substantial review and a Refuse to File letter was issued on October 11, 2012, mainly due to clinical deficiencies. Provepharm SAS was granted Orphan Drug Designation (12-3855) for methylene blue for the treatment of hereditary methemoglobinemia on December 18, 2012 and the designation was amended on June 28, 2013 to include the treatment of acquired methemoglobinemia. The current submission is a resubmission of the NDA submitted on September 13, 2013.

3 Studies Submitted

3.1 Studies Reviewed

Study#	Title	Module
NTP report TR 540 2008	NTP technical report on the toxicology and carcinogenesis studies of methylene blue trihydrate (CAS No. 7220-79-3) in F344/N rats and B6C3F$_1$ mice	4.3
36110TSC	4-week toxicity study by slow intravenous infusion to beagle dogs	4.2.3.2
35913 MMO	Bacterial reverse mutation test Proveblue	4.2.3.3.1
NTP report TER 92124 1993	Developmental toxicity of methylene blue trihydrate (CAS No. 7220-79-3) administered by gavage to Sprague-Dawley (CD®) rats	4.3
NTP report TER 92125 1994	Developmental toxicity of methylene blue trihydrate (CAS No. 7220-79-3) in New Zealand White (NZW) rabbits	4.3

3.2 Studies Not Reviewed

Study#	Title	Module
36109TSC	Two-phase dose range-finding study by slow intravenous infusion in Beagle dogs	4.2.3.2
35914 MMO	Bacterial reverse mutation test Methylene Blue Cooper officinal	4.2.3.3.1
35915 MMO	Bacterial reverse mutation test Methylene Blue Alfa Aesar	4.2.3.3.1
100033	Comparative study on the toxicity of three methylthionium chloride compounds in zebrafish	4.2.3.5.2
2009-IC-CTLT02-001.V4	Compared toxicity profiles of Proveblue and USP refence standard grade methylene blue	4.2.3.7.7

3.3 Previous Reviews Referenced

- Nonclinical review of 2-year carcinogenicity studies based on NTP report TR 540 2008 (Archival Date: 3/25/2014).

4 Pharmacology

4.1 Primary Pharmacology

A summary of the published literature related to the mechanism of action of methylene blue in treating methemoglobinemia is provided below.

Methemoglobinemia

Methemoglobinemia is an increase in the blood concentration of methemoglobin (MetHb). Methemoglobin is an altered hemoglobin condition, resulting from the oxidation of the ferrous moiety (Fe^{2+}) within the hemoglobin molecule to the ferric state (Fe^{3+}). The heme group containing the ferric state has reduced ability to bind to an oxygen molecule. In addition, the change in the conformation of the methemoglobin increases the affinity for the oxygen molecule, therefore, not allowing the release of oxygen molecules from the remaining ferrous hemes. This will reduce the oxygen-carrying capacity, impairing the oxygen delivery to tissue (tissue hypoxia), and producing metabolic acidosis. Methemoglobin levels of about 25% to 30% cause acute hypoxia (Camp, 2007), and severe untreated methemoglobinemia can lead to delirium and death. In the normal state, approximately 1% of hemoglobin is auto-oxidized to methemoglobin (Lunenfeld and Kane, 2004).

Acquired methemoglobinemia can be induced by exposure to various drugs and chemicals such as oxidizing agents (nitrates, nitrites, aniline products, and pesticides), anesthetic agents (lidocaine, benzocaine, pilocaine), and several other products (Hersh, 2004; Camp, 2007; Kane *et al*., 2007). Elevations in methemoglobin levels can also be observed in patients with sepsis (Krafte-Jacobs *et al*., 1997 and Ohashi *et al*., 1998). The hereditary forms of methemoblobinemia include infants that develop severe metabolic acidosis (blue baby syndrome), recessive congenital methemoglobinemia (NADH-cytochrome b_5 reductase deficiency), and rare congenital metabolic anomalies (glucose phosphate dehydrogenase deficiency; Rehman, 2001; Percy and Lappin, 2008). Figure below shows mechanisms causing methemoglobinemia.

Figure 1: Common causes of methemoglobinemia

(excerpted from Kane *et al*., 2007)

Mechanism of action of methylene blue in treating methemoglobinemia

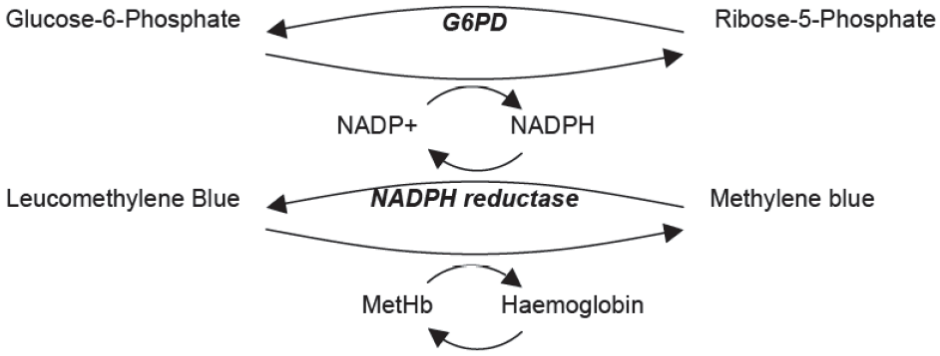
Methylene blue has been used clinically to treat methemoglobinemia. Methylene blue is administered typically in doses of 1 to 2 mg/kg of body weight intravenously over 5 minutes for methemoglobinemia; repeated doses may be indicated if symptoms persist, which may occur if there is continued absorption of the methemoglobin-inducer (Camp, 2007).

Methylene blue is quickly reduced to leucomethylene blue in the body in the presence of NADPH reductase. Leucomethylene blue is able to then rapidly transfer the added electron (as an electron donor) to reduce methemoglobin to hemoglobin, a non-enzymatic redox reaction. Methylene blue itself is an oxidizing agent, and if given in large doses it can cause methemoglobinemia (Camp, 2007). Methylene blue and leucomethylene blue exist in equilibrium to form a reversible redox system, and can continuously enter the cycle of reduction and oxidation until they are biotransformed or eliminated (Umbreit, 2007; see figure below).

In the pentose phosphate pathway, G6PD recycles NADP+ into NADPH, which is necessary for NADPH reductase. Therefore, methylene blue is contraindicated in people who have a genetic defect in natural reduction systems, such as G6PD deficiency (Ryan *et al*., 1998, cited in Camp, 2007).

Figure 2: Reduction of methemoglobin (MetHb) to hemoglobin by NADPH reductase (via methylene blue)

(excerpted from the Applicant's submission)



NADPH: reduced nicotinamide adenine dinucleotide phosphate.

4.2 Secondary Pharmacology

No secondary pharmacology studies were reviewed.

4.3 Safety Pharmacology

No safety pharmacology studies were submitted.

5 Pharmacokinetics/ADME/Toxicokinetics

5.1 PK/ADME

No pharmacokinetics studies were submitted for Provepharma's methylene blue and the literature for the pharmacokinetics of methylene blue in animals was not reviewed.

6 General Toxicology

6.1 Single-Dose Toxicity

No single-dose toxicology studies were submitted.

6.2 Repeat-Dose Toxicity

Study title: NTP technical report on the toxicology and carcinogenesis studies of methylene blue trihydrate (CAS No. 7220-79-3) in F344/N rats and B6C3F$_1$ mice

Study no.:	NTP report TR 540 2008
Study report location:	eCTD 4.3
Conducting laboratory and location:	Battelle Columbus Operations, Columbus, OH 43201
Date of study initiation:	Exact initiation dates not provided; first dose administered October 5, 1993 in rats
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Methylene blue trihydrate, from Aldrich Chemical Company, lot # 10306AF, Purity: ~96.8%

This report contained results of repeat-dose toxicology studies (1-month and 3-month studies) and 2-year carcinogenicity studies in both mice and rats. The 3-month rat study is reviewed below. The 2-year carcinogenicity studies for mice and rats were fully reviewed in a separate review for NDA 204630 (archival date: March 25, 2014) and are summarized in this review.

3-month study in rats

Key Study Findings

- Hematological responses included anemia as indicated by decreases in erythrocytes, hematocrit, and hemoglobin and increases in reticulocytes and methemoglobinemia as indicated by increases in methemoglobin.
- Organs of toxicity included the bone marrow (hyperplasia), liver, and spleen (hematopoietic cell proliferation, congestion, lymphoid depletion of lymphoid follicles and capsular fibrosis).
- The no observed adverse effect level (NOAEL) of methylene blue in rats was not determined and is below the low dose of 25 mg/kg when administered orally once daily 5 days per week for 14 weeks.

Methods

Doses:	0, 25, 50, 100, and 200 mg/kg
Frequency of dosing:	Once daily, 5 days per week (Monday-Friday) for 14 weeks
Route of administration:	Oral gavage
Dose volume:	5 mL/kg
Formulation/Vehicle:	0.5% aqueous methylcellulose solution
Species/Strain:	F344/N rats
Number/Sex/Group:	10/sex/group
Age:	7 weeks on first day of study
Weight:	Males: 123-125 g (group average) Females: 104-106 g (group average)
Satellite groups:	Clinical pathology: 20/sex/group; 10/sex/group bled for hematology and clinical chemistry assessment on each of Weeks 1 and 6; 5 control and 5 high dose (200 mg/kg) females maintained until study completion and then necropsied with tissue collection
Unique study design:	None

Observations and Results

Mortality:	Twice daily
Clinical signs:	Observed twice daily; detailed examinations weekly
Body weights:	Initially, weekly, and at the end of the studies
Food consumption:	Not conducted
Ophthalmoscopy:	Not conducted
Hematology:	Weeks 1 and 6 in satellite groups, and Week 13 in main study animals and 5 females each from the control and 200 mg/kg satellite groups
Clinical chemistry:	Weeks 1 and 6 in satellite groups, and Week 13 in main study animals and 5 females each from the control and 200 mg/kg satellite groups
Coagulation:	Weeks 1, 6, and 13
Urinalysis:	Not specified
Gross pathology:	At necropsy*
Organ weights:	At necropsy*
Histopathology:	At necropsy*
Toxicokinetics:	Not conducted
Sperm Mortality:	Collected at end of study in control, 50, 100 and 200 mg/kg groups
Vaginal Cytology:	Collected at end of study in control, 50, 100 and 200 mg/kg groups and remaining clinical pathology 200 mg/kg females

*Necropsy conducted on Day 92; the start of the treatment phase appears to be Day 0

Mortality

- No drug-related mortalities were reported.
- According to the report, there were 6 rats that died during the study due to gavage accidents: one male treated with 25 mg/kg died during Week 11, one female treated with 100 mg/kg died during Week 2, and 4 females treated with 200 mg/kg died (one during Week 2, two during Week 4, and one during Week 11).

Clinical Signs

- Blue staining of the urine, urogenital area, tail and fur were observed in all methylene blue-treated groups. Since the drug is a blue dye, this finding demonstrates exposure to the drug and presence of the drug in urine.

Body Weights

- A minimal decrease in body weight gain (6%) occurred only in males treated with 200 mg/kg. No changes in body weight or body weight gain were noted in female rats.

Table 1: Body weights of male rats in the 3-month study of methylene blue trihydrate

Interval	Mean body weight (g)				
	Males				
Dose (mg/kg)	0 Control	25	50	100	200
Initial (g)	125 ± 5	125 ± 4	123 ± 4	124 ± 5	125 ± 4
Final (g)	330 ± 4	337 ± 7	325 ± 6	321 ± 7	309 ± 6*
Change (g)	205 ± 4	213 ± 6	202 ± 5	197 ± 5	183 ± 3**
Final weight change/control (%)	0	↑2	↓2	↓3	↓6

*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SE.
Subsequent calculations are based on animals that survived to the end of the study.

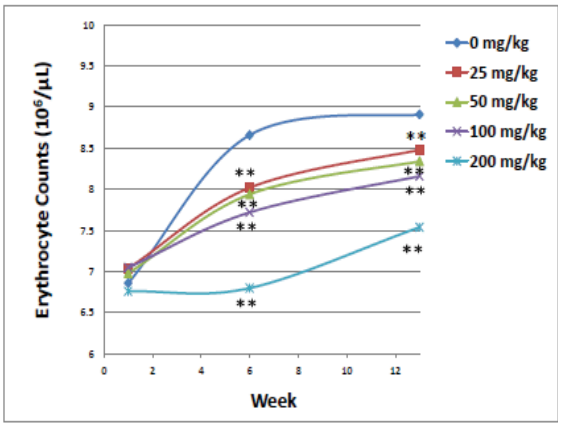
Hematology

Drug-related changes in hematological parameters included anemia in all methylene blue-treated groups as indicated by decreases in erythrocytes, hematocrit, and hemoglobin and increases in reticulocytes and methemoglobinemia as indicated by increases in methemoglobin. Increases in reticulocyte counts and increases in the incidence of Heinz bodies were also observed.

- Erythrocyte Counts: Drug-related decreases in erythrocyte counts were noted in both male and female rats at doses ≥ 25 mg/kg.

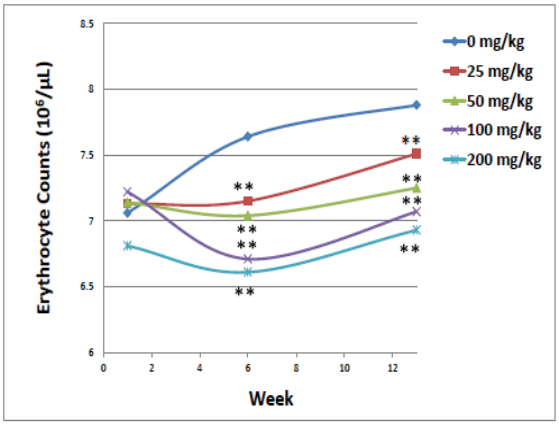
Figure 3: Drug-related changes in erythrocyte counts in rats

Erythrocyte counts (10^6/µL) in males



*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

Erythrocyte counts (10^6/µL) in females

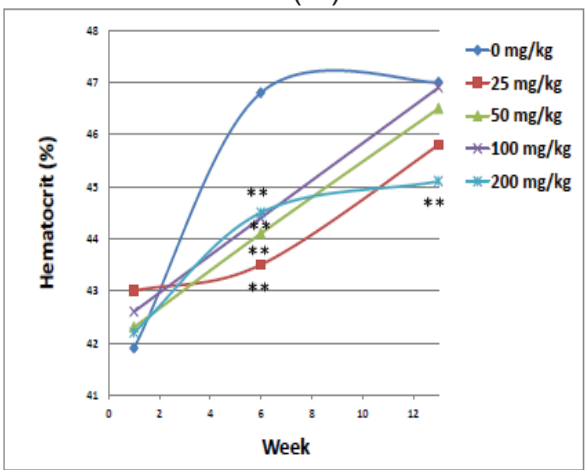


*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

- Hematocrit: Drug-related decreases in hematocrit were noted in male and female rats at doses ≥ 25 mg/kg; however, it reached the statistical significance in females only at doses ≥50 mg/kg.

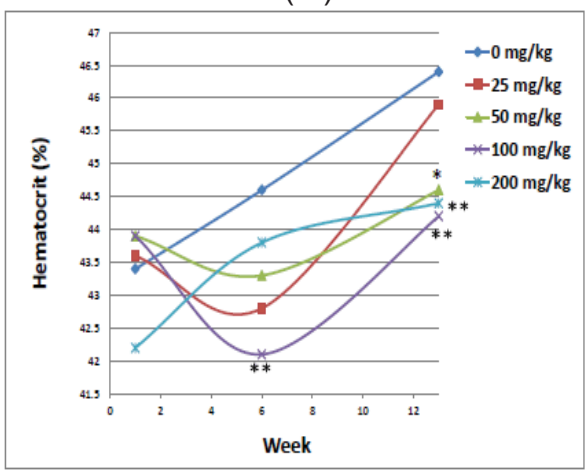
Figure 4: Drug-related changes in hematocrit in rats

Hematocrit (%) in males



*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

Hematocrit (%) in females

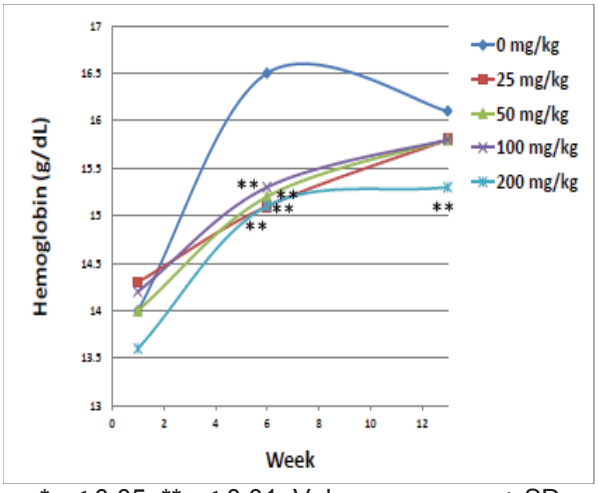


*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

- Hemoglobin: Drug-related decreases in hemoglobin were noted in both male and female rats at doses ≥ 25 mg/kg.

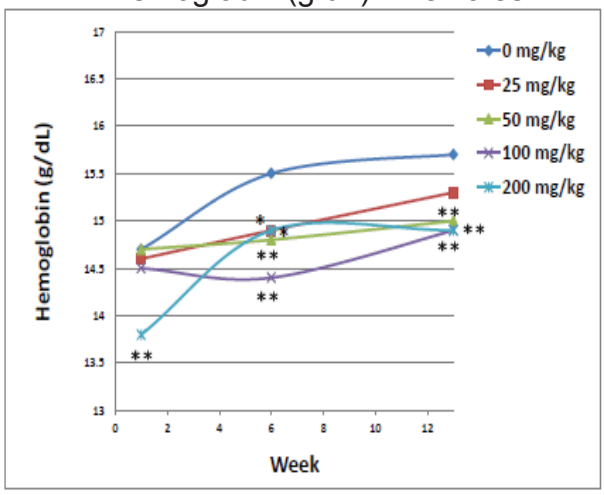
Figure 5: Drug-related changes in hemoglobin in rats

Hemoglobin (g/dL) in males



*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

Hemoglobin (g/dL) in females

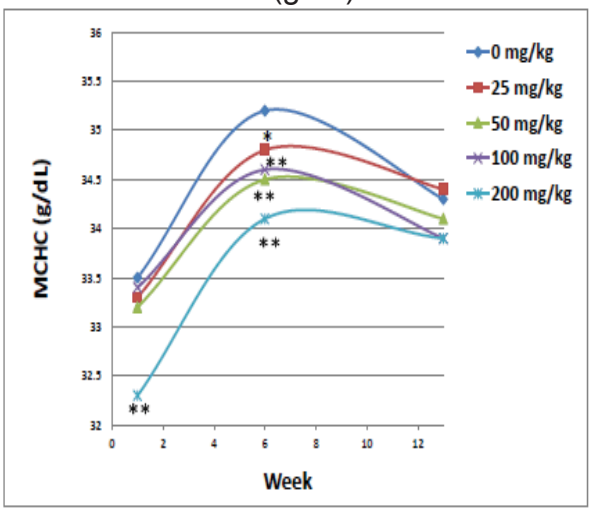


*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

- Mean corpuscular hemoglobin concentration (MCHC): Drug-related decreases in MCHC were noted in male and female rats at doses ≥ 25 mg/kg when measured at Week 6; however, at the end of Week 13, there were no differences from controls.

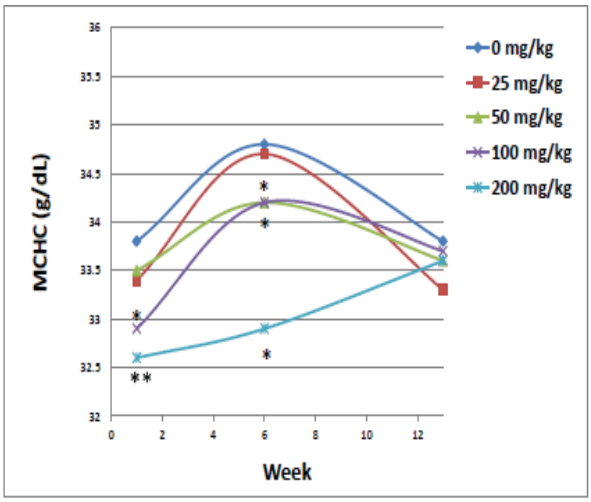
Figure 6: Drug-related changes in MCHC in rats

MCHC (g/dL) in males



*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

MCHC (g/dL) in females

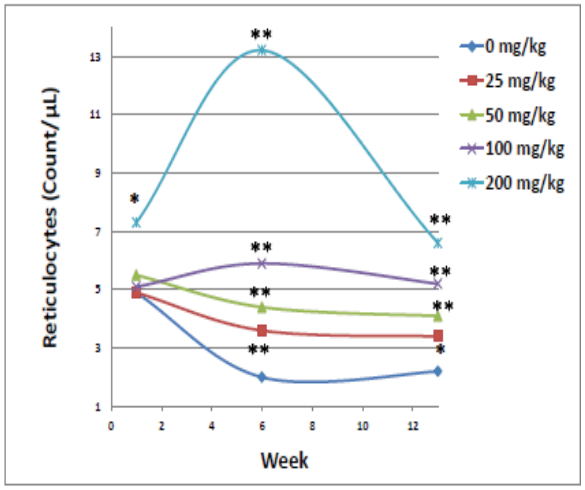


*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

- Reticulocyte counts: Drug-related increases in reticulocyte count were noted in both male and female rats at doses ≥ 25 mg/kg.

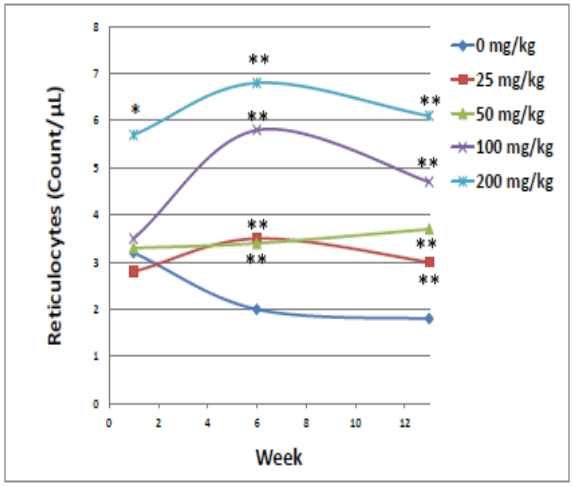
Figure 7: Drug-related changes in reticulocyte counts in rats

Retculocyte count (10^5/µL) in males



*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

Retculocyte count (10^5/µL) in females

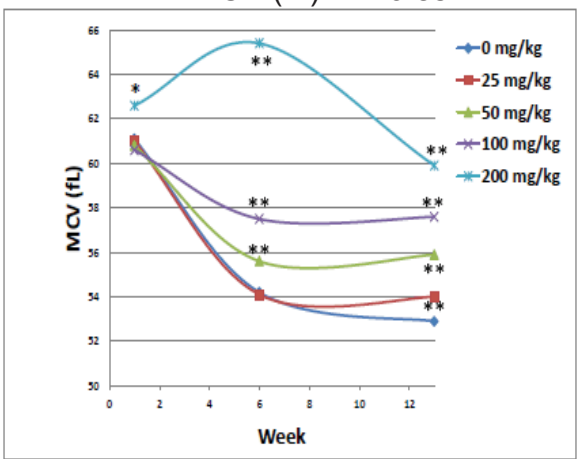


*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

- Mean corpuscular volume (MCV): Drug-related increases in MCV were noted in both male and female rats at doses ≥ 25 mg/kg.

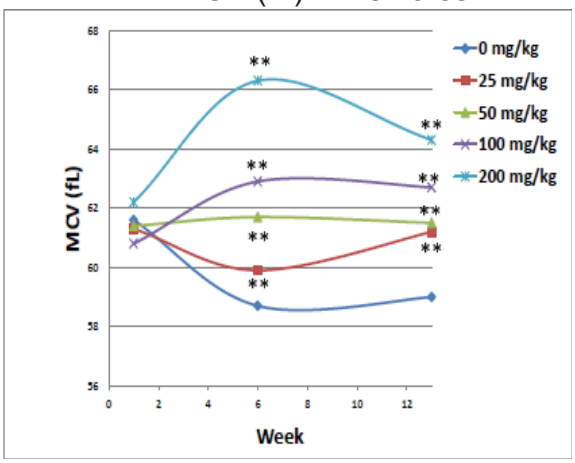
Figure 8: Drug-related changes in MCV in rats

MCV (fL) in Males



*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

MCV (fL) in Females



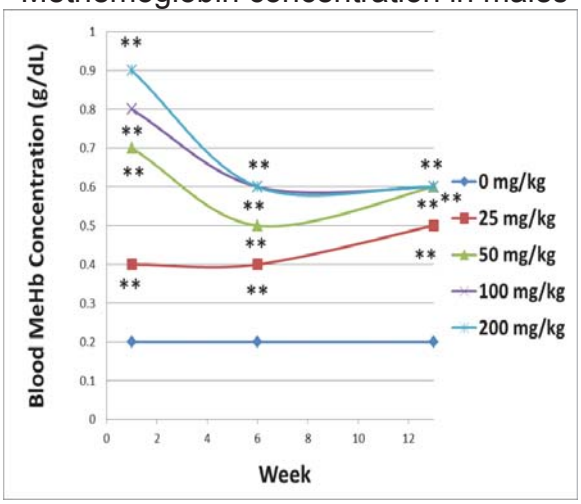
*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

Drug-related increases in blood methemoglobin concentrations and Heinz Body incidences were measured, and results are presented in the figures below.

- Blood methemoglobin concentrations: Drug-related increases in blood methemoglobin concentration were noted in both males and females at doses ≥ 25 mg/kg.

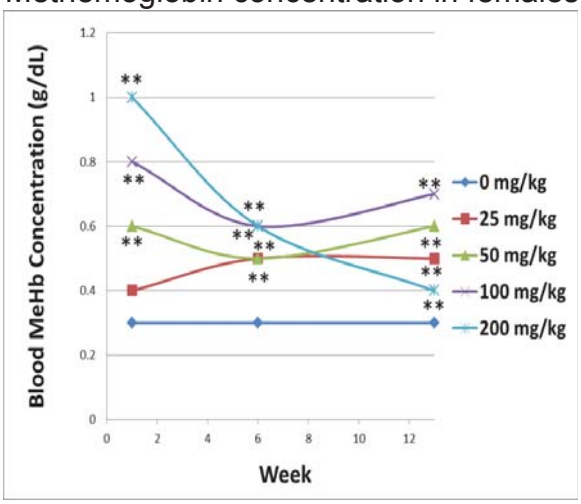
Figure 9: Drug-related changes in blood methemoglobin concentrations in rats

Methemoglobin concentration in males



*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

Methemoglobin concentration in females

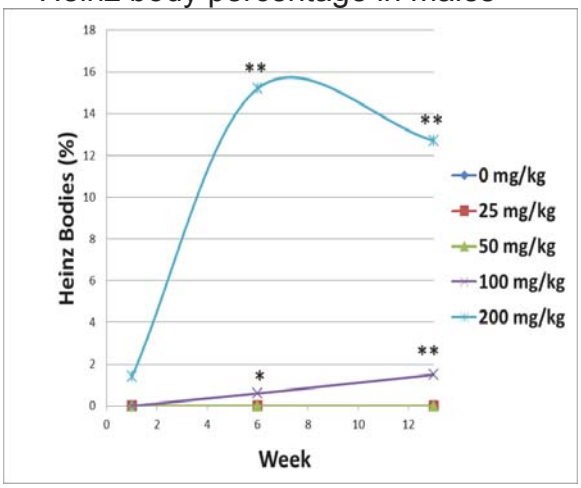


*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

- Heinz body incidence: Drug-related increases in Heinz body percentage were noted in both male and female rats at doses ≥ 100 mg/kg.

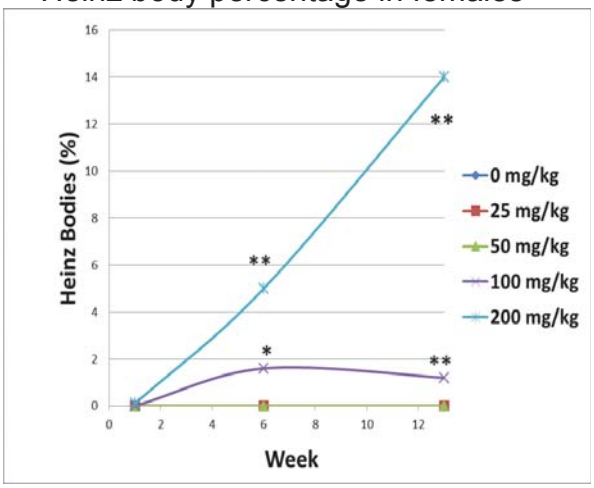
Figure 10: Heinz body incidence (%) in rats

Heinz body percentage in males



*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

Heinz body percentage in females



*p ≤ 0.05; **p ≤ 0.01; Values are mean ± SD

Table 2: Hematology changes in 3-month rat study

Index	Mean		Percentage deviation from Control							
	Control 0 mg/kg		25 mg/kg		50 mg/kg		100 mg/kg		200 mg/kg	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Erythrocytes (10^6/ µL)										
Week 1	6.86	7.06	-	-	-	-	-	-	-	↓4
Week 6	8.66	7.64	↓7**	↓6**	↓8**	↓8**	↓11**	↓12**	↓22**	↓14**
Week 13	8.91	7.88	↓5*	↓5**	↓6**	↓8**	↓8**	↓10**	↓15**	↓12**
Hematocrit (%)										
Week 1	41.9	43.4	-	-	-	-	-	-	-	↓3
Week 6	46.8	44.6	↓7**	↓4	↓6**	↓3	↓5**	↓6**	↓5**	↓2
Week 13	47.0	46.4	↓3	-	-	↓4*	-	↓5**	↓4*	↓4**
Hemoglobin (g/dL)										
Week 1	14.0	14.7	-	-	-	-	-	-	↓3	↓6**
Week 6	16.5	15.5	↓9**	↓4*	↓8**	↓5**	↓7**	↓7**	↓9**	↓4*
Week 13	16.1	15.7	-	↓3	-	↓5**	-	↓5**	↓5**	↓5**
MCHC (g/dL)										
Week 1	33.5	33.8	↓1	↓1	↓1	↓1	-	↓3*	↓4**	↓4**
Week 6	35.2	34.8	↓1*	-	↓2**	↓2	↓2**	↓2	↓3*	↓6*
Week 13	34.3	33.8	-	↓2	↓1	↓1	↓1	-	↓1	↓1
Reticulocytes (10^5/µL)										
Week 1	4.9	3.2	-	-	↑12	-	-	↑9	↑49	↑78*
Week 6	2.0	2.0	↑80**	↑75**	↑120**	↑70**	↑195**	↑190**	↑560**	↑240**
Week 13	2.2	1.8	↑55*	↑67**	↑86**	↑106**	↑136**	↑161**	↑200**	↑239**
MCV (fL)										
Week 1	61.1	61.6	-	-	-	-	-	-	↑3*	-
Week 6	54.2	58.7	-	↑2**	↑3**	↑5**	↑6**	↑7**	↑21**	↑13**
Week 13	52.9	59.0	↑2**	↑4**	↑6**	↑4**	↑9**	↑6**	↑13**	↑9**
Methemoglobin (g/dL)										
Week 1	0.2	0.3	↑100**	↑33	↑250**	↑100**	↑300**	↑167**	↑350**	↑233**
Week 6	0.2	0.3	↑100**	↑67**	↑150**	↑67**	↑200**	↑100**	↑200**	↑100**
Week 13	0.2	0.3	↑150**	↑67**	↑200**	↑100**	↑200**	↑133**	↑200**	↑33**

↑= increase ↓=decrease - = no test-article related changes
* P≤0.05; ** P≤0.01

Index	Mean									
	Control 0 mg/kg		25 mg/kg		50 mg/kg		100 mg/kg		200 mg/kg	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Heinz Bodies (%)										
Week 1	0	0	-	-	-	-	-	-	1.4	-
Week 6	0	0	-	-	-	-	0.6*	1.6*	15.2**	5.0**
Week 13	0	0	-	-	-	-	1.5**	1.2**	12.7**	14.0**

- = no test-article related changes
* P≤0.05; ** P≤0.01

Clinical Chemistry

Table 3: Clinical chemistry changes in 3-month rat study

Index	Mean		Percentage deviation from Control							
	Control 0 mg/kg		25 mg/kg		50 mg/kg		100 mg/kg		200 mg/kg	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Bile acids (µmol/L)										
Week 1	25.4	26.0	-	-	-	-	↑48	-	↑72	-
Week 6	14.1	25.0	-	-	↑89*	-	↑131**	-	↑231**	-
Week 13	16.9	20.9	-	↑44*	-	↑100**	-	↑144**	↑60*	↑188**
Creatine kinase (IU/L)										
Week 1	527	680	-	-	-	↑91	-	↑58	-	↑196*
Week 6	380	473	-	-	-	-	-	-	-	-
Week 13	268	315	-	-	-	-	-	-	-	-

↑= increase ↓=decrease - = no test-article related changes
* P≤0.05; ** P≤0.01

Gross Pathology

- Enlarged spleen was observed in males at 100 mg/kg and males and females at 200 mg/kg methylene blue trihydrate. This finding was mentioned in the NTP report, but the data were not provided

Organ Weights

- Dose-dependent increases in both absolute and relative spleen weight were observed in both male and female rats in methylene blue-treated groups with doses ≥ 25 mg/kg at the end of Week 13. These increases in spleen weight correlated with microscopic findings of congestion and hematopoiesis.
- Increases in absolute and relative liver weight were also observed, particularly at 200 mg/kg.

Table 4: Absolute and relative spleen and liver weights in 3-month rat study

Group and Dose		Mean Control	Percentage deviation from Control			
		0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Necropsy		Terminal	Terminal	Terminal	Terminal	Terminal
Number of animals examined (Males/Females)		10/10	9/10	10/10	10/9	10/6
Spleen						
Males	Absolute (g)	0.731	↑14**	↑23**	↑47**	↑103**
	Relative BW (%)	2.152	↑12**	↑26**	↑53**	↑116**
Females	Absolute (g)	0.487	↑10**	↑24*	↑58**	↑122**
	Relative BW (%)	2.432	↑10**	↑25*	↑61**	↑121**
Liver						
Males	Absolute (g)	12.89	-	-	-	↑9
	Relative BW (%)	37.890	-	↑9**	↑7**	↑16**
Females	Absolute (g)	7.119	-	-	↑7	↑14**
	Relative BW (%)	35.390	-	-	↑10**	↑14**

↑= increase - = no test-article related changes
*: p≤0.05; **: p≤0.01

Histopathology

Adequate Battery: Yes

Peer Review: Not reported

Histological Findings

Table 5: Drug-related microscopic findings in 3-month rat study

(excerpted from NTP report TR 540 2008)

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
Spleen[a]	10	10	10	10	10
Hematopoietic Cell Proliferation[b]	0	0	10** (2.0)	9** (2.0)	10** (2.0)
Congestion	0	9** (1.6)[c]	10** (2.0)	10** (2.0)	10** (1.9)
Lymphoid Follicle, Depletion Cellular	0	0	0	2 (1.0)	5* (1.4)
Capsule, Fibrosis	1 (1.0)	2 (1.0)	0	1 (1.0)	9** (1.0)
Bone Marrow	10	10	10	10	10
Hyperplasia	0	0	8** (2.0)	10** (2.0)	10** (3.0)
Female					
Spleen	10	10	10	10	10
Hematopoietic Cell Proliferation	0	0	8** (1.9)	9** (2.0)	10** (2.0)
Congestion	0	9** (1.7)	10** (2.0)	9** (2.0)	9** (1.8)
Lymphoid Follicle, Depletion Cellular	0	0	0	1 (2.0)	9** (1.6)
Capsule, Fibrosis	0	0	0	3 (1.0)	7** (1.0)
Bone Marrow	10	10	10	10	10
Hyperplasia	0	0	4* (1.3)	9** (1.8)	10** (2.9)

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Fisher exact test
** $P \leq 0.01$
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

Special Evaluation

Sperm Mortality

Unremarkable

Vaginal Cytology

Unremarkable

Toxicokinetics

Not conducted

Dosing Solution Analysis

Analytical results from the pre-administration and mid-study post-administration samples indicated that dosing solutions were within 10% of the target concentration.

Study title: 4-week toxicity study by slow intravenous infusion to beagle dogs

Study no.:	36110TSC
Study report location:	eCTD 4.2.3.2
Conducting laboratory and location:	(b) (4) [redacted]
Date of study initiation:	September 29, 2009
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Methylene blue from Provepharma SAS, batch # 9280, Purity: ~98% Comparator drug: Methylene blue injection USP 1% w/v, Martindale batch # 1042990, Purity: Not provided

Key Study Findings

- Hematological responses included anemia in all methylene blue-treated groups as indicated by decreases in erythrocytes, hemoglobin, and hematocrit and increases in reticulocytes and platelets.
- Organs of toxicity included the bone marrow, injection site (cephalic vein), kidney, liver, and spleen.
- The no observed adverse effect level (NOAEL) of methylene blue in dogs was not determined and is below the low dose of 0.25 mg/kg/day when administered intravenously once daily for 4 weeks.
- Provepharma's methylene blue (5 mg/mL) had a similar toxicological and toxicokinetic profile in dogs as the comparator drug, methylene blue injection USP 1% w/v (Martindale), at a dose of 1.0 mg/kg/day when administered intravenously once daily for 4 weeks.

Methods

Doses:	See table below for doses. Doses based on results of dose range-finding study (4 or 14 days of dosing) in dogs with Provepharma's methylene blue.
Frequency of dosing:	Once daily for 4 weeks
Route of administration:	Intravenous infusion via cephalic or saphenous vein; flow rate of 0.5 mL/minute
Dose volume:	Control, high dose, and the comparator arm: 0.2 mL/kg/day Low dose: 0.05 mL/kg/day Mid dose: 0.1 mL/kg/day
Formulation/Vehicle:	Water for injection
Species/Strain:	Beagle dogs
Number/Sex/Group:	3/sex/group

Age:	6-11 months at beginning of treatment period
Weight:	Males: 7.7-9.2 kg Females: 6.2-8.5 kg
Satellite groups:	None
Unique study design:	None
Deviation from study protocol:	One male treated with 0.25 mg/kg/day (Animal # U50004) was treated one additional day (29 days vs. 28 days) and was necropsied on Day 30; see toxicokinetics for more details

Table 6: Doses of methylene blue in 1-month dog study

Drug	Doses
Provepharma's methylene blue (concentration of 5 mg/mL)	0, 0.25, 0.50, or 1.0 mg/kg/day
Methylene blue injection USP 1% w/v (Martindale) (comparator drug)	1.0 mg/kg/day

Observations and times:

Mortality:	Twice daily
Clinical signs:	At least once daily
Body weights:	Twice before group allocation, Day -1, weekly, and prior to necropsy
Food consumption:	Daily
ECG:	During the pretreatment period (Predose) and at least 2 hours on Day 24
Ophthalmoscopy:	During the pretreatment period (Predose) and Week 4
Hematology:	During pretreatment period (Predose) and Days 24 for all animals, Day 14 for Control and 1.0 mg/kg/day Provepharma's methylene blue and comparator drug groups
Clinical chemistry:	During pretreatment period (Predose) and Day 24
Coagulation:	During pretreatment period (Predose) and Days 24 for all animals, Day 14 for Control and 1.0 mg/kg/day Provepharma's methylene blue and comparator drug groups
Urinalysis:	During pretreatment period (Predose) and Day 24
Gross pathology:	At necropsy*
Organ weights:	At necropsy*
Histopathology:	At necropsy*
Toxicokinetics:	Controls: • Days 1 and 25 for males and Days 1 and 27 for females at 5 minutes and 2 hours after dosing Methylene blue treated groups: • Day 1 at 5 minutes and 0.5, 1, 2, 4, 6, 8, and 24 hours

	after dosing • Day 28 at predose, 5 minutes and 0.5, 1, 2, 4, 6, and 8 hours after dosing

* Necropsy on Day 29, Day 30 for one male treated with 0.25 mg/kg/day (Animal # U50004)

Results

Mortality

No mortality was observed in this study.

Clinical Signs

- Greenish abnormal color of urine and/or feces was observed in some animals treated with 0.50 or 1.0 mg/kg/day Provepharma's methylene blue or 1.0 mg/kg/day of the comparator drug.
- According to the report, one male treated with 1.0 mg/kg/day Provepharma's methylene blue had one injection site (right cephalic vein) that was increased in size (Days 8 to 20) and/or indurated (Days 14 to 26). One female treated with 0.5 mg/kg/day Provepharma's methylene blue had one injection site (right cephalic vein) that was increased in size from Day 23. Of note, there was no definition in the report regarding "increase in size".

Body Weights

Unremarkable

Food Consumption

Unremarkable

Ophthalmoscopy

Unremarkable

ECG

Unremarkable

Hematology

- Hematological responses included anemia in all methylene blue-treated groups as indicated by decreases in erythrocytes, hemoglobin, and hematocrit and increases in reticulocytes and platelets.
- According to the report, Heinz bodies were observed in the groups treated with 1.0 mg/kg/day Provepharma's methylene blue and 1.0 mg/kg/day of comparator drug on Days 14 and 24, suggesting the formation of methemoglobinemia. The data was not provided.
- Methemoglobin levels were not measured in this study.

Table 7: Hematology changes in 1-month dog study

Index	Mean		Percentage deviation from Control							
	Control 0 mg/kg/day		0.25 mg/kg/day		0.50 mg/kg/day		1.00 mg/kg/day		1.00 mg/kg/day Comparator drug	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Erythrocytes (10^{12}/L)										
Day 14	7.02	7.53	NE	NE	NE	NE	↓29	↓30	↓34*	↓29
Day 24	6.82	7.14	↓17	↓10	↓18	↓20	↓25	↓25	↓24	↓22
Hemoglobin (g/dL)										
Day 14	15.6	16.7	NE	NE	NE	NE	↓27	↓26	↓31*	↓27*
Day 24	15.2	15.8	↓13	↓7	↓18	↓15	↓23	↓22*	↓21	↓22*
Hematocrit (L/L)										
Day 14	0.46	0.49	NE	NE	NE	NE	↓22	↓22	↓26*	↓22
Day 24	0.45	0.46	↓13	↓4	↓16	↓9	↓16	↓13	↓13	↓13
MCHC (g/dL)										
Day 14	33.7	34.2	NE	NE	NE	NE	↓7	↓5	↓7*	↓6*
Day 24	33.9	33.9	-	-	↓4	↓6	↓9*	↓8	↓9*	↓10*
MCV (fL)										
Day 14	65.9	64.9	NE	NE	NE	NE	↑11	↑11*	↑13*	↑9
Day 24	65.8	65.3	↑5	↑5	↑4	↑13*	↑13*	↑13	↑14*	↑11
Reticulocytes (%)										
Day 14	0.76	0.82	NE	NE	NE	NE	↑546	↑394	↑618*	↑422
Day 24	0.54	0.66	↑81	-	↑117	↑105	↑498	↑241	↑613*	↑324*
Platelet count (10^{9}/L)										
Day 14	340	361	NE	NE	NE	NE	↑68	↑91*	↑68	↑59
Day 24	337	391	↑42	↑38	↑60	↑41	↑62	↑73*	↑65	↑53
Fibrinogen (g/L)										
Day 14	1.89	2.65	NE	NE	NE	NE	↑39	↑31	↑79*	↑44*
Day 24	3.01	2.70	↑5	↑19	-	↑14	↑20	↑36	↑8	↑20

↑= increase ↓=decrease - = no test-article related changes
* P≤0.05; ** P≤0.01
NE= Hematology not evaluated in this group on Day 14

Clinical Chemistry

- During the last week of treatment on Day 24, increases in total bilirubin were observed in dogs treated with methylene blue at doses ≥ 0.50 mg/kg/day in males and ≥ 0.25 mg/kg/day in females compared to controls.

Table 8: Clinical chemistry changes on Day 24 in 1-month dog study

Index	Mean		Mean (Percentage deviation from Control)							
	Control 0 mg/kg/day		0.25 mg/kg/day		0.50 mg/kg/day		1.00 mg/kg/day		1.00 mg/kg/day Comparator drug	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Total bilirubin (µmol/L)	1	1	1	3 (↑200)	3 (↑200)	3 (↑200)	2 (↑100)	3 (↑200)	2 (↑100)	3 (↑200)

↑= increase

Urinalysis

- During the last week of treatment on Day 24, a higher incidence of moderate bilirubin levels in the urine were observed in the dogs treated with methylene blue compared to controls.
- Moderate bilirubin levels correlated with dark yellow or yellow/green colored urine.

Table 9: Urinalysis findings on Day 24 in 1-month dog study

Index	No. of animals with observation									
	Control 0 mg/kg/day		0.25 mg/kg/day		0.50 mg/kg/day		1.00 mg/kg/day		1.00 mg/kg/day Comparator drug	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Number of animals examined	3	3	3	3	3	3	3	3	3	3
Bilirubin										
Negative	1	3	1	0	0	1	1	0	0	0
Low	2	0	1	1	0	0	0	0	0	0
Moderate	0	0	1	2	3	2	2	3	3	3
Color										
Pale yellow	0	1	2	0	0	1	1	0	0	0
Yellow	3	2	1	1	3	0	1	0	0	0
Dark Yellow	0	0	0	2	0	0	1	0	1	0
Yellow/green	0	0	0	0	0	2	0	3	2	3

Gross Pathology

Table 10: Macroscopic findings in 1-month dog study

Treatment-Related Macroscopic Findings		No. of animals affected									
		Males					Females				
Dose (mg/kg/day)		0	0.25	0.50	1.0	1.0 CD	0	0.25	0.50	1.0	1.0 CD
Number of animals examined		3	3	3	3	3	3	3	3	3	3
Inj. Site left Cephalic vein L3	Thickened subcutaneous tissue	-	-	-	1	1	-	-	-	-	-
Inj. Site right Cephalic vein L2	Thickened subcutaneous tissue	-	-	-	-	-	-	-	1	2	-
Inj. Site right Cephalic vein L3	Thickened subcutaneous tissue	-	-	-	2	-	1	-	2	2	1
Spleen	Enlarged	-	-	-	-	1	-	-	-	1	2

CD= comparator drug (Methylene blue injection USP 1% w/v)
- = no test-article related changes
L2= Level 2 L3= Level 3
Definition of levels of the injection sites:

- Level 1: About 1 cm upstream from the tip of the catheter
- Level 2: At the tip of the catheter
- Level 3: About 1 cm downstream from the tip of the catheter

Organ Weights

- Increases in absolute and relative spleen weight were observed in both male and female dogs treated with 0.50 and 1.0 mg/kg/day Provepharma's methylene blue and 1.0 mg/kg/day of the comparator drug.

Table 11: Absolute and relative spleen weights in 1-month dog study

Group and Dose (mg/kg/day)		Mean Control	Percentage deviation from Control			
		0	0.25	0.50	1.0	1.0 CD
Number of animals examined (Males/Females)		3/3	3/3	3/3	3/3	3/3
Spleen						
Males	Absolute (g)	31.59	-	↑7	↑62	↑75
	Relative BW (%)	0.35723	-	↑14	↑57	↑75
Females	Absolute (g)	25.76	-	↑48	↑103	↑126
	Relative BW (%)	0.34362	-	↑50	↑115	↑130*

CD= comparator drug (Methylene blue injection USP 1% w/v)
↑= increase; - = no test-article related changes; *: p≤0.05

Histopathology

Microscopic examination was performed on all tissues for dogs in the control group, the 1.0 mg/kg/day Provepharma's methylene blue group, and the 1.0 mg/kg/day comparator drug group. Liver, kidney, spleen, and injection sites were also examined in all dogs in the 0.25 and 0.50 mg/kg/day groups. In selected animals from the control, the 1.0 mg/kg/day Provepharma's methylene blue, and the 1.0 mg/kg/day comparator groups, additional sections of liver and kidney were stained by Perls's (both tissues) and/or Hall's (liver only) stains to characterize pigment noted in slides stained by hematoxylin-eosin.

Adequate Battery: Yes

Peer Review: No

Histological Findings

- Findings are those observed in all animals, both scheduled and early (unscheduled) deaths.
- Microscopic findings observed in the gallbladder, kidneys, liver, and spleen are in the table below.
- Brown pigment observed in the cytoplasm of proximal tubular cells in the kidney and green/brown pigment observed in Küpffer cells in the liver were positive with Perl's stain, which is consistent with hemosiderin (an iron-storage complex) secondary to hemolysis and increased catabolism of the hemoglobin. Green/brown pigment was also observed in the cytoplasm of macrophages in the spleen and was also secondary to hemolysis and increased catabolism of the hemoglobin.
- In the bone marrow, there was increased cellularity of the hematopoietic component of the bone marrow in all methylene blue-treated groups, indicated by the absence of adipocytes in the bone marrow compared to the small numbers of adipocytes (slight severity) observed in all controls.
- There was a trend towards an increased incidence and/or severity of inflammatory findings at the injection sites in dogs treated with 1.0 mg/kg/day Provepharma's methylene blue and 1.0 mg/kg/day of the comparator drug

compared to controls. The findings were more prominent in levels 2 and 3 of the injection sites (see footnote to Table 10 for definition of levels of the injection sites). The main perivenous findings were hemorrhage, mixed inflammatory cell infiltrates, brown pigment-laden macrophages, fibroplasia/fibrosis, edema, and degeneration/necrosis. At the level of the injected veins (right or left cephalic veins), the findings included intimal and/or medial thickening, degeneration/necrosis of the venous wall, thrombi, and inflammatory cells in the venous wall.

Table 12: Microscopic findings in 1-month dog study

Treatment-Related Microscopic Findings			No. of animals affected									
			Males					Females				
Dose (mg/kg/day)			0	0.25	0.50	1.0	1.0 CD	0	0.25	0.50	1.0	1.0 CD
Number of animals examined			3	3	3	3	3	3	3	3	3	3
Organ	Finding											
Gal bladder	Green/brown pigment	Minimal	-	NE	NE	1	1	-	NE	NE	1	-
Kidneys	Increased brown pigment	Minimal	-	-	-	2	2	-	-	-	2	3
Liver	Green/brown pigment	Total	-	2	3	3	3	-	2	3	3	3
		Minimal	-	2	3	2	2	-	2	2	-	1
		Slight	-	-	-	1	1	-	-	1	2	2
		Moderate	-	-	-	-	-	-	-	-	1	-
	Inflammatory cell foci	Minimal	-	1	1	3	2	1	-	2	3	3
Spleen	Hemopoiesis	Total	2	3	3	3	3	1	3	3	3	3
		Minimal	2	2	-	-	-	1	2	-	-	-
		Slight	-	1	3	-	-	-	1	2	-	-
		Moderate	-	-	-	3	3	-	-	1	3	3
	Congestion	Total	1	-	3	3	3	-	3	3	3	3
		Slight	-	-	3	1	2	-	3	3	2	1
		Moderate	1	-	-	2	1	-	-	-	1	2
	Increased green/brown pigment	Minimal	1	1	1	1	2	-	1	3	3	2

CD= comparator drug (Methylene blue injection USP 1% w/v)
NE= Tissue not examined in this group
- = no test-article related changes

Toxicokinetics

In dogs, the toxicokinetics of intravenous administration of Provepharma's methylene blue (0.25, 0.50, or 1.0 mg/kg/day) and the comparator drug, Methylene blue injection USP (1.0 mg/kg/day), were evaluated on Days 1 and 28.

On Day 28, one male treated with 0.25 mg/kg/day (Animal # U50004) was infused for 108 seconds instead of for 48 seconds, so this animal received 0.56 mg/kg of the drug instead of 0.25 mg/kg. Blood sampling was stopped after 1 hour and an additional day of treatment with blood sampling for toxicokinetics was scheduled (Day 29).

- Absorption of Provepharma's methylene blue and the comparator drug was rapid with t_{max} at 0.083 hours (5 minutes) for both males and females at 0.5 and 1.0 mg/kg/day and females at 0.25 mg/kg/day on Days 1 and 28.
- The drug levels were below the level of quantification in many animals at various time points. Methylene blue was last quantifiable at 1 or 2 hours at 0.25 mg/kg/day, between 1 and 4 hours at 0.5 mg/kg/day, between 1 and 8 hours in at 1.0 mg/kg/day Provepharma's methylene blue, and between 2 and 6 hours for the comparator drug at 1.0 mg/kg/day. On Day 28, there was not enough quantifiable plasma to perform toxicokinetic evaluation in males at 0.25 mg/kg/day.
- Half-life ($t_{1/2}$) was similar at all dose levels and ranged from 0.8 to 1.5 hours with the exception of a $t_{1/2}$ of 5 hours in females treated with 1.0 mg/kg/day Provepharma's methylene blue on Day 1.
- Exposures (C_{max} and $AUC_{(0-t)}$) for Provepharma's methylene blue at 1.0 mg/kg/day were slightly lower but similar to the comparator drug at 1.0 mg/kg/day in both males and females on Day 1 and in females on Day 28. This suggests similar toxicokinetics for the two compounds. Exposures in males at 1.0 mg/kg/day on Day 28 were lower than those with the comparator drug.
- C_{max} and $AUC_{(0-t)}$ for Provepharma's methylene blue increased with an increase in dose. In general, increases were approximately dose proportional or slightly greater than dose proportional. An exception was the increase in $AUC_{(0-t)}$ from 0.5 to 1.0 mg/kg/day in males on Day 28, which was less than dose proportional.
- Following repeated administration of methylene blue, C_{max} and $AUC_{(0-t)}$ were higher on Day 28 than on Day 1 at 0.25 mg/kg/day, similar on Days 1 and 28 at 0.5 mg/kg/day, and were lower on Day 28 than on Day 1 at 1.0 mg/kg/day.
- Overall, exposures were slightly higher in females than males at 0.5 and 1.0 mg/kg/day Provepharma's methylene blue.

Table 13: Toxicokinetics of methylene blue in 1-month dog study

(excerpted from report for study 36110TSC)

			Reference item		Test item					
		Dose-level (mg/kg)	1		0.25		0.5		1	
Sampling Period	Parameters	Unit	M	F	M	F	M	F	M	F
Day 1	C_{max}	(ng/mL)	150	124	25.5	19.8	47.6	56.4	116	114
	t_{max}^{∇}	(h)	0.083	0.083	0.5	0.083	0.083	0.083	0.083	0.083
	$t_{1/2}$	(h)	1.17	2.88	nc	1.26	0.77	1.21	0.83	5.19
	λ_z	(1/h)	0.60	0.34	nc	0.55	0.91	0.84	0.92	0.39
	R^2		0.98	0.97	nc	0.99	0.97	0.92	0.99	0.94
	$AUC_{(0-t)}$	(ng.h/mL)	169	201	22.0	16.3	32.2	65.6	134	172
	$AUC_{(0-24)}$	(ng.h/mL)	nc	nc	nc	nc	nc	nc	nc	nc
	$AUC_{(0-\infty)}$	(ng.h/mL)	199	246	nc	38.1	55.2	92.6	151	254
	AUC extrapolation	%	15.4	17.4	nc	57.3	41.4	27.1	11.7	24.6
Day 28	C_{max}	(ng/mL)	103	112	nc	27.9	36.1	57.1	69.1	91.5
	t_{max}^{∇}	(h)	0.083	0.083	nc	0.083	0.083	0.083	0.083	0.083
	$t_{1/2}$	(h)	1.94	1.57	nc	1.53	1.23	1.00	0.70	1.12
	λ_z	(1/h)	0.52	0.60	nc	0.72	0.76	0.72	1.03	0.64
	R^2		0.93	0.99	nc	0.94	0.95	0.97	1.00	0.97
	$AUC_{(0-t)}$	(ng.h/mL)	121	134	nc	27.3	40.5	74.8	63.9	129
	$AUC_{(0-24)}$	(ng.h/mL)	nc	nc	nc	nc	nc	nc	nc	nc
	$AUC_{(0-\infty)}$	(ng.h/mL)	153	169	nc	55.8	61.7	89.8	79.7	151
	AUC extrapolation	%	19.4	21.3	nc	44.9	35.6	18.3	20.9	15.4

M: male; F: female

nc: not calculated

∇: value correspond to the median

Histopathology inventory for general toxicology studies

Study	NTP report TR 540	36110TSC
Species	Rat	Dog
Study length	3 months	1 month
Adrenals	X	X*
Aorta	X	X
Bone Marrow smear	X	X
Bone	X	X
Brain	X	X*
Cecum	X	X
Cervix		X
Clitoral gland	X	
Colon	X	X
Duodenum	X	X
Epididymis	X	X*
Esophagus	X	X
Eye	X	X
Fallopian tube		X
Gall bladder		X
Gross lesions	X	
Harderian gland	X	
Heart	X*	X*
Ileum	X	X
Injection site		X
Jejunum	X	X
Kidneys	X*	X*
Lacrimal gland		
Larynx		X
Liver	X*	X*
Lungs	X*	X
Lymph nodes, cervical		
Lymph nodes mandibular	X	X
Lymph nodes, mesenteric	X	X
Mammary Gland	X	X
Masses in tissue	X	
Nasal cavity	X	
Optic nerves		X
Ovaries	X	X*

Study	NTP report TR 540	36110TSC
Species	Rat	Dog
Study length	3 months	1 month
Pancreas	X	X
Parathyroid	X	X*
Peripheral nerve		
Pharynx		
Pituitary	X	X
Preputial gland	X	
Prostate	X	X
Rectum	X	X
Salivary gland	X	X
Sciatic nerve		X
Seminal vesicles	X	
Skeletal muscle		X
Skin		X
Spinal cord		X
Spleen	X*	X
Sternum		X
Stomach	X	X
Teeth		
Testes	X*	X*
Thymus	X*	X*
Thyroid	X	X*
Tongue		X
Trachea	X	X
Urinary bladder	X	X
Uterus	X	X
Vagina		X
Zymbal gland		

X, histopathology performed
*, organ weight obtained

7 Genetic Toxicology

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

Study title: NTP technical report on the toxicology and carcinogenesis studies of methylene blue trihydrate (CAS No. 7220-79-3) in F344/N rats and B6C3F$_1$ mice

Study no.:	NTP report TR 540 2008
Study report location:	eCTD 4.3
Conducting laboratory and location:	First assay: BioReliance Corporation Second assay and adjunct studies for metabolites: SITEK Research Laboratories Locations not provided
Date of study initiation:	Not provided
GLP compliance:	Not clear
QA statement:	No
Drug, lot #, and % purity:	Methylene blue trihydrate, from Sigma Chemical Company, lot # 68H3728, Purity: >91%; another lot used, but not specified

Key Study Findings

- Methylene blue trihydrate and three metabolites (Azure A, Azure B, and Azure C) were positive for mutagenicity both in the presence and absence of S9 in *Salmonella typhimurium* strains TA98 and TA100 and in *Escherichia coli* strain WP2 *uvrA*/pKM101.

Methods

Strains:	*Salmonella typhimurium* strains TA97, TA98, TA100, TA1535, and TA1537 *Escherichia coli* strain WP2 *uvrA*/pKM101
Concentrations in definitive study:	Study 1: 1-200 µg/plate Study 2: 0.25-150 µg/plate for TA98 and TA100; 0.25-1,500 µg/plate for WP2 *uvrA*/pKM101
Basis of concentration selection:	High dose was limited by toxicity
Negative control:	Vehicle (solvent) control
Positive control:	See table below
Formulation/Vehicle:	Not specified; 0.5% aqueous methylcellulose used in other studies in report
Incubation & sampling time:	Tester strains incubated with methylene blue in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C; top agar supplemented with $_{L}$-histidine and d-biotin was added and contents of tubes placed on agar plate; mutant colonies counted following incubation for 2 days at 37° C

Mutagenicity assays in bacterial cells were conducted by NTP as part of the toxicology assessment of methylene blue trihydrate and these results were reported in the same report as the repeat-dose toxicology and carcinogenicity studies.

Based on the report, two independent mutagenicity assays were conducted with methylene blue trihydrate. Testing for the first assay was performed as reported by Zeiger *et al*. (1988). The second assay was conducted with the same lot of methylene blue tested in the 2-year carcinogenicity study (lot # 68H3728) and used a slightly modified protocol (activation only with rat liver S9). It also used *Eschericha coli* strain WP2 *uvrA*/pKM101in addition to the *Salmonella typhimurium* strains.

Adjunct studies for the Ames assay were conducted with three metabolites of methylene blue trihydrate: Azure A, Azure B, and Azure C. All three compounds were tested in the protocol used in the second assay.

Each assay consisted of triplicate plates of concurrent positive and negative controls and at least five concentrations of methylene blue trihydrate.

Table 14: Summary of positive control agents for Ames assay for methylene blue trihydrate

Assay	Chemicals	Responding strains
Nonactivation	9-aminoacridine	TA97 and TA1537
	Sodium azide (SA)	TA100, TA1535
	4-nitro-o-phenylenediamine	TA98
	Methyl methanesulfonate	WP2uvrA/pKM101
S9 activation	2-aminoanthracene (2-AA)	TA97, TA98, TA100, TA1535, TA1537

Study Validity

Test is valid based on the information and data provided in the report. The positive and negative controls fulfilled the requirements of a valid test.

Criteria for positive response

A positive response for mutagenicity was defined as a reproducible, concentration-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. There was no minimum percentage or fold increase required for the drug to be considered positive

Results

Two different lots of methylene blue trihydrate were tested independently at two laboratories for mutagenicity.

- In the first study, methylene blue trihydrate was mutagenic in *Salmonella typhimurium* strains TA98 and TA100 in the presence of 30% rat or hamster S9 activation. In the absence of S9, mutagenicity was seen only in TA98.
- In the second study, methylene blue trihydrate was mutagenic both in the presence and absence of 10% rat liver S9 in *Salmonella typhimurium* strains TA98 and TA100 and in *Escherichia coli* strain WP2 *uvrA*/pKM101.
- The three metabolites (Azure A, Azure B, and Azure C) were positive for mutagenicity both in the presence and absence of 10% rat liver S9 in *Salmonella typhimurium* strains TA98 and TA100 and in *Escherichia coli* strain WP2 *uvrA*/pKM101.

Table 15: Mutagenicity in first Ames assay of methylene blue trihydrate

(excerpted from NTP report TR 540 2008)

Strain	Dose (µg/plate)	Revertants/Plate[b] –S9 Trial 1	–S9 Trial 2	+30% hamster S9 Trial 1	+30% hamster S9 Trial 2	+30% rat S9 Trial 1	+30% rat S9 Trial 2
TA100	0	119 ± 6.8		136 ± 15.2	137 ± 2.0	147 ± 3.1	156 ± 3.2
	1	97 ± 5.9					
	3.3	132 ± 6.3			157 ± 2.4		155 ± 2.0
	10	143 ± 2.2		130 ± 11.5	213 ± 12.6	179 ± 1.5	176 ± 1.5
	33	159 ± 5.6		246 ± 25.7	792 ± 31.6	339 ± 9.7	603 ± 41.0
	67			407 ± 14.5	351 ± 35.7	478 ± 26.0	703 ± 41.4
	100	45 ± 5.8[c]		475 ± 16.5	146 ± 29.1[c]	509 ± 29.2	192 ± 41.6[c]
	200			180 ± 30.8[c]	341 ± 78.2[c]	146 ± 21.8[c]	153 ± 31.5[c]
Trial summary		Equivocal		Positive	Positive	Positive	Positive
Positive control[d]		543 ± 11.6		623 ± 23.7	881 ± 16.5	1,412 ± 33.5	506 ± 45.3
TA98	0	16 ± 1.5	18 ± 4.2	22 ± 0.3	28 ± 3.4	26 ± 4.9	33 ± 2.6
	1	22 ± 2.0	20 ± 0.7				
	3.3	19 ± 4.3	27 ± 3.9		79 ± 6.0		78 ± 3.8
	10	25 ± 3.8	33 ± 1.2	54 ± 8.1	85 ± 3.2	77 ± 4.2	107 ± 11.9
	33	37 ± 1.5	50 ± 5.0	287 ± 26.0	155 ± 6.2	119 ± 9.0	175 ± 17.4
	50		21 ± 1.5				
	67			191 ± 6.1	58 ± 10.3	176 ± 13.0	149 ± 6.4
	100	6 ± 1.8[c]	9 ± 4.7[c]	161 ± 11.7	40 ± 8.9[c]	214 ± 17.4	31 ± 7.5[c]
	200			61 ± 9.6[c]	47 ± 1.2[c]	43 ± 10.7[c]	23 ± 6.1[c]
Trial summary		Weakly Positive	Positive	Positive	Positive	Positive	Positive
Positive control		342 ± 7.1	298 ± 48.8	560 ± 12.2	806 ± 64.7	356 ± 8.1	137 ± 4.4

[a] Study was performed at BioReliance Corporation. The detailed protocol is presented by Zeiger *et al.* (1988). 0 µg/plate was the solvent control.

[b] Revertants are presented as mean ± standard error from three plates.

[c] Slight toxicity

[d] The positive controls in the absence of metabolic activation were sodium azide (TA100) and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with both strains was 2-aminoanthracene.

Table 16: Mutagenicity in second Ames assay of methylene blue trihydrate

(excerpted from NTP report TR 540 2008)

Strain	Dose (µg/plate)	Revertants/Plate[b] –S9 Trial 1	–S9 Trial 2	+ 10% rat S9 Trial 1	+ 10% rat S9 Trial 2
TA100	0	52 ± 3	55 ± 2	67 ± 5	66 ± 8
	0.25		107 ± 5		
	0.5		133 ± 2		
	1	105 ± 10	193 ± 5		
	2.5		271 ± 20		
	5	124 ± 8	115 ± 9	95 ± 2	87 ± 4
	10	42 ± 5		194 ± 2	111 ± 15
	15	Toxic			
	25	Toxic		238 ± 11	187 ± 9
	50			265 ± 7	253 ± 6
	100			342 ± 5	313 ± 1
	150				Toxic
Trial summary		Positive	Positive	Positive	Positive
Positive control[c]		365 ± 13	396 ± 10	649 ± 22	798 ± 14
TA98	0	22 ± 2	29 ± 6	34 ± 2	46 ± 1
	0.25		33 ± 2		
	0.5		64 ± 5		
	1	151 ± 27	155 ± 3		
	2.5		124 ± 6		
	5	44 ± 2	73 ± 1	59 ± 3	73 ± 9
	10	Toxic		122 ± 2	109 ± 10
	15	Toxic			
	25	Toxic		201 ± 8	146 ± 6
	50			203 ± 14	234 ± 4
	100			87 ± 6	110 ± 2
	150			40 ± 2	
Trial summary		Positive	Positive	Positive	Positive
Positive control		615 ± 34	476 ± 32	1,314 ± 23	1,078 ± 20
***Escherichia coli* WPM *uvrA*/pKM101 (Analogous to TA102)**					
	0	141 ± 18	105 ± 1	220 ± 8	178 ± 12
	0.25	158 ± 9			
	0.5	255 ± 11			
	1	376 ± 2	570 ± 14		
	2.5	426 ± 6			
	5		491 ± 39		
	10		166 ± 21	243 ± 16	198 ± 31
	15		124 ± 6		
	25		Toxic	275 ± 5	338 ± 38
	100			310 ± 18	437 ± 31
	500			462 ± 15	368 ± 34
	1,500			193 ± 17	186 ± 8
Trial summary		Positive	Positive	Positive	Positive
Positive control		2,149 ± 59	2,035 ± 15	1,162 ± 19	1,069 ± 7

[a] Study was performed at SITEK Research Laboratories. 0 µg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (WPM *uvrA*/pKM101). The positive control for metabolic activation with all strains was 2-aminoanthracene.

Table 17: Mutagenicity in Ames assay of Azure A

(excerpted from NTP report TR 540 2008)

Strain	Dose (µg/plate)	Revertants/Plate[b] –S9 Trial 1	–S9 Trial 2	+ 10% rat S9 Trial 1	+ 10% rat S9 Trial 2
TA100	0	100 ± 4	79 ± 11	69 ± 9	67 ± 3
	5	140 ± 6			
	10	252 ± 5			
	25	520 ± 46	557 ± 27	87 ± 8	93 ± 8
	50	482 ± 24	191 ± 53	112 ± 2	125 ± 6
	100	144 ± 47	123 ± 21	110 ± 9	245 ± 38
	250		Toxic	144 ± 5	131 ± 3
	500			Toxic	79 ± 14[c]
	1,000				
Trial summary		Positive	Positive	Positive	Positive
Positive control[d]		438 ± 27	636 ± 34	1,114 ± 95	1,053 ± 35
TA98	0	31 ± 4	30 ± 1	26 ± 2	30 ± 1
	5		47 ± 7		
	10		121 ± 16		
	25	180 ± 12	322 ± 3	31 ± 3	33 ± 2
	50	143 ± 29	350 ± 21	44 ± 4	48 ± 5
	100	131 ± 44	111 ± 40	39 ± 2	95 ± 16
	200	35 ± 11		67 ± 4	62 ± 4
	500	3 ± 2[c]		66 ± 6	69 ± 8
	1,000				64 ± 7[e]
Trial summary		Positive	Positive	Positive	Positive
Positive control		418 ± 49	257 ± 31	1,059 ± 53	818 ± 101
***Escherichia coli* WPM *uvrA*/pKM101 (Analogous to TA102)**					
	0	178 ± 10	164 ± 2	194 ± 10	203 ± 13
	50	646 ± 5		211 ± 13	
	100	552 ± 15		297 ± 23	
	250	577 ± 82	540 ± 119	543 ± 72	311 ± 8
	500	540 ± 5	531 ± 28	344 ± 31	414 ± 32
	1,500	249 ± 13	161 ± 23[c]	488 ± 38	381 ± 19
	2,500		113 ± 7[c]		392 ± 26
	3,000	7 ± 3[c]		129 ± 13	
	3,500		8 ± 7[c]		178 ± 10
Trial summary		Positive	Positive	Positive	Positive
Positive control		1,714 ± 58	1,895 ± 117	737 ± 79	1,148 ± 40

[a] Study was performed at SITEK Research Laboratories. 0 µg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] Slight toxicity and precipitate on plate
[d] The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (WPM *uvrA*/pKM101). The positive control for metabolic activation with all strains was 2-aminoanthracene.
[e] Precipitate on plate

Table 18: Mutagenicity in Ames assay of Azure B

(excerpted from NTP report TR 540 2008)

Strain	Dose (µg/plate)	Revertants/Plate[b] –S9 Trial 1	–S9 Trial 2	+ 10% rat S9 Trial 1	+ 10% rat S9 Trial 2
TA100	0	95 ± 1	79 ± 11	69 ± 9	78 ± 13
	1	102 ± 8			
	5	134 ± 3			
	10	196 ± 4	229 ± 3	164 ± 6	
	25	232 ± 3	317 ± 16	175 ± 18	124 ± 11
	50	314 ± 4	250 ± 28	165 ± 19	202 ± 35
	100	359 ± 24	47 ± 7	94 ± 11	99 ± 6
	250		Toxic	51 ± 11	49 ± 5
	500				7 ± 3[c]
Trial summary		Positive	Positive	Positive	Positive
Positive control[d]		322 ± 29	636 ± 34	1,114 ± 95	743 ± 40
TA98	0	31 ± 4	23 ± 1	28 ± 1	32 ± 1
	1		29 ± 3		
	5		45 ± 7		
	10	104 ± 4	41 ± 4	57 ± 7	70 ± 1
	25	107 ± 15	79 ± 4	59 ± 4	75 ± 2
	50	86 ± 13	82 ± 9	46 ± 5	81 ± 3
	100	27 ± 6	85 ± 1	44 ± 6	49 ± 5
	250	Toxic		45 ± 5	44 ± 3
	500				50 ± 11
Trial summary		Positive	Positive	Positive	Positive
Positive control		451 ± 16	250 ± 3	1,059 ± 53	592 ± 24
***Escherichia coli* WPM *uvrA*/pKM101 (Analogous to TA102)**					
	0	174 ± 7	164 ± 2	218 ± 2	203 ± 13
	1	174 ± 14			
	5	232 ± 10			
	10	297 ± 28			
	25	357 ± 6			
	50	464 ± 20	675 ± 36		279 ± 20
	100	460 ± 21	650 ± 67	292 ± 8	356 ± 16
	250		436 ± 26	392 ± 37	288 ± 23
	500		228 ± 21	369 ± 33	378 ± 18
	750		169 ± 31	420 ± 29	543 ± 28
	1,000			425 ± 31	
Trial summary		Positive	Positive	Positive	Positive
Positive control		1,333 ± 110	1,895 ± 117	759 ± 22	1,148 ± 40

[a] Study was performed at SITEK Research Laboratories. 0 µg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] Slight toxicity and precipitate on plate
[d] The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (WPM *uvrA*/pKM101). The positive control for metabolic activation with all strains was 2-aminoanthracene.

Table 19: Mutagenicity in Ames assay of Azure C

(excerpted from NTP report TR 540 2008)

Strain	Dose (µg/plate)	Revertants/Plate[b] –S9 Trial 1	–S9 Trial 2	+ 10% rat S9 Trial 1	+ 10% rat S9 Trial 2
TA100	0	100 ± 4	79 ± 11	69 ± 9	67 ± 3
	1	94 ± 4			
	5	129 ± 5			
	10	153 ± 4			
	25	229 ± 37	270 ± 23	85 ± 6	
	50	260 ± 11	320 ± 14	84 ± 6	
	100		311 ± 57	124 ± 6	112 ± 4
	250		198 ± 24	113 ± 6	127 ± 2
	500		58 ± 9	100 ± 7	66 ± 5
	750		Toxic	84 ± 4	55 ± 4[c]
	1,000				71 ± 4[c]
Trial summary		Positive	Positive	Positive	Positive
Positive control[d]		438 ± 27	636 ± 34	1,114 ± 95	1,053 ± 35
TA98	0	31 ± 4	23 ± 5	28 ± 1	30 ± 1
	1		22 ± 2		
	5		40 ± 5		
	10		62 ± 5		
	25	67 ± 2	94 ± 8	31 ± 3	
	50	93 ± 7	100 ± 7	35 ± 3	
	100	69 ± 10		46 ± 7	48 ± 1
	250	73 ± 13		62 ± 0	88 ± 15
	500	39 ± 4		69 ± 1	66 ± 5
	750	32 ± 5		63 ± 4	68 ± 2
	1,000				35 ± 2[c]
Trial summary		Positive	Positive	Positive	Positive
Positive control		418 ± 49	257 ± 31	1,059 ± 53	818 ± 101
***Escherichia coli* WPM *uvrA*/pKM101 (Analogous to TA102)**					
	0	178 ± 10	164 ± 2	194 ± 10	203 ± 13
	5	207 ± 18			
	10	266 ± 31			
	25	320 ± 9			
	50	384 ± 6		266 ± 11	
	100	496 ± 49	616 ± 31	315 ± 8	292 ± 4
	250		588 ± 20		312 ± 19
	500		398 ± 52	354 ± 30	393 ± 29
	1,000		156 ± 9	597 ± 29[c]	441 ± 17
	1,500			500 ± 78[c]	
	2,500		130 ± 12		346 ± 52[c]
	3,500		58 ± 36		212 ± 20[c]
	5,000		1 ± 1[d]		134 ± 11[c]
Trial summary		Positive	Positive	Positive	Positive
Positive control		1,714 ± 58	1,895 ± 117	737 ± 79	1,148 ± 40

[a] Study was performed at SITEK Research Laboratories. 0 µg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] Precipitate on plate
[d] The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (WPM *uvrA*/pKM101). The positive control for metabolic activation with all strains was 2-aminoanthracene.
[e] Slight toxicity and precipitate on plate

Study title: Bacterial reverse mutation test

Study no.:	35913 MMO
Study report location:	eCTD 4.2.3.3.1
Conducting laboratory and location:	(b) (4) (b) (4)
Date of study initiation:	May 29, 2009
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Methylene blue from Provepharma SAS, batch # HT CER 01040, Purity: 97.9%

Key Study Findings

- Methylene blue was positive for mutagenicity in *Salmonella typhimurium* strains TA1535, TA98, TA100, and TA102 in the absence of S9 activation and in strainsTA98 and TA102 in the presence of S9 activation.

Methods

Strains:	*Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537
Concentrations in definitive study:	TA98, TA100, TA1535, and TA1537: 0, 3.9, 7.8, 15.6, 31.3, 62.5, and 125 µg/plate TA102: 0, 15.6, 31.3, 62.5, 125, 250, and 500 µg/plate
Basis of concentration selection:	Results of preliminary test, highest concentration based on level of toxicity; moderate to strong toxicity observed at concentrations ≥100 µg/plate in the TA100 and ≥500 µg/plate in the TA102 strain both with and without S9 activation
Negative control:	Vehicle control
Positive control:	See table below
Formulation/Vehicle:	Water for injection
Incubation & sampling time:	48 to 72 hours of incubation at 37°C

A bacterial reverse mutation (Ames) test was conducted with Provepharma's methylene blue. A preliminary test and two mutagenicity experiments were conducted. The preliminary test, both experiments without S9 activation and the first experiment with S9 activation were performed according to the direct incorporation method. The second experiment with S9 activation was performed according to the pre-incubation method with methylene blue, the S9 mix, and the bacterial suspension incubated for 60 minutes at 37°C before adding the overlay agar and pouring onto the surface of a minimum agar plate. Each strain was exposed to at least five concentration levels of methylene blue with three plates per concentration level.

Table 20: Summary of positive control agents for Ames assay for Provepharma's methylene blue

Assay	Chemicals	Vehicle	Concentration (µg/plate)	Responding strains
Nonactivation	2-nitrofluorene (2NF)	DMSO	0.5	TA98
	Sodium azide (NaN_3)	DMSO	1.0	TA100, TA1535
	9-aminoacridine (9AA)	DMSO	50	TA1537
	Mitomycin C (MMC)	Distilled water	0.5	TA102
S9 activation	2-anthramine (2AM)	DMSO	2	TA98, TA1535, TA1537
	2-anthramine (2AM)	DMSO	10	TA102
	Benzo[a]pyrene (BAP)	DMSO	5	TA100

DMSO= dimethylsulfoxide

Study Validity
Test is valid based on the information and data provided in the report. The number of revertants in the vehicle and positive controls was consistent with the historical data of the testing facility, and the number of revertants in the positive controls was higher than the negative controls.

Criteria for positive response
A positive response for mutagenicity was defined as a reproducible 2-fold increase in the number of revertants compared with the vehicle controls in the TA98, TA100, and TA102 strains or 3-fold increase in the number of revertants compared with the vehicle controls in the TA1535 and TA1537 strains.

Results

Cytotoxicity

- In the preliminary toxicity test, moderate to strong toxicity demonstrated by thinning of the bacterial lawn and a decrease of the number of revertants was observed at concentrations ≥100 µg/plate in the TA100 and ≥500 µg/plate in the TA102 strain, both with and without S9 activation.
- In the mutagenicity experiments, a moderate toxicity demonstrated by thinning of the bacterial lawn and/or a decrease of the number of revertants was observed at 125 µg/plate in the TA1535, TA1537, TA100 and TA98 strains and at 500 µg/plate in the TA102 strain, both with and without S9 activation.

Mutagenicity

- Without S9 activation:
  - There were increases in the number of revertants that exceeded the criteria for positivity in the TA 1535, TA98, TA100, and TA 102 strains in both experiments. This was a positive response.
  - In the TA1537 strain, there were increases in the number of revertants above the threshold for positivity of 3-fold the vehicle control value in the

second experiment. Since these increases were not observed in the first experiment, and were therefore not reproducible, the increases were considered as equivocal.

- With S9 activation:
  - There were increases in the number of revertants that exceeded the criteria for positivity in the TA98 and TA 102 strains in both experiments. This was a positive response.
  - A slight (up to 2.9-fold the vehicle control value) concentration-related increase in the number of revertants was observed in the TA 1535 strain in the first experiment, but was not reproduced in the second experiment using the preincubation method; the finding was considered equivocal.
  - An increase in the number of revertants that exceeded the criteria for positivity was observed in the TA100 stain in the first experiment, but was not reproduced in the second experiment using the preincubation method; the finding was considered equivocal.

Table 21: Mutagenicity of Provepharma's methylene blue in first experiment

Phase	Dose (µg/plate)	Revertant colony counts									
		TA1535		TA1537		TA98		TA100		TA102	
		Mean	Fold of control	Mean	Fold of control	Mean	Fold of control	Mean	Fold of control	Mean	Fold of control
S9 -	Vehicle Control	11		10		30		121		371	
	3.9	18	1.6	9	0.9	42	1.4	145	1.2	NA	NA
	7.8	22	1.9	8	0.8	44	1.5	174	1.4	NA	NA
	15.6	35	3.1	8	0.9	101	3.4	320	2.6	592	1.6
	31.3	31	2.7	11	1.1	75	2.5	307	2.5	810	2.2
	62.5	50	4.4	16	1.6	129	4.3	464	3.8	1036	2.8
	125	42	3.7	13	1.3	134	4.5	250	2.1	567	1.5
	250	NA	NA	NA	NA	NA	NA	NA	NA	475	1.3
	500	NA	NA	NA	NA	NA	NA	NA	NA	56	0.2
	Positive Control	720	63.5	437	45.2	175	5.9	769	6.4	2576	6.9
S9+	Vehicle Control	18		16		33		100		440	
	3.9	11	0.6	19	1.2	69	2.1	97	1.0	NA	NA
	7.8	7	0.4	11	0.7	89	2.7	89	0.9	NA	NA
	15.6	21	1.2	15	1.0	92	2.8	120	1.2	938	2.1
	31.3	24	1.3	15	1.0	67	2.0	108	1.1	116	2.5
	62.5	29	1.6	11	0.7	153	4.6	237	2.4	2007	4.6
	125	51	2.9	17	1.1	341	10.3	178	1.8	1639	3.7
	250	NA	NA	NA	NA	NA	NA	NA	NA	1751	4.0
	500	NA	NA	NA	NA	NA	NA	NA	NA	50	0.1
	Positive Control	268	15.2	121	7.7	1186	35.9	327	3.3	3137	7.1

NA= Not available, concentration not tested for this strain

Table 22: Mutagenicity of Provepharma's methylene blue in second experiment

Phase	Dose (µg/plate)	Revertant colony counts									
		TA1535		TA1537		TA98		TA100		TA102	
		Mean	Fold of control	Mean	Fold of control	Mean	Fold of control	Mean	Fold of control	Mean	Fold of control
S9 -	Vehicle Control	20		5		23		138		372	
	3.9	19	1.0	7	1.4	36	1.6	180	1.3	NA	NA
	7.8	31	1.6	9	1.7	44	1.9	162	1.2	NA	NA
	15.6	33	1.6	6	1.1	63	2.8	253	1.8	1068	2.9
	31.3	38	1.9	6	1.1	70	3.0	342	2.5	1583	4.3
	62.5	74	3.7	17	3.5	127	5.5	559	4.0	1813	4.9
	125	81	4.0	15	3.1	194	8.4	206	1.5	2991	8.0
	250	NA	NA	NA	NA	NA	NA	NA	NA	1980	5.3
	500	NA	NA	NA	NA	NA	NA	NA	NA	219	0.6
	Positive Control	623	31.2	917	183.3	140	6.1	673	4.9	2124	5.7
S9+	Vehicle Control	24		12		25		105		440	
	3.9	15	0.6	16	1.4	55	2.2	101	1.0	NA	NA
	7.8	22	0.9	8	0.7	58	2.3	88	0.8	NA	NA
	15.6	28	1.2	10	0.8	39	1.6	107	1.0	647	1.5
	31.3	20	0.8	14	1.2	38	1.5	101	1.0	846	1.9
	62.5	23	1.0	16	1.4	43	1.7	133	1.3	1402	3.2
	125	25	1.0	19	1.6	82	3.2	96	0.9	1279	2.9
	250	NA	NA	NA	NA	NA	NA	NA	NA	905	2.1
	500	NA	NA	NA	NA	NA	NA	NA	NA	65	0.1
	Positive Control	176	7.3	126	10.8	951	37.5	248	2.4	1567	3.6

NA= Not available, concentration not tested for this strain

7.2 In Vitro Assays in Mammalian Cells

Study title: NTP technical report on the toxicology and carcinogenesis studies of methylene blue trihydrate (CAS No. 7220-79-3) in F344/N rats and B6C3F$_1$ mice

Study no.:	NTP report TR 540 2008
Study report location:	eCTD 4.3
Conducting laboratory and location:	SITEK Research Laboratories, location not specified
Date of study initiation:	Initiation dates not provided
GLP compliance:	Not clear
QA statement:	No
Drug, lot #, and % purity:	Methylene blue trihydrate, coded aliquot from Radian Corporation (Austin, Texas)

Key Study Findings

- Methylene blue trihydrate induced sister chromatid exchanges and chromosomal aberrations both in the presence and absence of S9 activation.

Methods

Cell line:	Chinese hamster ovary (CHO) cells
Concentrations in definitive study:	Sister chromatid exchange test Without S9: 0.17, 0.5, 0.63, 1.3, 1.7, and 2.5 µg/mL With S9: 0.5, 1.7, and 5 µg/mL Chromosomal aberrations test Without S9: 4.7, 7.5, 10, 15, 22, and 25 µg/mL With S9: 1.0, 2.2, and 4.7 µg/mL
Basis of concentration selection:	High dose was limited by toxicity
Negative control:	Solvent control (water)
Positive control:	Without S9: Mitomycin C (0.001 and 0.004 µg/mL for sister chromatid exchange test and 0.4 µg/mL for chromosomal aberration test) With S-9: Cyclophosphamide (0.125 and 0.5 µg/mL for sister chromatid exchange test and 20 µg/mL for chromosomal aberration test)
Formulation/Vehicle:	Water listed as solvent control
Incubation & sampling time:	Sister chromatid exchange test Incubation with methylene blue: 26 hours without S9 and 2 hours with S9. Bromodeoxyuridine (BrdU) was added 2 hours after culture initiation for a total of 26 hours with and without S9, with the exception 31 hours for 5 µg/mL methylene blue due to cell cycle delay. Harvest time: At 28 hours Chromosomal aberrations test Incubation with methylene blue: 11.7 hours without S9 and 2 hours with S9 Harvest time: At 13.7 hours without S9 and at 13.5 hours with S9

In vitro genetic toxicology assays in Chinese hamster ovary (CHO) cells were conducted by NTP as part of the toxicology assessment of methylene blue trihydrate and these results were reported in the same report as the repeat-dose toxicology and carcinogenicity studies.

Methylene blue trihydrate from Radian Corporation was tested in CHO cells for induction of sister chromatid exchanges and chromosomal aberrations both in the presence and absence of Aroclor 1254-induced male Sprague-Dawley rat liver S9 and cofactor mix. Testing was performed as reported in Galloway *et al.* (1987).

For the sister chromatid exchange test, 50 second-division metaphase cells were scored for frequency of sister chromatid exchanges/cell from each concentration level. A positive response was defined as an increase in sister chromatid exchange of ≥20% above the concurrent solvent control value at two or more concentrations. An increase of ≥20% at any single concentration was considered weak evidence of activity (weak positive).

For the chromosomal aberrations test, 200 first-division metaphase cells were scored at each concentration level. Classes of aberrations included simple (breaks and terminal deletions), complex (rearrangements and translocations), and other (pulverized cells, despiralized chromosomes, and cells containing 10 or more aberrations). A positive response was defined as a statistically significant difference at two or more concentrations. A statistically significant difference at a single concentration and a significant trend were considered a weak positive response.

Study Validity

The positive and negative controls fulfilled the requirements of a valid test.

Results

- In the sister chromatid exchange test, methylene blue trihydrate induced sister chromatid exchanges at concentrations of 0.63 to 2.5 µg/mL in the absence of S9 activation and at 5.0 µg/mL in the presence of S9 activation.
- In the chromosomal aberrations test, methylene blue trihydrate induced chromosomal aberrations at concentrations of 7.5 to 25.0 µg/mL in the absence of S9 activation and at 4.7 µg/mL in the presence of S9 activation.

Table 23: Sister chromatid exchanges in CHO cells

(excerpted from NTP report TR 540 2008)

Compound	Dose (µg/mL)	Total Cells Scored	No. of Chromo-somes	No. of SCEs	SCEs/ Chromo-some	SCEs/ Cell	Hrs in BrdU	Relative Change of SCEs/ Chromosome[b] (%)
–S9								
Trial 1								
Summary: Weakly positive								
Water[c]		50	1,048	365	0.35	7.3	26.0	
Methylene blue trihydrate	0.17	50	1,047	385	0.37	7.7	26.0	5.58
	0.5	50	1,050	406	0.39	8.1	26.0	11.02
	1.7	50	1,047	445	0.43	8.9	26.0	22.03*
	5	0					31.0[d]	
						P=0.002[e]		
Mitomycin-C[f]	0.001	50	1,050	514	0.49	10.3	26.0	40.55*
	0.004	10	210	203	0.97	20.3	26.0	177.55*
Trial 2								
Summary: Positive								
Water		50	1,049	344	0.33	6.88	26.0	
Methylene blue trihydrate	0.63	50	1,048	470	0.45	9.40	26.0	36.76*
	1.3	50	1,048	497	0.47	9.94	26.0	44.61*
	2.5	50	1,046	559	0.53	11.18	26.0	62.97*
	5	Toxic					31.0[d]	
						P≤0.001		
Mitomycin-C	0.001	50	1,050	549	0.52	10.98	26.0	59.44*
	0.004	10	210	243	1.16	24.30	26.0	252.86*

Compound	Dose (µg/mL)	Total Cells Scored	No. of Chromo-somes	No. of SCEs	SCEs/ Chromo-some	SCEs/ Cell	Hrs in BrdU	Relative Change of SCEs/ Chromosome (%)
+S9								
Trial 1								
Summary: Weakly positive								
Water		50	1,048	395	0.38	7.90	26.0	
		50	1,050	412	0.39	8.24	31.0[d]	
Methylene blue trihydrate	0.5	50	1,047	399	0.38	7.98	26.0	1.11
	1.7	50	1,050	452	0.43	9.04	26.0	14.21
	5[d]	50	1,048	770	0.73	15.40	31.0[d]	94.94*
	17	Toxic					31.0[d]	
						P≤0.001		
Cyclophosphamide[f]	0.125	50	1,050	479	0.46	9.58	26.0	21.03*
	0.5	10	210	199	0.95	19.90	26.0	151.42*

* Positive (≥20% increase over solvent control)
[a] Study was performed at SITEK Research Laboratories. The detailed protocol is presented by Galloway *et al.* (1987).
SCE=sister chromatid exchange; BrdU=bromodeoxyuridine
[b] SCEs/chromosome in treated cells versus SCEs/chromosome in solvent control cells
[c] Solvent control
[d] Due to cell cycle delay, harvest time was extended to maximize the number of second-division metaphase cells available for analysis.
[e] Significance of SCEs/chromosome tested by the linear regression trend test versus log of the dose
[f] Positive control

Table 24: Chromosomal aberrations in CHO cells

(excerpted from NTP report TR 540 2008)

Compound	Dose (µg/mL)	Total Cells Scored	Number of Aberrations	Aberrations/ Cell	Cells with Aberrations (%)
–S9					
Trial 1					
Harvest time: 13.7 hours					
Summary: Positive					
Water[b]		200	0	0.00	0.0
Methylene blue trihydrate	7.5	200	6	0.03	3.0*
	10	200	8	0.04	4.0*
	15	200	14	0.07	7.0*
	25	50	30	0.60	34.0*
	35	Toxic			0.0
				P≤0.001[c]	
Mitomycin-C[d]	0.4	25	17	0.68	52.0
Trial 2					
Harvest time: 13.7 hours					
Summary: Positive					
Water		200	0	0.00	0.0
Methylene blue trihydrate	4.7	200	3	0.02	0.5
	10	200	5	0.03	2.5*
	22	200	50	0.25	18.5*
	47	Toxic			
	100	Toxic			
				P≤0.001	
Mitomycin-C	0.4	25	18	0.72	48.0*

Compound	Dose (µg/mL)	Total Cells Scored	Number of Aberrations	Aberrations/ Cell	Cells with Aberrations (%)
+S9					
Trial 1					
Harvest time: 13.5 hours					
Summary: Weakly positive					
Water		200	3	0.02	1.5
Methylene blue trihydrate	1.0	200	3	0.02	1.5
	2.2	200	5	0.03	2.5
	4.7	200	42	0.21	12.0*
	10	0			
					P≤0.001
Cyclophosphamide[d]	20	25	12	0.48	24.0

* Positive response (P≤0.05) versus the solvent control
[a] Study was performed at SITEK Research Laboratories. The detailed protocol is presented by Galloway *et al.* (1987).
[b] Solvent control
[c] Significance of percent cells with aberrations tested by the linear regression trend test versus log of the dose
[d] Positive control

7.3 In Vivo Clastogenicity Assay in Rodent (Micronucleus Assay)

Study title: NTP technical report on the toxicology and carcinogenesis studies of methylene blue trihydrate (CAS No. 7220-79-3) in F344/N rats and B6C3F$_1$ mice

Study no:	NTP report TR 540 2008
Study report location:	eCTD 4.3
Conducting laboratory and location:	Single dose study: ILS, Inc., location not specified 3 month study: SITEK Research Laboratories, location not specified
Date of study initiation:	Initiation dates not provided
GLP compliance:	Not clear
QA statement:	No
Drug, lot #, and % purity:	Methylene blue trihydrate Single dose study: Not provided 3-month study: Lot # 10306AF from Aldrich Chemical Company, Purity: ~96.8%

Key Study Findings

- Methylene blue trihydrate was negative for micronucleus induction in bone marrow or peripheral blood in male mice treated with a single dose of methylene blue trihydrate (25, 50, or 150 mg/kg).
- Methylene blue trihydrate was also negative for micronucleus induction in peripheral blood samples from male and female mice at the end of a 3-month repeat-dose toxicity study of methylene blue trihydrate (25, 50, 100, or 200 mg/kg).

In vivo micronucleus tests in the mouse were conducted by NTP as part of the toxicology assessment of methylene blue trihydrate and these results were reported in the same report as the repeat-dose toxicology and carcinogenicity studies. A single-dose micronucleus test study using bone marrow and peripheral blood from B6C3F$_1$ mice administered methylene blue as an intraperitoneal injection was conducted at ILS, Inc. Methods of Tice *et al*. (1990) were used for this study. Additionally, peripheral blood samples were collected from the male and female mice at the end of the 3-month mouse repeat-dose toxicity study (last dose administered January 6 or 7, 1994) for micronucleus assessment and the study was conducted by SITEK Research Laboratories. Methods of MacGregor *et al*. (1990) were used for this study.

Single-dose study

Methods

Doses in definitive study:	25, 50, or 150 mg/kg*
Frequency of dosing:	Single dose
Route of administration:	Intraperitoneal injection
Dose volume:	0.4 mL per animal listed in Tice *et al*. (1990)
Formulation/Vehicle:	Corn oil
Species/Strain:	B6C3F$_1$ mice
Number/Sex/Group:	4 or 5 males/group
Satellite groups:	None
Basis of dose selection:	Range-finding studies
Negative control:	Vehicle (solvent control), corn oil
Positive control:	Cyclophosphamide (25 mg/kg)

*The NTP (TR 540 2008) report states, that preliminary range-finding studies were performed (data not provided in the report). Factors affecting dose selection included chemical solubility and toxicity and the extent of cell cycle delay induced by methylene blue trihydrate exposure.

Bone marrow and peripheral blood were collected for micronucleus assessment at a single time point of 48 hours after the single-dose of methylene blue trihydrate. The frequency of micronucleated cells was scored in 2,000 polychromatic erythrocytes (PCEs) per animal and the percentage of PCEs among the total erythrocyte population was scored in both bone marrow and peripheral blood as a measure of bone marrow toxicity.

Study Validity

The positive and negative controls fulfilled the requirements of a valid test. The study is valid.

Results

- No increase in the frequency of micronucleated erythrocytes was observed in bone marrow or peripheral blood collected from male mice 48 hours after a single intraperitoneal injection of 25, 50, or 150 mg/kg methylene blue trihydrate. Positive controls showed a significant increase in the mean percentage of micronucleated polychromatic erythrocytes compared to vehicle controls.

Table 25: Micronuclei in polychromatic erythrocytes of male mice treated with single-dose of methylene blue trihydrate

(excerpted from NTP report TR 540 2008)

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated PCEs/1,000 PCEs[b]	P Value[c]	PCEs[b] (%)
Bone Marrow (48 hours)					
Corn oil[d]	0	5	1.1 ± 0.40		41.7 ± 4.71
Methylene blue trihydrate	25	5	1.9 ± 0.53	0.0719	30.1 ± 4.77
	50	4	1.4 ± 0.24	0.2999	25.5 ± 3.20
	150	4	1.5 ± 0.20	0.2277	33.4 ± 3.22
			P=0.401[e]		
Cyclophosphamide[f]	25	5	2.8 ± 0.58	0.0032	26.7 ± 5.01
Peripheral Blood (48 hours)					
Corn oil	0	5	2.8 ± 0.51		2.7 ± 0.15
Methylene blue trihydrate	25	5	4.3 ± 0.75	0.0373	4.5 ± 0.34
	50	4	2.6 ± 0.97	0.5886	3.8 ± 0.51
	150	4	1.5 ± 0.35	0.9672	2.8 ± 0.81
			P=0.994		
Cyclophosphamide	25	5	8.4 ± 1.21		3.9 ± 0.46

[a] Study was performed at ILS, Inc. The detailed protocol is presented by Tice *et al.* (1990). PCE=polychromatic erythrocyte
[b] Mean ± standard error
[c] Pairwise comparison with the vehicle control; dosed group values are significant at P≤0.008; positive control values are significant at P≤0.05 (ILS, 1990)
[d] Vehicle control
[e] Significance of micronucleated PCEs/1,000 PCEs tested by the one-tailed trend test; significant at P≤0.025 (ILS, 1990)
[f] Positive control

3-month Study

Methods

Doses in definitive study:	25, 50, 100, or 200 mg/kg*
Frequency of dosing:	5 days/week for 14 weeks
Route of administration:	Oral gavage
Dose volume:	10 mL/kg
Formulation/Vehicle:	0.5% aqueous methylcellulose
Species/Strain:	B6C3F$_1$ mice
Number/Sex/Group:	5/sex/group
Satellite groups:	None
Basis of dose selection:	Animals were dosed in the 3-month mouse toxicology study and doses were based on the results of the 1-month toxicology study
Negative control:	Vehicle control, methylcellulose
Positive control:	Not conducted

*Based of the effects on the hematopoietic system and the early deaths at doses of 250 mg/kg or greater in the 1-month study, doses of 25, 50, 100, and 200 mg/kg were selected for the 3-month study in mice.

The frequency of micronucleated cells in 2,000 normochromatic erythrocytes (NCEs) per mouse and the percentage of PCBs (reticulocytes) in a population of 1,000 erythrocytes was determined as a measure of chemical-related bone marrow toxicity. While micronucleated cells were scored in NCEs instead of PCEs, it is important to note that the study was conducted in 1994, when this practice was acceptable.

Study Validity

Positive control was not included and the negative control fulfilled the study requirement. The study is valid.

Results

- No increases in micronucleated erythrocytes were observed in peripheral blood samples collected from male and female mice at the end of a 3-month mouse repeat-dose toxicity study with oral administration of 25, 50, 100, or 200 mg/kg methylene blue trihydrate.

Table 26: Micronuclei in mouse peripheral blood erythrocytes following treatment with methylene blue trihydrate by oral gavage for 3 months

(excerpted from NTP report TR 540 2008)

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs[b] (%)
Male					
Methylcellulose[d]	0	5	0.0 ± 0.0		3.6 ± 0.43
Methylene blue trihydrate	25	5	0.3 ± 0.20	0.0416	5.0 ± 0.41
	50	5	0.0 ± 0.00	0.5000	10.0 ± 1.20
	100	5	0.1 ± 0.10	0.1586	11.0 ± 0.76
	200	5	0.2 ± 0.12	0.0786	36.9 ± 2.10
			P=0.235[e]		
Female					
Methylcellulose	0	5	0.6 ± 0.19		1.9 ± 0.11
Methylene blue trihydrate	25	5	0.1 ± 0.10	0.9706	3.4 ± 0.20
	50	5	0.3 ± 0.12	0.8414	6.8 ± 0.97
	100	5	0.1 ± 0.10	0.9706	13.6 ± 1.22
	200	5	0.1 ± 0.10	0.9706	19.7 ± 1.64
			P=0.959		

[a] Study was performed at SITEK Research Laboratories. The detailed protocol is presented by MacGregor *et al.* (1999).
[b] NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte
[c] Mean ± standard error
[d] Pairwise comparison with the vehicle control; dosed group values are significant at P≤0.006 (ILS, 1990)
[e] Vehicle control
Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test; significant at P≤0.025 (ILS, 1990)

7.4 Other Genetic Toxicity Studies

None

8 Carcinogenicity

Study title: NTP technical report on the toxicology and carcinogenesis studies of methylene blue trihydrate (CAS No. 7220-79-3) in F344/N rats and B6C3F$_1$ mice

Study no.:	NTP report TR 540 2008
Study report location:	eCTD 4.3
Conducting laboratory and location:	Southern Research Institute Birmingham, Alabama
Date of study initiation:	Exact initiation dates not provided; first dose administered June 26, 2000 in rats and July 10, 2000 in mice
GLP compliance:	Yes
QA statement:	Quality assurance conducted but signed statement not provided; quality assurance reports on file at NIEHS
Drug, lot #, and % purity:	Methylene blue trihydrate, from Sigma Chemical Company, lot # 68H3728, Purity: >91%
CAC concurrence:	N/A: No ECAC meeting was held

This report contained repeat-dose toxicology studies (1-month and 3-month studies) and 2-year carcinogenicity studies in both mice and rats. The 3-month rat repeat-dose study is reviewed above in the general toxicology section. The 2-year carcinogenicity studies for mice and rats were fully reviewed in a separate review for NDA 204630 (archival date: March 25, 2014). The key findings and the conclusions for the 2-year carcinogenicity studies are provided below.

Key Study Findings

2-year carcinogenicity study in mice

- Survival in the methylene blue-treated groups was similar to that of controls.
- There were no statistically significant neoplastic findings using the FDA criteria for a positive response.
- Non-neoplastic findings included hematopoietic cell proliferation in the spleen at 12.5 and 25 mg/kg in both males and females and inflammation of the nose at all doses of methylene blue.

2-year carcinogenicity study in rats

- Survival in the methylene blue-treated groups was similar to that of controls.
- Methylene blue caused pancreatic islet adenomas or carcinomas (combined) in male rats.
- Non-neoplastic findings included hyperplasia in pancreatic islets and focal hyperplasia in the acinus of the pancreas at 25 and 50 mg/kg methylene blue in males and hematopoietic cell proliferation and capsule fibrosis in the spleen at all doses of methylene blue in both males and females.

Conclusions for 2-year mouse and rat carcinogenicity studies

In the NTP report for the 2-year carcinogenicity studies in the mouse and rat, the conclusion was made that there was some evidence of carcinogenic activity of methylene blue based on increased incidences of pancreatic islet cell adenoma and adenoma or carcinoma (combined) in male rats and increased incidences of carcinoma and adenoma or carcinoma in the small intestine in male mice. The increased incidence of malignant lymphoma in male and female mice was considered possibly related to methylene blue trihydrate administration.

A statistical review of the NTP carcinogenicity studies for methylene blue trihydrate was performed by the FDA, with a particular focus on the findings that were reported in the NTP report.

Based on the analyses and the FDA criteria for a positive carcinogenicity response, FDA's conclusion is that there are no statistically significant neoplastic findings in the 2-year mouse carcinogenicity study. Additional information was taken into consideration for the finding of pancreatic islet adenoma or carcinoma (combined) in male rats. According to the historical incidence for all routes of administration provided in the NTP report, adenoma or carcinoma (combined) in pancreatic islets has been observed in 6.4% of controls, with a mean of 6.8% and a range of 0-14%. Further, the incidences of hyperplasia in pancreatic islets were also increased in males treated with 25 and 50 mg/kg methylene blue. Although the findings were not statistically significant, the FDA concluded that methylene blue caused pancreatic islet adenomas or carcinomas (combined) in male rats based on the incidences exceeding the historical control incidence, particularly at the mid dose of 25 mg/kg.

The results of the 2-year mouse and rat carcinogenicity studies were presented to the Executive CAC on March 18, 2014 and the following recommendations and conclusions were made:
Rat:

- The Committee concurred that the study was acceptable, although daily dosing would have been more representative of any future chronic usage.

- The Committee concurred that pancreatic islet adenomas or carcinomas (combined) in the males were drug related because the incidence exceeded the historical control, particularly in the mid dose group. Furthermore, it was noted that the animals were not dosed on weekends (and thus had time to recover from drug-related effects). The terminal half-life of the drug is reported to be around 5 hours.

Mouse:

- The Committee concurred that the study was acceptable.

- The Committee concurred that there were no drug-related neoplasms in the study.

9 Reproductive and Developmental Toxicology

9.1 Fertility and Early Embryonic Development

No report for fertility and early embryonic development studies were submitted.

9.2 Embryonic Fetal Development

Study title: Developmental toxicity of methylene blue trihydrate (CAS No. 7220-79-3) administered by gavage to Sprague-Dawley (CD®) rats

Study no.:	NTP report TER 92124 1993
Study report location:	eCTD 4.3
Conducting laboratory and location:	(b) (4) [redacted] 27709-2194
Date of study initiation:	April 24, 1992
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	Methylene blue trihydrate supplied by Aldrich Chemical Co., lot # AY03010 KW, Purity: >94%

Key Study Findings

- Malformations including enlarged lateral ventricles were observed mainly at 125, 200, and 350 mg/kg of methylene blue. Other fetal abnormalities included anasarca (edema).
- Maternal toxicity was observed at all doses of methylene blue tested (50, 125, and 200 mg/kg/day) based on increases in maternal spleen weights.
- Treatment with methylene blue significantly increased resorptions and post-implantation loss (reported as nonlive implants) at doses of 200 and 350 mg/kg/day.
- Fetal body weights were decreased at 200 and 350 mg/kg/day; the decreases were statistically significant at 200 mg/kg/day in the second replicate.

Methods

Doses:	Original doses were 0, 50, 200, or 350 mg/kg/day based on dose range-finding study from NTP. The results of the first replicate indicated that the 350 mg/kg dose caused resorption of nearly 100% of fetuses in all litters, therefore, in the second replicate doses of 0, 50, 125, or 200 mg/kg/day were used.

Frequency of dosing:	Once daily gestation days (GD) 6-15
Dose volume:	5 mL/kg
Route of administration:	Oral gavage
Formulation/Vehicle:	Distilled, deionized water
Species/Strain:	Sprague-Dawley (CD®) rat
Number/Sex/Group:	At least 20 confirmed pregnant rats/group; two study replicates with each replicate containing a minimum of 10 time-mated females assigned to each dose group
Satellite groups:	None
Study design:	26 females/group were dosed with vehicle or methylene blue (doses for the second replicate were 50, 125, or 200 mg/kg/day) GD 6-15 and euthanized on GD 20. The day of sperm detection was designated as GD 0.
Parameters and endpoints evaluated:	Females: Clinical signs, body weight, spleen, liver, and gravid uterine weights, food consumption, necropsy, and number of corpora lutea, implantation sites, resorptions, and viable and dead fetuses Fetuses: Fetal weight and external, visceral, and skeletal examinations (malformations and variations)

Note: In this review, data for all parameters are presented for the second replicate with doses of 0, 50, 125, or 200 mg/kg/day. For the first replicate with doses of 0, 50, 200, or 350 mg/kg/day, only the results of the Cesarean section (uterine examination) and offspring (fetal weights and malformations/variations) are presented.

Results

Mortality

- There were no mortalities in this study. One female in the 125 mg/kg/day group was removed because of a dosing error.

Clinical Signs

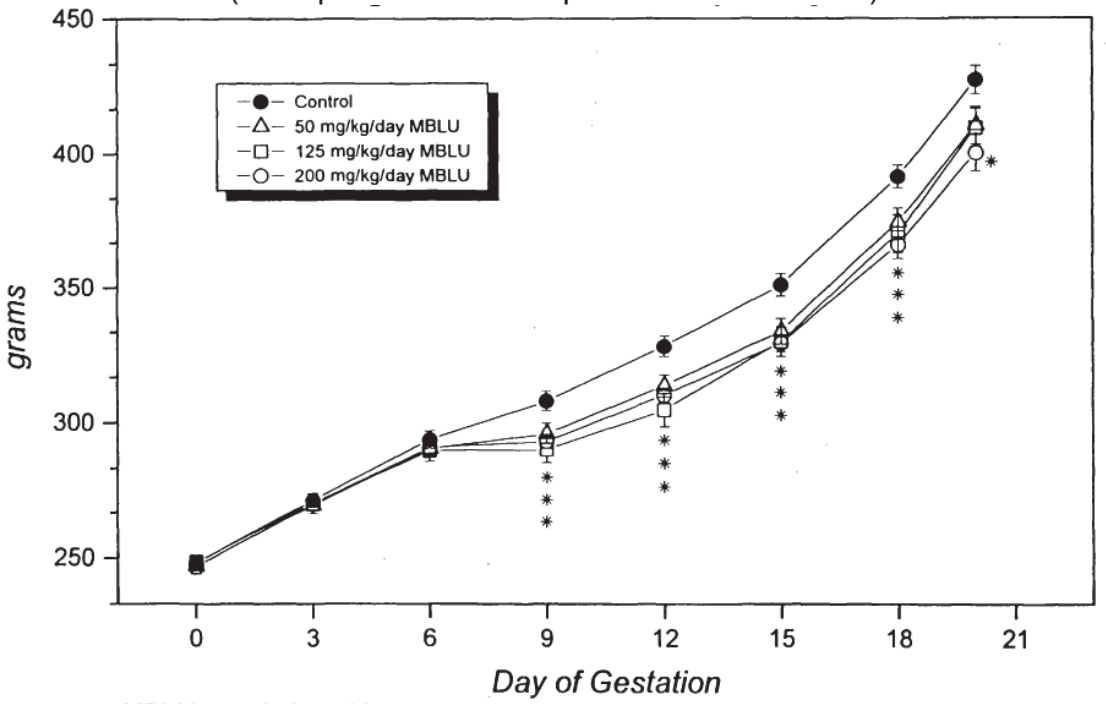
- The predominant clinical sign was blue colored feces, which were observed in 90-100% of females in the methylene blue-treated groups. After the end of the dosing period on GD 15, the incidence of blue feces gradually decreased in all methylene-blue treated groups until none were found on GD 20. Since the drug is a blue dye, this finding demonstrates exposure to the drug and presence of the drug in feces.
- Incidences of rooting post-dosing and alopecia (on abdomen and/or limbs) were observed in the methylene-blue treated groups.

Body Weight

- Mean maternal body weights were significantly lower in the methylene blue-treated groups compared to controls. Lower body weights were observed starting on GD 9 through GD 18 at all doses of methylene blue and on GD 20 at 200 mg/kg/day. However, the reduced body weight appears to be secondary to reduced uterine weight; the corrected body weights are comparable among treated groups.
- The mean maternal body weight gain during the treatment period (GD 6-15) was reduced by 23-32% in the methylene-blue treated groups compared to controls.
- The corrected body weight gain (weight gain during gestation minus the gravid uterine weight) was not significantly lower in the methylene blue-treated groups compared to controls.
- The gravid uterine weight was lower in the 200 mg/kg/day group than controls, but the difference was not statistically significant.

Figure 11: Maternal body weight in rats

(excerpted from NTP report TER 92124 1993)

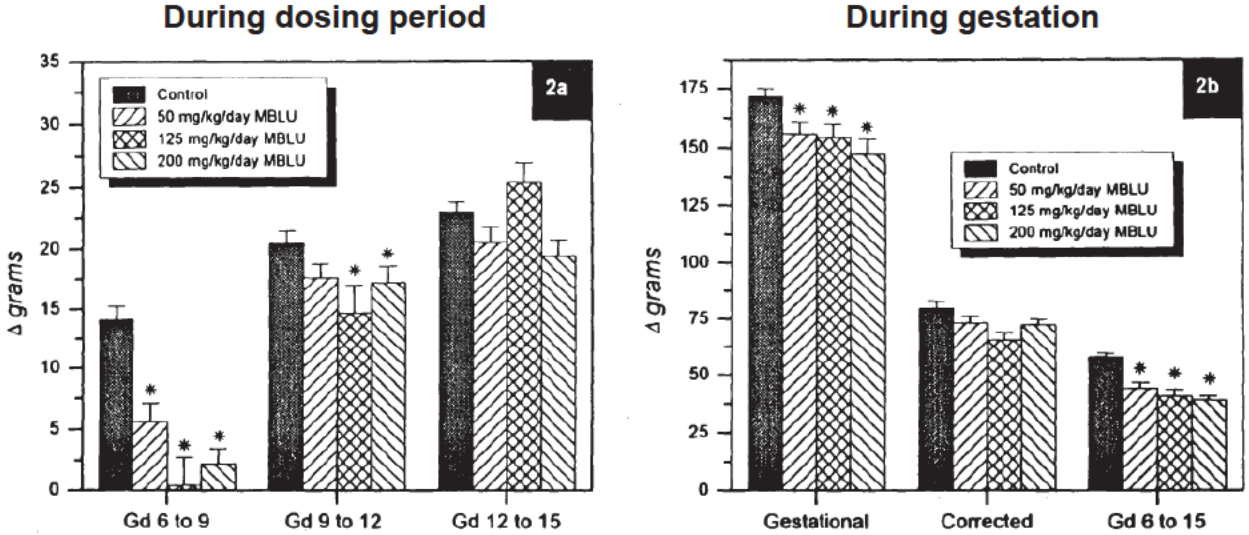


MBLU= methylene blue

*= Significantly different from control, P<0.05

Figure 12: Maternal body weight gain in rats

(excerpted from NTP report TER 92124 1993)



MBLU= methylene blue

*= Significantly different from control, P<0.05

Table 27: Maternal body weight changes

Parameter	Dose (mg/kg/day)			
	0	50	125	200
Gestation weight gain (GD 0-20; g)	171	156*	154*	147*
Treatment weight gain (GD 6-15; g)	57	44*	40*	39*
Corrected weight gain (g)	79	73	65	72
Gravid uterine weight (g)	92	83	89	75

* Significantly different from control (p<0.05)

Corrected weight gain= gestation weight gain minus the gravid uterine weight

Food Consumption

- Maternal absolute and relative food consumption was significantly decreased in methylene blue-treated groups compared to controls GD 6-9 and GD 9-12. The food consumption was similar to controls by GD 12-15; however, on GD 18-20, maternal food consumption was significantly higher in the 125 and 200 mg/kg/day methylene blue groups than controls.

Toxicokinetics

Not conducted

Necropsy

- Maternal spleen weights were increased at all doses and liver weights were increased at 125 and 200 mg/kg/day methylene blue compared to controls.

Table 28: Maternal organ weights for rat embryo-fetal development study

Group and Dose		Mean	Mean (Percentage deviation from control)		
		Control 0 mg/kg/day	50 mg/kg/day	125 mg/kg/day	200 mg/kg/day
Number of animals examined		26	25	25	26
Liver	Absolute (g)	19.1	19.0	19.4	20.3* (↑6)
	Relative body weight (%)	4.5	4.7	4.8* (↑7)	5.2* (↑16)
Spleen	Absolute (g)	0.77	0.98* (↑27)	1.29* (↑68)	1.75* (↑127)
	Relative body weight (%)	0.18	0.24* (↑33)	0.32* (↑78)	0.45* (↑150)

↑=Increase; * Significantly different from control ($p<0.05$)

Cesarean Section Data

- Treatment with methylene blue significantly increased resorptions at doses of 200 and 350 mg/kg/day. The resorption of all fetuses from 10 of 11 litters and only 4 viable fetuses in the remaining litter at 350 mg/kg/day in the first replicate lead to a reduction in doses to 125 mg/kg/day for the mid-dose and 200 mg/kg/day for the high-dose. The dose of 200 mg/kg/day produced an increase in resorptions in both replicates.

Table 29: Uterine examination data in rats: Replicate 1

Dose (mg/kg/day)	0	50	200	350
Number of pregnant females (litters)	13	12	13	11
Number of females with all resorptions	0	0	3	10
Number of females with viable fetuses	13	12	10	1
Corpora lutea				
Mean number per female	16.15	16.00	17.62	15.73
Implantation sites				
Mean number per litter	16.15	15.50	17.15	14.64
Pre-implantation loss				
Mean % per litter	2.26	5.34	2.98	8.38
Resorptions				
Number of litters with resorptions	8	7	10	11
% litters with resorptions	61.54	58.33	76.92	100.00
Mean number per litter	0.85	1.42	5.15	14.27
Mean % per litter	5.90	12.76	30.56*	97.58*
Late fetal deaths				
Number of litters with late fetal deaths	1	0	0	0
% litters with late fetal deaths	7.69	0	0	0
Mean number per litter	0.08	0	0	0
Mean % per litter	0.38	0	0	0
Post implantation loss (nonlive implants)				
Number of litters with nonlive implants	9	7	10	11
% litters with nonlive implants	69.23	58.33	76.92	100.00
Mean number per litter	0.92	1.42	5.15	14.27
Mean % per litter	6.29	12.76	30.56*	97.58*

Dose (mg/kg/day)	0	50	200	350
Viable fetuses				
Number of litters with viable fetuses	13	12	10	1
Total number of fetuses	198	169	156	4
Mean number per female/mean litter size	15.23	14.08	15.60	4.00*

* Significantly different from control (p<0.05)

Table 30: Uterine examination data in rats: Replicate 2

Dose (mg/kg/day)	0	50	125	200
Number of pregnant females (litters)	26	25	25	26
Number of females with all resorptions	0	0	0	4
Number of females with viable fetuses	26	25	25	22
Corpora lutea				
Mean number per female	16.46	16.00	16.72	17.96*
Implantation sites				
Mean number per litter	16.35	15.56	16.32	17.65
Pre-implantation loss				
Mean % per litter	2.33	5.87	4.28	2.46
Resorptions				
Number of litters with resorptions	12	15	16	19
% litters with resorptions	46.15	60.00	64.00	73.08
Mean number per litter	0.62	1.28	1.32	4.42
Mean % per litter	4.04	10.19	8.16	25.23*
Late fetal deaths				
Number of litters with late fetal deaths	1	0	0	1
% litters with late fetal deaths	3.85	0	0	3.85
Mean number per litter	0.04	0	0	0.04
Mean % per litter	0.19	0	0	0.23
Post implantation loss (nonlive implants)				
Number of litters with nonlive implants	13	15	16	19
% litters with nonlive implants	50.00	60.00	64.00	73.08
Mean number per litter	0.65	1.28	1.32	4.46
Mean % per litter	4.23	10.19	8.16	25.45*
Viable fetuses				
Number of litters with viable fetuses	26	25	25	22
Total number of fetuses	408	357	375	343
Mean number per female/mean litter size	15.69	14.28	15.00	15.59

* Significantly different from control (p<0.05)

Offspring

- Fetal body weights were decreased at 200 and 350 mg/kg/day; the decreases were statistically significant at 200 mg/kg/day in the second replicate.

Table 31: Fetal sex ratio and weights in rats: Replicate 1

Dose (mg/kg/day)	0	50	200	350
Fetal sex ratio				
Mean % male fetuses per litter	52.85	52.24	48.89	50.00
Mean fetal weight (g) per litter				
Males	3.778	3.809	3.472	3.144
Females	3.636	3.619	3.275	2.820
Males + females	3.716	3.718	3.366	2.982

Table 32: Fetal sex ratio and weights in rats: Replicate 2

Dose (mg/kg/day)	0	50	125	200
Fetal sex ratio				
Mean % male fetuses per litter	51.17	51.94	47.29	46.89
Mean fetal weight (g) per litter				
Males	3.811	3.872	3.834	3.418*
Females	3.622	3.636	3.642	3.206*
Males + females	3.719	3.757	3.742	3.302*

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

- Malformations and variations were observed in all groups including controls; those with a higher incidence in or observed only in methylene blue-treated groups are presented below.

Table 33: Malformations and variations in rats: Replicate 1

Dose (mg/kg/day)		0	50	200	350
Number of litters evaluated		13	12	10	1
Number of fetuses evaluated		198	169	156	4
Total malformations	Number of litters (%)	9 (69.23)	7 (58.33)	6 (60.00)	1 (100.00)
	Number of fetuses (%)	17 (8.59)	14 (8.28)	13 (8.33)	1 (25.00)
Total variations	Number of litters (%)	13 (100.00)	12 (100.00)	10 (100.00)	1 (100.00)
	Number of fetuses (%)	91 (45.96)	59 (34.01)	38 (24.36)	2 (50.00)
Malformations					
Fused ribs (skeletal)	Number of litters (%)	0 (0)	0 (0)	1 (10.00)	0 (0)
	Number of fetuses (%)	0 (0)	0 (0)	1 (0.64)	0 (0)
Enlarged lateral ventricle (partial)/bilateral (visceral)	Number of litters (%)	6 (46.15)	6 (50.00)	5 (50.00)	1 (100.00)
	Number of fetuses (%)	6 (3.03)	8 (4.73)	11 (7.05)	1 (25.00)
Variations					
Misaligned sternebrae (skeletal)	Number of litters (%)	1 (7.69)	0 (0)	3 (30.0)	0 (0)
	Number of fetuses (%)	1 (0.51)	0 (0)	3 (1.92)	0 (0)
Wavy rib (skeletal)	Number of litters (%)	0 (0)	0 (0)	2 (20.00)	1 (100.00)
	Number of fetuses (%)	0 (0)	0 (0)	10 (6.41)	2 (50.00)
Other abnormalities					
Anasarca (edema; external)	Number of litters (%)	0 (0)	0 (0)	0 (0)	1 (100.00)
	Number of fetuses (%)	0 (0)	0 (0)	0 (0)	1 (25.00)

Table 34: Malformations and variations in rats: Replicate 2

Dose (mg/kg/day)		0	50	125	200
Number of litters evaluated		26	25	25	22
Number of fetuses evaluated		408	357	375	343
Total malformations	Number of litters (%)	21 (80.8)	17 (68.00)	24 (96.00)	17 (77.3)
	Number of fetuses (%)	67 (16.4)	52 (14.6)	91 (24.3)	56 (16.3)
Total variations	Number of litters (%)	26 (100.00)	24 (96.00)	25 (100.00)	22 (100.00)
	Number of fetuses (%)	168 (41.2)	116 (32.5)	129 (34.4)	94 (27.4)
Malformations					
Fused ribs (skeletal)	Number of litters (%)	0 (0)	0 (0)	0 (0)	1 (4.55)
	Number of fetuses (%)	0 (0)	0 (0)	0 (0)	1 (0.29)
Enlarged lateral ventricle (full)/bilateral (visceral)	Number of litters (%)	1 (3.85)	2 (8.00)	3 (12.00)	3 (13.64)
	Number of fetuses (%)	1 (0.25)	2 (0.56)	3 (0.80)	4 (1.17)
Enlarged lateral ventricle (full)/left (visceral)	Number of litters (%)	1 (3.85)	0 (0)	4 (16.00)	1 (4.55)
	Number of fetuses (%)	1 (0.25)	0 (0)	5 (1.33)	1 (0.29)
Enlarged lateral ventricle (full)/right (visceral)	Number of litters (%)	0 (0)	0 (0)	4 (16.00)	1 (4.55)
	Number of fetuses (%)	0 (0)	0 (0)	4 (1.07)	1 (0.29)
Enlarged lateral ventricle (partial)/bilateral (visceral)	Number of litters (%)	17 (65.38)	16 (64.00)	21 (84.00)	15 (68.18)
	Number of fetuses (%)	44 (10.78)	40 (11.20)	56 (14.93)	38 (11.08)
Enlarged lateral ventricle (partial)/left (visceral)	Number of litters (%)	4 (15.38)	3 (12.00)	7 (28.00)	2 (9.09)
	Number of fetuses (%)	4 (0.98)	3 (0.84)	7 (1.87)	2 (0.58)
Enlarged lateral ventricle (partial)/right (visceral)	Number of litters (%)	4 (15.38)	3 (12.00)	11 (44.00)	6 (27.27)
	Number of fetuses (%)	4 (0.98)	3 (0.84)	13 (3.47)	6 (1.75)
Left common carotid arises from innominate artery (visceral)	Number of litters (%)	0 (0)	0 (0)	1 (4.00)	1 (4.55)
	Number of fetuses (%)	0 (0)	0 (0)	2 (0.53)	1 (0.29)
Variations					
Misaligned sternebrae (skeletal)	Number of litters (%)	1 (3.85)	0 (0)	2 (8.00)	3 (13.64)
	Number of fetuses (%)	1 (0.25)	0 (0)	2 (0.53)	3 (0.87)
Wavy rib (skeletal)	Number of litters (%)	0 (0)	0 (0)	2 (8.00)	6 (27.27)
	Number of fetuses (%)	0 (0)	0 (0)	8 (2.13)	23 (6.71)

Dose (mg/kg/day)		0	50	125	200
Number of litters evaluated		26	25	25	22
Number of fetuses evaluated		408	357	375	343
Agenesis of innominate artery (visceral)	Number of litters (%)	0 (0)	0 (0)	1 (4.00)	4 (18.18)
	Number of fetuses (%)	0 (0)	0 (0)	3 (0.8)	13 (3.79)
Left carotid arises adjacent to innominate artery (visceral)	Number of litters (%)	3 (11.54)	1 (4.00)	6 (24.00)	6 (27.27)
	Number of fetuses (%)	3 (0.74)	2 (0.56)	23 (6.13)	13 (3.79)
Left carotid adjacent to right carotid (visceral)	Number of litters (%)	0 (0)	0 (0)	1 (4.00)	2 (9.09)
	Number of fetuses (%)	0 (0)	0 (0)	2 (0.53)	9 (2.62)
Short aortic arch (visceral)	Number of litters (%)	1 (3.85)	1 (4.00)	2 (8.00)	3 (13.64)
	Number of fetuses (%)	1 (0.25)	1 (0.28)	9 (2.40)	5 (1.46)
Other abnormalities					
Anasarca (edema; external)	Number of litters (%)	0 (0)	1 (4.00)	1 (4.00)	1 (4.55)
	Number of fetuses (%)	0 (0)	1 (0.28)	1 (0.27)	1 (0.29)

Study title: Developmental toxicity of methylene blue trihydrate (CAS No. 7220-79-3) in New Zealand White (NZW) rabbits

Study no.: NTP report TER 92125 1994
Study report location: eCTD 4.3
Conducting laboratory and location: (b) (4)
Date of study initiation: July 29, 1992
GLP compliance: Yes
QA statement: Yes
Drug, lot #, and % purity: Methylene blue trihydrate supplied by Aldrich Chemical Co., lot # AY03010 KW, Purity: >94%

Key Study Findings

- Maternal toxicity observed with methylene blue included maternal death at 100 mg/kg/day.
- Treatment with methylene blue caused spontaneous abortion at all doses.
- Resorptions and post-implantation loss (reported as nonlive implants) were increased in methylene blue-treated groups compared to the controls.

- A malformation of umbilical hernia was observed at 100 and 150 mg/kg/day.

Methods

Doses:	0, 50, 100, or 150 mg/kg/day
Frequency of dosing:	Once daily gestation days (GD) 6-19
Dose volume:	5 mL/kg
Route of administration:	Oral gavage
Formulation/Vehicle:	Distilled, deionized water
Species/Strain:	New Zealand White rabbit
Number/Sex/Group:	25 females for control group, 26 females/group for methylene blue groups
Satellite groups:	None
Study design:	25 females were dosed with vehicle and 26 females/group were dosed with methylene blue (50, 100, or 150 mg/kg/day) GD 6-19 and euthanized on GD 30. The day of insemination was considered to be GD 0. Study was performed in two replicates (13 females/group in each replicate) which were 35 days apart.
Parameters and endpoints evaluated:	Females: Clinical signs, body weight, spleen, liver, and gravid uterine weights, food consumption, necropsy, and number of corpora lutea, implantation sites, resorptions, and viable and dead fetuses Fetuses: Fetal weight and external, visceral, and skeletal examinations (malformations and variations)

Results

Mortality

There were 4 females treated with the mid-dose of 100 mg/kg/day methylene blue that died.

- Three of the deaths were considered the result of dosing errors since blue fluid was discovered in the trachea and lungs at necropsy.
- One death on GD 12 may have been drug-related; at the necropsy the lungs were free of dye and the esophagus was not punctured. Blue dye was in the intestines and liver of the animal, indicating that there was exposure to methylene blue.

Clinical Signs

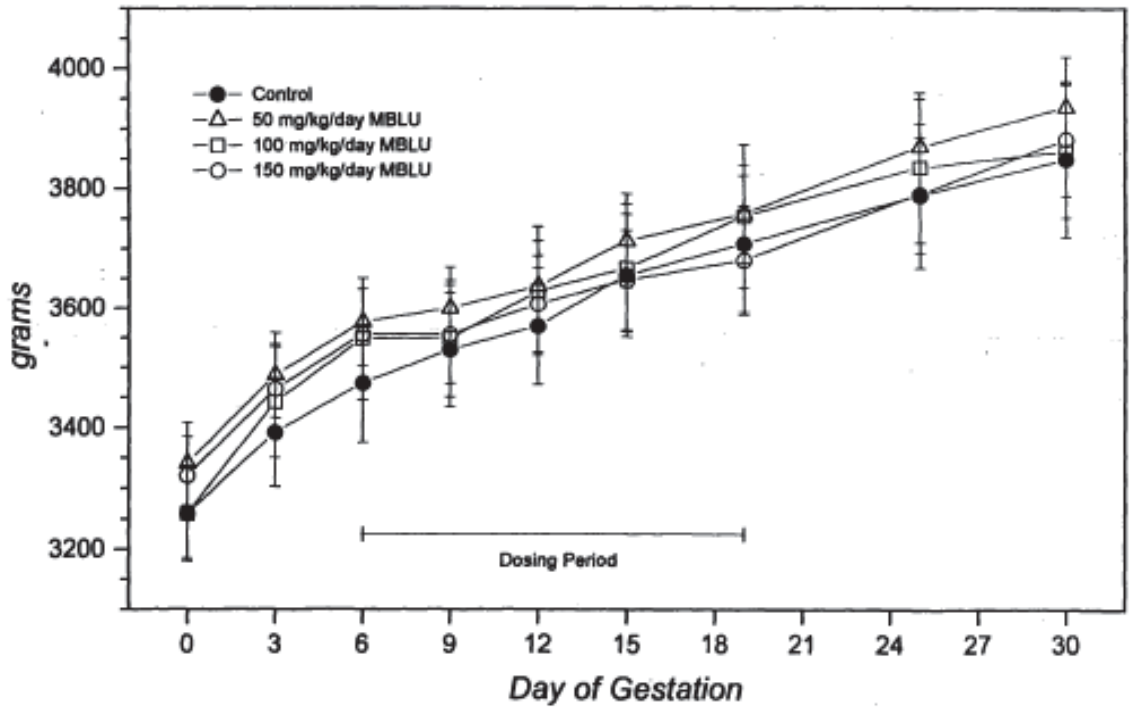
- The predominant clinical sign was blue or green colored urine or feces, which were observed in the females in the methylene blue-treated groups. Since the drug is a blue dye, this finding demonstrates exposure to the drug and presence of the drug in the urine and feces.

Body Weight

- Maternal body weight gain was significantly decreased during the first 3 days of dosing (GD 6-9) in females treated with 100 and 150 mg/kg/day compared to controls.
- Although not statistically significant, maternal body weight gain was lower GD 12-15 in the 100 and 150 mg/kg/day groups and throughout the dosing period (GD 6-19) for all methylene blue-treated groups compared to controls.
- The gravid uterine weight was lower in the 100 and 150 mg/kg/day groups than controls, but the differences were not statistically significant.
- The corrected body weight gain (weight gain during gestation minus the gravid uterine weight) were not lower in the methylene blue-treated groups compared to controls, therefore, the reduced body weight appears to be secondary to reduced uterine weight.

Figure 13: Maternal body weight in rabbits

(excerpted from NTP report TER 92125 1994)

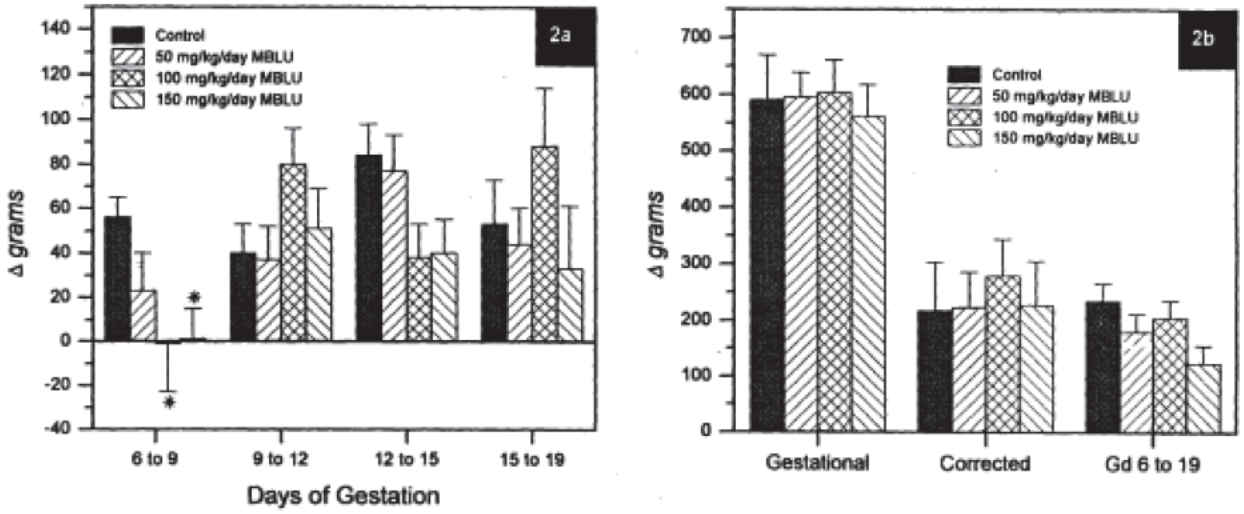


MBLU= methylene blue

Figure 14: Maternal body weight gain in rabbits

(excerpted from NTP report TER 92125 1994)

During dosing period **During gestation**



MBLU= methylene blue

*= Significantly different from control, P<0.05

Table 35: Maternal body weight changes in rabbits

Parameter	Dose (mg/kg/day)			
	0	50	100	150
Gestation weight gain (GD 0-30; g)	591	595	603	561
Treatment weight gain (GD 6-19; g)	234	180	205	124
Corrected weight gain (g)	218	223	279	227
Gravid uterine weight (g)	373	372	324	334

* Significantly different from control (p<0.05)

Food Consumption

- Maternal food consumption in the 150 mg/kg/day group began to decrease compared to controls GD 12-15 and continued to decrease until 15-19, however, the differences were not statistically significant. After the end of the dosing period, food intake returned to normal in the 150 mg/kg/day group.

Toxicokinetics

Not conducted

Necropsy

Unremarkable

Cesarean Section Data

- Spontaneous abortion was observed at all doses of methylene blue, but not the control group. A total of 7 out of 26 treated female rabbits aborted at the high dose of 150 mg/kg/day.
- There were no statistically significant differences between the control group and the methylene blue-treated groups for any of the parameters, however, there were trends for an increase in the mean percent resorptions per litter and percent nonlive implants (resorptions plus late fetal deaths) per litter for the methylene blue-treated groups compared to the controls. The percent of litters with resorptions or nonlive implants were also higher in the methylene blue-treated groups.

Table 36: Uterine examination data in rabbits

Dose (mg/kg/day)	0	50	100	150
Number of females treated	25	26	26	26
Number dead due to dosing error	0	0	3	0
Number dead due to drug	0	0	1	0
Number of females aborted	0	4	2	7
Number of females pregnant at necropsy*	16	20	16	17
Corpora lutea				
Mean number per female	9.25	8.70	8.94	9.76
Implantation sites				
Mean number per litter	5.88	6.35	5.81	6.82
Pre-implantation loss				
Mean % per litter	36.58	30.66	34.45	28.56
Resorptions				
Number of litters with resorptions	6	11	9	13
% litters with resorptions	37.50	55.00	56.25	76.47
Mean number per litter	0.75	1.15	1.50	2.53
Mean % per litter	16.35	25.23	31.89	41.08
Late fetal deaths				
Number of litters with late fetal deaths	0	0	0	1
% litters with late fetal deaths	0	0	0	5.88
Mean number per litter	0	0	0	0.06
Mean % per litter	0	0	0	0.59
Post implantation loss (nonlive implants)				
Number of litters with nonlive implants	6	11	9	13
% litters with nonlive implants	37.50	55.00	56.25	76.47
Mean number per litter	0.75	1.15	1.50	2.59
Mean % per litter	16.35	25.23	31.89	41.66
Total loss of litter (100% nonlive implants)				
Number of litters with 100% nonlive implants	2	3	3	3
% litters with100% nonlive implants	12.50	15.00	18.75	17.65
Viable fetuses				
Number of litters with viable fetuses	14	17	13	14
Total number of fetuses	82	104	69	72
Mean number per female/mean litter size	5.86	6.12	5.31	5.14

* The number of females pregnant at necropsy does not include the number of females aborted

Offspring

Table 37: Fetal sex ratio and weights in rabbits

Dose (mg/kg/day)	0	50	100	150
Fetal sex ratio				
Mean % male fetuses per litter	47.63	54.66	46.15	44.63
Mean fetal weight (g) per litter				
Males	55.06	49.98	52.87	53.33
Females	51.72	50.52	51.05	50.91
Males + females	53.71	50.79	52.04	51.40

- There was a higher incidence of malformations in the 100 and 150 mg/kg/day methylene blue groups compared to controls, but the differences were not statistically significant.
- A malformation of umbilical hernia was observed at 100 and 150 mg/kg/day.

Table 38: Malformations and variations in rabbits

Dose (mg/kg/day)		0	50	100	150
Number of litters evaluated		14	17	13	14
Number of fetuses evaluated		82	104	69	72
Total malformations	Number of litters (%)	2 (14.29)	1 (5.88)	3 (23.08)	5 (35.71)
	Number of fetuses (%)	2 (2.44)	1 (0.96)	5 (7.25)	7 (9.72)
Total variations	Number of litters (%)	13 (92.86)	16 (94.12)	11 (84.62)	11 (78.57)
	Number of fetuses (%)	45 (54.88)	68 (65.38)	45 (65.22)	42 (58.33)
Malformations					
Umbilical hernia	Number of litters (%)	0 (0)	0 (0)	1 (7.69)	2 (14.29)
	Number of fetuses (%)	0 (0)	0 (0)	1 (1.45)	4 (5.56)
Enlarged lateral ventricles of the brain	Number of litters (%)	1 (7.14)	0 (0)	2 (15.38)	0 (0)
	Number of fetuses (%)	1 (1.22)	0 (0)	3 (4.35)	0 (0)

9.3 Prenatal and Postnatal Development

No prenatal and postnatal development studies were submitted.

10 Special Toxicology Studies

No special toxicology studies were reviewed.

11 Integrated Summary and Safety Evaluation

Methylene blue is a thiazine dye that promotes a non-enyzmatic redox conversion of methemoglobin to hemoglobin in erythrocytes. Methylene blue is quickly reduced to leucomethylene blue in the body in the presence of NADPH reductase. Leucomethylene blue is able to then rapidly transfer the added electron (as an electron donor) to reduce methemoglobin to hemoglobin. To support the approval of methylene blue for the treatment of acquired methemoglobinemia, the Applicant submitted results of toxicology studies conducted with their product (b) (4) along with literature on the pharmacology, pharmacokinetics and toxicology of methylene blue, and studies of methylene blue trihydrate conducted by the National Toxicology Program (NTP).

General Toxicology

General toxicology studies of methylene blue include 1- and 3-month repeat-dose toxicology studies conducted by NTP with oral administration of methylene blue trihydrate in mice and rats and a 1-month repeat-dose toxicology study with intravenous administration of Provepharma's methylene blue in dogs. For this review, the 3-month rat study conducted by NTP with oral methylene blue trihydrate and the 1-month dog study with intravenous administration of Provepharma's methylene blue were reviewed.

In the 3-month repeat-dose toxicology study in rats, F344/N rats were administered methylene blue trihydrate (0, 25, 50, 100, or 200 mg/kg; 0, 150, 300, 600, and 1200 mg/m^2) by oral gavage at a dose volume of 5 mL/kg once daily 5 days per week for 14 weeks. Additional groups of animals that were administered the same doses of methylene blue trihydrate were used for the assessment of hematology and clinical chemistry on Weeks 1 and 6. In the 1-month repeat-dose toxicology study in dogs, Beagle dogs were administered Provepharma's methylene blue (0, 0.25, 0.50, or 1.0 mg/kg/day; 0, 5, 10, or 20 mg/m^2/day; concentration 5 mg/mL) or a comparator drug of methylene blue injection USP 1% w/v from Martindale (1.0 mg/kg/day; 20 mg/m^2) by intravenous infusion into the cephalic or saphenous vein at a flow rate of 0.5 mL/minute once daily for 4 weeks. There was no mortality attributed to the administration of methylene blue in either study. Despite the difference in the routes of administration, similar toxicities were observed in rats and dogs. Hematological responses in both species included anemia as indicated by decreases in erythrocytes, hematocrit, and hemoglobin and increases in reticulocytes and methemoglobinemia as indicated by increases in methemoglobin and/or Heinz bodies (inclusions within red blood cells composed of denatured hemoglobin). Liver and spleen were organs of toxicity in both rats and dogs. Hyperplasia was reported in the bone marrow of rats and there was increased cellularity of the hematopoietic component of the bone marrow in dogs; this may be secondary to anemia to produce more blood cells. Liver toxicity consisted of increased bile acids and increased liver weights in rats, while increases of total bilirubin in the blood and bilirubin in urine and microscopic findings of green/brown pigment and inflammatory foci in the liver were observed in dogs. Increases in absolute and relative spleen weights, enlarged spleen, and congestion in the spleen were observed in both species. Additional microscopic findings in the spleen were hematopoietic cell

proliferation, lymphoid depletion of lymphoid follicles, and capsular fibrosis in rats and hemopoiesis and increased green/brown pigment in dogs.

Additional toxicities were observed with intravenous administration of methylene blue in dogs. Injection site toxicity observed in the cephalic vein included clinical signs of increased size and/or indurated right cephalic vein, macroscopic findings of thickened subcutaneous tissue of the left or right cephalic vein, and various microscopic inflammatory findings including degeneration/necrosis and inflammatory cells in the venous wall. Increased brown pigment was observed in the kidney at 1.0 mg/kg/day (20 mg/m^2/day) methylene blue.

In the 1-month repeat-dose dog study, the toxicokinetics of intravenous administration of Provepharma's methylene blue (0.25, 0.50, or 1.0 mg/kg/day; 5, 10, or 20 mg/m^2/day) and the reference drug, Methylene blue injection USP (1.0 mg/kg/day; 20 mg/m^2/day), were evaluated on Days 1 and 28. Absorption of Provepharma's methylene blue and the comparator drug was rapid with t_{max} at 0.083 hours (5 minutes) for most animals. Half-life ($t_{1/2}$) was similar at all dose levels and ranged from 0.8 to 1.5 hours with the exception of a $t_{1/2}$ of 5.19 hours in females treated with 1.0 mg/kg/day (20 mg/m^2/day) Provepharma's methylene blue on Day 1. C_{max} and $AUC_{(0\text{-}t)}$ for Provepharma's methylene blue increased with an increase in dose. In general, increases were approximately dose proportional or slightly greater than dose proportional. Provepharma's methylene blue had a similar toxicological and toxicokinetic profile in dogs as the comparator drug Methylene blue injection USP 1% w/v (Martindale) at a dose of 1.0 mg/kg/day (20 mg/m^2/day) when administered intravenously once daily for 4 weeks.

Genetic Toxicology

The genotoxicity of methylene blue has been evaluated in both in vitro and in vivo studies conducted by NTP with methylene blue trihydrate and in an in vitro bacterial reverse mutation assay (Ames test) conducted with Provepharma's methylene blue. In the in vitro reverse mutation assay in bacterial cells conducted by NTP, methylene blue trihydrate and three metabolites (Azure A, Azure B, and Azure C) were positive for mutagenicity both in the presence and absence of S9 activation in *Salmonella typhimurium* strains TA98 and TA100 and in *Escherichia coli* strain WP2 *uvrA*/pKM101. In the Ames test conducted with the Provepharma methylene blue, methylene blue was positive for mutagenicity in *Salmonella typhimurium* strains TA1535, TA98, TA100, and TA102 in the absence of S9 activation and in strainsTA98 and TA102 in the presence of S9 activation. In an in vitro sister chromatid exchange test and an in vitro structural chromosome aberration test in CHO cells conducted by NTP, methylene blue trihydrate was genotoxic both in the presence and absence of S9 activation. In the sister chromatid exchange test, methylene blue trihydrate induced sister chromatid exchanges at concentrations of 0.63 to 2.5 µg/mL in the absence of S9 activation and at 5.0 µg/mL in the presence of S9 activation. In the chromosomal aberrations test, methylene blue trihydrate was clastogenic by inducing chromosomal aberrations at concentrations of

7.5 to 25.0 µg/mL in the absence of S9 activation and at 4.7 µg/mL in the presence of S9 activation.

Methylene blue trihydrate was negative in the in vivo micronucleus tests in the mouse conducted by NTP. Methylene blue trihydrate was negative for micronucleus induction in bone marrow or peripheral blood collected from male mice 48 hours after a single intraperitoneal injection of methylene blue trihydrate (25, 50, or 150 mg/kg; 75, 150, or 450 mg/m^2). Methylene blue trihydrate was also negative for micronucleus induction in peripheral blood samples from male and female mice at the end of a 3-month repeat-dose toxicity study of methylene blue trihydrate (25, 50, 100, or 200 mg/kg; 75, 150, 300, or 600 mg/m^2).

Carcinogenicity

Two-year carcinogenicity studies in mice and rats conducted by NTP with methylene blue trihydrate from Sigma Chemical Company were fully reviewed in a separate review for NDA 204630 (archival date: March 25, 2014). In both studies, methylene blue trihydrate was administered by oral gavage once daily, 5 days per week for 2 years. In the mouse study, B6C3F$_1$ mice were administered doses of 0, 2.5, 12.5, or 25 mg/kg (0, 7.5, 37.5, or 75 mg/m^2) at a dose volume of 10 mL/kg. In the rat study, F344/N rats were administered doses of 0, 5, 25, or 50 mg/kg (0, 30, 150, or 300 mg/m^2) at a dose volume of 5 mL/kg. The survival of both mice and rats treated with methylene blue was similar to vehicle controls. In female mice treated with 25 mg/kg (75 mg/m^2), the body weights were 5-9% lower than controls Weeks 45 through 73. Hematological responses of anemia as indicated by decreases in erythrocytes, hematocrit, and hemoglobin and methemoglobinemia as indicated by increases in methemoglobin, were observed in both species. Heinz bodies, which are inclusions within red blood cells composed of denatured hemoglobin, were also observed in both species. Non-neoplastic histopathology findings included hematopoietic cell proliferation in the spleen in both mice (12.5 and 25 mg/kg; 37.5 and 75 mg/m^2) and rats (25 and 50 mg/kg; 150 and 300 mg/m^2), capsule fibrosis in the spleen at all doses in rats, hyperplasia in pancreatic islets and focal hyperplasia in the acinus of the pancreas at 25 and 50 mg/kg (150 and 300 mg/m^2) in male rats, and inflammation of the nose at all doses in mice.

Based on the FDA criteria for a positive carcinogenicity response, FDA's conclusion is that there are no statistically significant neoplastic findings in the 2-year mouse carcinogenicity study. The FDA concluded that methylene blue caused pancreatic islet adenomas or carcinomas in male rats in the 2-year carcinogenicity study based on the incidences exceeding the historical control incidence, particularly at the mid dose of 25 mg/kg (150 mg/m^2). In addition, there was a dose-related increase in pancreatic islet hyperplasia.

Reproductive and Developmental Toxicology

Embryo-fetal development studies in rats and rabbits were conducted by NTP with methylene blue trihydrate supplied by Aldrich Chemical Co. In the rat study, female

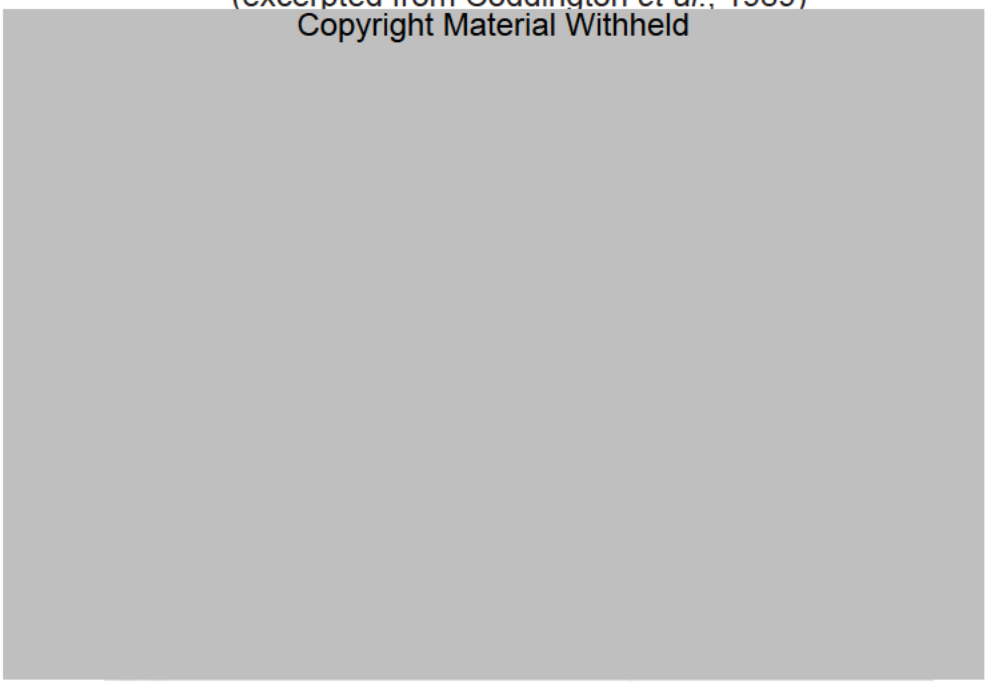
Sprague-Dawley rats were administered methylene blue by oral gavage once daily on GD 6-15 and euthanized on GD 20. The original doses were (0, 50, 200, or 350 mg/kg/day; 0, 300, 1200, or 2100 mg/m^2/day) based on a dose range finding study. The resorption of all fetuses from 10 of 11 litters and only 4 viable fetuses in the remaining litter at 350 mg/kg/day in the first replicate, lead to a reduction in the mid- and high doses. Therefore, in the second replicate doses of 0, 50, 125, or 200 mg/kg/day (0, 300, 750, or 1200 mg/m^2/day) methylene blue were administered. Maternal toxicity was observed at all doses of methylene blue tested (50, 125, and 200 mg/kg/day; 300, 750, or 1200 mg/m^2/day) based on increases in maternal spleen weights. The gravid uterine weight was lower in the 200 mg/kg/day (1200 mg/m^2/day) group compared to controls, but the difference was not statistically significant. Treatment with methylene blue significantly increased resorptions and post-implantation loss at 200 and 350 mg/kg/day (1200 and 2100 mg/m^2/day). Since there was an increase in the number of corpora lutea at 200 mg/kg/day (1200 mg/m^2/day) in the second replicate, the number of viable fetuses was not affected even tough post-implantation loss was increased. Fetal body weights were decreased at 200 and 350 mg/kg/day (1200 and 2100 mg/m^2/day); the decreases were statistically significant at 200 mg/kg/day (1200 mg/m^2/day) in the second replicate. While malformations were observed in all groups including controls, there was an increase in the incidence of some malformations including enlarged lateral ventricles in the brain in the methylene-blue treated groups, particularly at the 125, 200 and 350 mg/kg (750, 1200 and 2100 mg/m^2/day) doses. Other fetal abnormalities included anasarca (edema).

In the rabbit embryofetal development study, female New Zealand White rabbits were administered methylene blue (0, 50, 100, or 150 mg/kg/day; 0, 600, 1200, or 1800 mg/m^2/day) by oral gavage once daily on GD 6-19 and euthanized on GD 30. Methylene blue was maternally toxic with a maternal death at 100 mg/kg/day (1200 mg/m^2/day). The gravid uterine weight was lower in the 100 and 150 mg/kg/day (1200 and 1800 mg/m^2/day) groups than controls, but the differences were not statistically significant. Treatment with methylene blue caused spontaneous abortion at all doses with a total of 7 out of 26 treated female rabbits aborted at the high dose (150 mg/kg/day; 1800 mg/m^2/day). Although there was no statistical significance, resorptions and post-implantation loss were increased in methylene blue-treated groups compared to the controls. The incidence of total malformations was higher in the 100 and 150 mg/kg/day (1200 and 1800 mg/m^2/day) groups compared to controls, but the differences were not statistically significant. A malformation of umbilical hernia was observed at 100 and 150 mg/kg/day (1200 and 1800 mg/m^2/day). Methylene blue produced post-implantation loss and was teratogenic at maternally toxic doses in both the rat and rabbit. The clinical dose of methylene blue will be titrated to doses that will reduce methemoglobin and hypoxia, and only in an overdose situation would adverse embryo-fetal effects be expected. Since the embryo-fetal toxicities including teratogenicity were observed at maternally toxic doses and fetal toxicities are not expected at the therapeutic range, pregnancy category C is recommended. Toxicokinetics were not conducted in the NTP embryo-fetal studies for methylene blue, therefore, dose to dose comparisons to the clinical dose will be used for the animal-to-human conversions for methylene blue labeling.

Fertility studies with methylene blue have not been conducted. According to published literature (Coddington *et al.*, 1989), methylene blue reduced motility of human sperm in a time- and concentration-dependent manner in an in vitro study conducted in semen from men with normal fertility. Sperm that were not exposed to methylene blue (0%) retained excellent motility throughout the experiment, while exposure to methylene blue rapidly decreased motility of the sperm (see table below). Sperm that were exposed to methylene blue for 45 or 60 minutes were unable to recover motility, but appeared to be alive. The data were published in an article (Coddington *et al.*, 1989) in a peer-reviewed journal that was submitted with the NDA, therefore, the data were not fully reviewed for this NDA.

Table 39: Effects of methylene blue on sperm motility

(excerpted from Coddington *et al.*, 1989)



12 Appendix/Attachments

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/s/

BRENDA J GEHRKE
05/27/2014

CHING-JEY G CHANG
05/28/2014

HALEH SABER
05/28/2014

CARCINOGENICITY ASSESSMENT COMMITTEE (CAC/CAC-EC) REPORT
AND
FDA-CDER RODENT CARCINOGENICITY DATABASE FACTSHEET

P/T REVIEWER:	Brenda J. Gehrke, Ph.D.
ECAC MEETING DATE:	March 18, 2014
NDA:	204630
DRUG CODE#:	None
CAS #:	Methylene blue: 61-73-4 Methylene blue trihydrate: 7220-79-3
DIVISION(s):	Division of Hematology Oncology Toxicology For Division of Hematology Products
DRUG NAME(s):	Proposed drug: Methylene blue ([redacted] (b) (4) Methylene blue trihydrate was used in carcinogenicity studies conducted by NTP
SPONSOR:	Provepharma is Applicant for NDA; the National Toxicology Program (NTP) sponsored the carcinogenicity studies
LABORATORY:	Southern Research Institute Birmingham, Alabama

CARCINOGENICITY STUDY REPORT DATE: May 2008

THERAPEUTIC CATEGORY: Proposed indication is for the treatment of acquired methemoglobinemia

PHARMACOLOGICAL/CHEMICAL CLASSIFICATION: To be determined

MECHANISM OF ACTION: Methylene blue is a thiazine dye that promotes a non-enyzmatic redox conversion of methemoglobin to hemoglobin

MUTAGENIC/GENOTOXIC:

Methylene blue trihydrate was mutagenic in an in vitro bacterial cell assay (Ames test). In in vitro tests in Chinese hamster ovary (CHO) cells, methylene blue trihydrate induced sister chromatid exchanges and was clastogenic by inducing chromosomal aberrations. Methylene blue trihydrate was not genotoxic in an in vivo mouse micronucleus study.

MOUSE CARCINOGENICITY STUDY:

MOUSE STUDY DURATION (weeks): 106 weeks
STUDY STARTING DATE: First dose administered on July 10, 2000
STUDY ENDING DATE: Necropsy on July 16, 2002
MOUSE STRAIN: $B6C3F_1$
ROUTE: Oral gavage
DOSING COMMENTS: None

NUMBER OF MICE:
- Control: 50
- Low Dose: 50
- Middle Dose: 50
- High Dose: 50

MOUSE DOSE LEVELS (mg/kg/day):
- Low Dose: 2.5
- Middle Dose: 12.5
- High Dose: 25

BASIS FOR DOSES SELECTED: Based on the results of 3-month mouse study conducted by NTP; 25 mg/kg was chosen as high dose since it produced some anemia but was not expected to affect longevity in a 2-year study; lowest dose selected because it was within the range of human therapeutic use of methylene blue trihydrate

PRIOR ECAC DOSE CONCURRENCE: No

MOUSE CARCINOGENICITY: Negative in both male and female mice

ADEQUACY OF THE STUDY: The study is considered acceptable based on drug-related toxicities at high dose, including the formation of methemoglobin.

MOUSE TUMOR FINDINGS (details):

- The incidences of carcinoma and adenoma or carcinoma combined in the small intestine were increased in males treated with methylene blue. Carcinoma in the small intestine was observed in 4/50 (8%) males treated with the high dose of methylene blue and was not observed in the males in the concurrent control group. Based on the lack of the neoplasm in the concurrent control (<1%), carcinoma in the small intestine was considered a rare tumor. While the trend test (p=0.018) for the FDA analyses unadjusted for survival was significant for a

rare tumor, the pairwise test comparing the high dose to control (p=0.059) was not significant. Both pairwise and trend analyses should be positive in order for the FDA to consider the finding to be positive.

- The incidences of malignant lymphoma were increased at 25 mg/kg in males and 12.5 and 25 mg/kg in females treated with methylene blue. Malignant lymphoma was observed in the control groups and is a common tumor.

- There were no statistically significant neoplastic findings in mice using the FDA criteria for a positive response.

Neoplastic findings for mice in the 2-year carcinogenicity study

Neoplastic findings		No. of animals affected (Percentage of animals affected)							
		Males				**Females**			
Dose (mg/kg)		0	2.5	12.5	25	0	2.5	12.5	25
Number of animals examined		50	50	50	50	50	50	50	50
Organ/tissue	Finding								
Small intestine	Carcinoma	0 (0%)	1 (2%)	2 (4%)	4 (8%)	1 (2%)	0 (0%)	0 (0%)	0 (0%)
	Adenoma or carcinoma	1 (2%)	2 (4%)	4 (8%)	6 (12%)	1 (2%)	1 (2%)	0 (0%)	0 (0%)
Various	Malignant lymphoma	2 (4%)	2 (4%)	2 (4%)	5 (10%)	6 (12%)	4 (8%)	9 (18%)	12 (24%)

Summary of FDA and NTP analysis results for mice

Sex	Tumor type	One-sided P-value			
		NTP survival-adjusted Poly-3 test		FDA survival-unadjusted exact permutation test	
		Trend test	Control-high pairwise test	Trend test	Control-high pairwise test
Males	Small intestine carcinoma	0.027	0.071	0.018	0.059
Males	Small intestine adenoma or carcinoma	0.029	0.071	0.019	0.056
Males	Malignant lymphoma	0.126	0.250	0.136	0.218
Females	Malignant lymphoma	0.025	0.126	0.027	0.096

MOUSE STUDY COMMENTS:

- Toxicokinetics were not measured in any of the mouse toxicology studies conducted by NTP with methylene blue trihydrate.
- Survival in the methylene blue-treated groups was similar to that of controls.

- Mean body weights of male mice treated with methylene blue were similar to controls throughout the study. In female mice, the mean body weights were similar to controls overall, however, at 25 mg/kg the body weights were 5-9% lower than controls Weeks 45 through 73.
- Hematological responses were observed including anemia in males at 25 mg/kg as indicated by decreases in erythrocytes, hematocrit, and hemoglobin and methemoglobinemia in females at 12.5 mg/kg and males and females at 25 mg/kg as indicated by increases in methemoglobin. Heinz bodies (inclusions within red blood cells composed of denatured hemoglobin) were also increased in both males and females at 12.5 and 25 mg/kg methylene blue.

RAT CARCINOGENICITY STUDY:

RAT STUDY DURATION (weeks): 106 weeks
STUDY STARTING DATE: First dose administered on June 26, 2000
STUDY ENDING DATE: Necropsy on July 2, 2002
RAT STRAIN: F344/N
ROUTE: Oral gavage
DOSING COMMENTS: None

NUMBER OF RATS:
- Control: 50
- Low Dose: 50
- Middle Dose: 50
- High Dose: 50

RAT DOSE LEVELS (mg/kg/day):
- Low Dose: 5
- Middle Dose: 25
- High Dose: 50

BASIS FOR DOSES SELECTED: Based on the results of the 3-month rat study conducted by NTP; 50 mg/kg was chosen as high dose since it produced some anemia but was not expected to affect longevity in a 2-year study; lowest dose selected because it was within the range of human therapeutic use of methylene blue trihydrate

PRIOR ECAC DOSE CONCURRENCE: No

RAT CARCINOGENICITY: Negative in female rats. Positive for pancreatic islet adenomas or carcinomas (combined) in male rats treated with methylene blue.

ADEQUACY OF THE STUDY: The study is acceptable based on drug-related toxicities, including reductions in the body weight and formation of methemoglobin at mid dose and high dose.

RAT TUMOR FINDINGS (details):

- The incidences of adenoma and adenoma or carcinoma (combined) in the pancreatic islets were increased in males treated with methylene blue. Adenoma or carcinoma (combined) in pancreatic islets was observed in 9/50 (18%), 14/50 (28%), and 8/50 (16%) males in the 5, 25, and 50 mg/kg methylene blue-treated groups, respectively. The neoplasm was observed in 4/50 (8%) males in the concurrent control group. According to the historical incidence for all routes of administration provided in the NTP report, adenoma or carcinoma (combined) in pancreatic islets has been observed in 92/1,448 (6.4%) of controls, with a mean of 6.8% and a range of 0-14%.
- Although the findings were not statistically significant, the FDA concluded that methylene blue caused pancreatic islet adenomas or carcinomas (combined) in male rats based on the incidences exceeding the historical control incidence, particularly at the mid dose of 25 mg/kg. In addition, there were dose-related increases in the pancreatic islet hyperplasia.

Neoplastic findings for rats in the 2-year carcinogenicity study

Neoplastic findings		No. of animals affected (Percentage of animals affected)							
		Males				**Females**			
Dose (mg/kg)		0	5	25	50	0	5	25	50
Number of animals examined		50	50	50	50	49	48	48	49
Organ/tissue	Finding								
Pancreatic islets	Adenoma	4 (8%)	9 (18%)	12 (24%)	8 (16%)	2 (4%)	0 (0%)	1 (2%)	1 (2%)
	Adenoma or carcinoma	4 (8%)	9 (18%)	14 (28%)	8 (16%)	2 (4%)	0 (0%)	1 (2%)	1 (2%)

Summary of FDA and NTP analysis results for rats

Sex	Tumor type	One-sided P-value			
		NTP survival-adjusted Poly-3 test		FDA survival-unadjusted exact permutation test	
		Trend test	Control-high pairwise test	Trend test	Control-high pairwise test
Males	Pancreatic islets adenoma	0.201	0.155	0.212	0.178
Males	Pancreatic islets adenoma or carcinoma	0.174	0.155	0.191	0.178

RAT STUDY COMMENTS:

- Toxicokinetics were not measured in any of the rat toxicology studies conducted by NTP with methylene blue trihydrate.
- Survival in the methylene blue-treated groups was similar to that of controls.
- Body weights were lower in both males and females treated with 25 and 50 mg/kg methylene blue compared to controls, with mean weights 9-13% lower than controls at Week 101.
- Hematological responses were observed at 25 and 50 mg/kg including anemia as indicated by decreases in erythrocytes, hematocrit, and hemoglobin and methemoglobinemia as indicated by increases in methemoglobin. Heinz bodies (inclusions within red blood cells composed of denatured hemoglobin) were also increased in females at 25 and 50 mg/kg methylene blue.

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:	204630
Supporting document/s:	9
Applicant's letter date:	September 13, 2013
CDER stamp date:	September 13, 2013
Product:	Methylene blue injection
Indication:	Treatment of acquired methemoglobinemia
Applicant:	Provepharma SAS
Review Division:	Division of Hematology Oncology Toxicology (for Division of Hematology Products)
Reviewer:	Brenda J. Gehrke, Ph.D.
Supervisor/Team Leader:	Haleh Saber, Ph.D.
Division Director:	John Leighton, Ph.D., DABT Ann Farrell, M.D. (DHP)
Project Manager:	Kim J. Robertson

Disclaimer

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1 Executive Summary

1.1 Introduction

Methemoglobinemia is a disease characterized by a reduced ability of the blood to carry oxygen due to a reduction in the levels of normal hemoglobin. Excessive levels of an altered form of hemoglobin (methemoglobin) can be caused by various genetic factors or substances (e.g. drugs, nitrates and nitrites, chemicals, and pesticides). Methylene blue ((b) (4) is a thiazine dye that promotes a non-enyzmatic redox conversion of methemoglobin (MetHb) to hemoglobin. Based on this mechanism of action, methylene blue is currently used to treat methemoglobinemia and marketed in the United States as unapproved products without an approved NDA. Provepharma SAS is developing a methylene blue product for the treatment of acquired methemoglobinemia. The proposed clinical doses of 1 mg/kg (b) (4) in adults and children (b) (4) will be administered by intravenous injection over a period of 5 minutes as an initial dose (b) (4) Toxicology studies conducted with the Provepharma methylene blue have been submitted along with literature on the pharmacology, pharmacokinetics and toxicology of methylene blue, and studies of methylene blue trihydrate conducted by the National Toxicology Program (NTP) to support the approval of methylene blue for the treatment of acquired methemoglobinemia.

1.2 Brief Discussion of Nonclinical Findings

Two-year carcinogenicity studies in mice and rats were conducted by NTP with methylene blue trihydrate from Sigma Chemical Company. In both studies, methylene blue trihydrate was administered by oral gavage once daily, 5 days per week for 2 years (106 weeks). In the mouse study, $B6C3F_1$ mice were administered doses of 0, 2.5, 12.5, or 25 mg/kg (0, 7.5, 37.5, or 75 mg/m^2) at a dose volume of 10 mL/kg. In the rat study, F344/N rats were administered doses of 0, 5, 25, or 50 mg/kg (0, 30, 150, or 300 mg/m^2) at a dose volume of 5 mL/kg. The survival of both mice and rats treated with methylene blue was similar to vehicle controls. In rats, body weights were lower at 25 and 50 mg/kg methylene blue compared to controls. Hematological responses of anemia as indicated by decreases in erythrocytes, hematocrit, and hemoglobin and methemoglobinemia as indicated by increases in methemoglobin, were observed in both species. Heinz bodies, which are inclusions within red blood cells composed of denatured hemoglobin, were also observed in both species. Non-neoplastic histopathology findings included hematopoietic cell proliferation in the spleen in both mice (12.5 and 25 mg/kg) and rats (25 and 50 mg/kg), capsule fibrosis in the spleen at all doses in rats, hyperplasia in pancreatic islets and focal hyperplasia in the acinus of the pancreas at 25 and 50 mg/kg in male rats, and inflammation of the

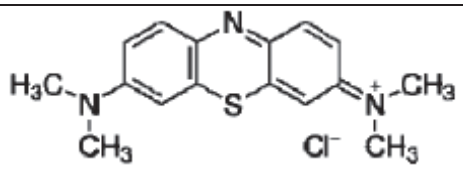
nose at all doses in mice. Methylene blue increased the incidences of adenomas or carcinomas of the small intestine in male mice and pancreatic islet adenomas or carcinomas in male rats. Additionally, the incidences of malignant lymphoma in mice were increased at 25 mg/kg in males and 12.5 and 25 mg/kg in females treated with methylene blue.

NTP concluded that there was some evidence of carcinogenic activity of methylene blue based on increased incidences of pancreatic islet cell adenoma or carcinoma in male rats and increased incidences of adenoma or carcinoma in the small intestine in male mice. The increased incidence of malignant lymphoma in male and female mice was considered an equivocal finding, possibly related to methylene blue trihydrate administration. Based on the FDA criteria for a positive carcinogenicity response, FDA's conclusion is that there are no statistically significant neoplastic findings in the 2-year mouse carcinogenicity study. Although the findings were not statistically significant, the FDA concluded that methylene blue caused pancreatic islet adenomas or carcinomas (combined) in male rats in the 2-year carcinogenicity study based on the incidences exceeding the historical control incidence, particularly at the mid dose of 25 mg/kg. In addition, there was a dose-related increase in pancreatic islet hyperplasia.

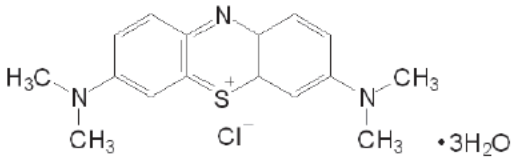
2 Drug Information

2.1 Drug

Provepharma methylene blue

CAS Registry Number	61-73-4
Generic Name	Methylene blue; (b) (4)
Chemical Name	3,7-bis(dimethylamino)-phenothiazin-5-ium chloride
Molecular Formula/ Molecular Weight	$C_{16}H_{18}N_3SCl$/ 319.86 g/mol
Structure or Biochemical Description	

Methylene blue trihydrate

CAS Registry Number	7220-79-3
Generic Name	Methylene blue trihydrate; methylthioninium chloride
Chemical Name	3,7-bis(dimethylamino)-phenothiazin-5-ium chloride trihydrate
Molecular Formula/ Molecular Weight	$C_{16}H_{24}N_3O_3SCl$/ 373.9 g/mol
Structure or Biochemical Description	

2.2 Relevant INDs, NDAs, BLAs and DMFs

Pre-IND 113942, Pre-IND 118156

2.3 Drug Formulation

The methylene blue injection (0.5%) is a sterile solution for intravenous administration supplied in 10 mL glass type 1 ampules. Each ampule contains 50 mg methylene blue and water for injection q.s., for a concentration of 5 mg/mL.

2.6 Proposed Clinical Population and Dosing Regimen

The proposed clinical population is patients with acquired methemoglobinemia. Methylene blue will be administered by intravenous injection over a period of 5 minutes as an initial dose (b) (4).

Table 1: Clinical doses
(excerpted from Applicant's submission)

(b) (4)

8 Carcinogenicity

Study title: NTP technical report on the toxicology and carcinogenesis studies of methylene blue trihydrate (CAS No. 7220-79-3) in F344/N rats and B6C3F$_1$ mice

Study no.:	NTP report TR 540 2008
Study report location:	eCTD 4.3
Conducting laboratory and location:	Southern Research Institute Birmingham, Alabama
Date of study initiation:	Exact initiation dates not provided; first dose administered June 26, 2000 in rats and July 10, 2000 in mice
GLP compliance:	Yes
QA statement:	Quality assurance conducted but signed statement not provided; quality assurance reports on file at NIEHS
Drug, lot #, and % purity:	Methylene blue trihydrate, from Sigma Chemical Company, lot # 68H3728, Purity: >91%
CAC concurrence:	N/A; No ECAC meeting was held

Adequacy of Carcinogenicity Studies:

- Since the studies were conducted by NTP for methylene blue trihydrate, the CAC was not consulted on the study design of these rat and mouse 2-year carcinogenicity studies. However, both studies were considered acceptable by the FDA, based on drug-induced toxicities at the high dose of methylene blue.

Appropriateness of Test Models:

- The studies evaluated three doses each based on the results of the 3-month mouse and rat studies. The high doses chosen produced some anemia in the mouse and rat studies but were not expected to affect longevity in the 2-year studies. The lowest doses selected were within the range of human therapeutic use of methylene blue trihydrate.
- The study report provided sufficient histopathology data from the designated organs and tissues to evaluate both the non-neoplastic and neoplastic effects at all dose levels including controls. Additionally, data on survival, body weights, and hematology were provided.
- Toxicokinetics were not conducted in any of the mouse or rat toxicology studies conducted by NTP, therefore, exposures to methylene blue trihydrate were not measured.

Evaluation of Tumor Findings:

2-year carcinogenicity study in mice

Key Study Findings

- Survival in the methylene blue-treated groups was similar to that of controls.
- There were no statistically significant neoplastic findings using the FDA criteria for a positive response.
- Non-neoplastic findings included hematopoietic cell proliferation in the spleen at 12.5 and 25 mg/kg in both males and females and inflammation of the nose at all doses of methylene blue.

Methods

Doses:	0, 2.5, 12.5, or 25 mg/kg
Frequency of dosing:	Once daily, 5 days per week for 2 years (106 weeks)
Dose volume:	10 mL/kg
Route of administration:	Oral gavage
Formulation/Vehicle:	0.5% aqueous methylcellulose
Basis of dose selection:	Results of 3-month mouse study: 25 mg/kg chosen as high dose since it produced some anemia but was not expected to affect longevity in a 2-year study; lowest dose selected because it was within the range of human therapeutic use of methylene blue trihydrate
Species/Strain:	$B6C3F_1$ mice
Number/Sex/Group:	50/sex/group
Age:	6 weeks old at beginning of study
Animal housing:	Males: 1/cage Females: 5/cage
Paradigm for dietary restriction:	Food and water available *ad libitum*
Dual control employed:	None
Interim sacrifice:	Not conducted
Satellite groups:	Hematology: Groups of 30/sex/group were administered the same doses for up to 18 months and were evaluated at 2 weeks, 3, 12, or 18 months for hematology

Observations and times:

Mortality:	Twice daily
Clinical signs:	Observed twice daily Clinical findings recorded monthly (every 4 weeks) beginning Week 5 for main study animals
Body weights:	Day 1, Weekly for first 13 weeks, at 4-week intervals thereafter, and at necropsy for main study animals
Food consumption:	Not conducted
Hematology:	At 2 weeks and 3, 12, and 18 months in satellite groups
Urinalysis:	At 3, 12, and 18 months in 5/sex/group main study animals; placed in metabolism cages for 24-hours for urine collection
Gross pathology:	At necropsy on main study animals
Histopathology:	Conducted for all main study animals

Results

Mortality

Table 2: Survival of mice in the 2-year carcinogenicity study

	Males				Females			
Dose (mg/kg)	0 Control	2.5	12.5	25	0 Control	2.5	12.5	25
Animals initially in study	50	50	50	50	50	50	50	50
Accidental deaths[a]	0	2	0	2	1	1	1	0
Moribund	4	4	7	4	7	4	5	1
Natural deaths	11	6	5	3	9	5	2	6
Animals surviving to study termination	35	38	38	41	33[c]	40	42[d]	43[e]
Mean survival (days)[b]	693	698	705	715	693	696	712	710

[a]= Censored from survival analyses by NTP

[b]= Mean of all deaths (uncensored, censored, and terminal sacrifice)

[c]= Includes four animals that died last week of study

[d]= Includes one animal that died last week of study

[e]= Includes two animals that dies last week of study

Clinical Signs

Unremarkable

Body Weights

- Mean body weights of male mice treated with methylene blue were similar to controls throughout the study. In female mice, the mean body weights were similar to controls overall, however, at 25 mg/kg the body weights were 5-9% lower than controls Weeks 45 through 73.

Figure 1: Mean body weight in male mice in the 2-year carcinogenicity study
(excerpted from NTP report TR 540 2008)

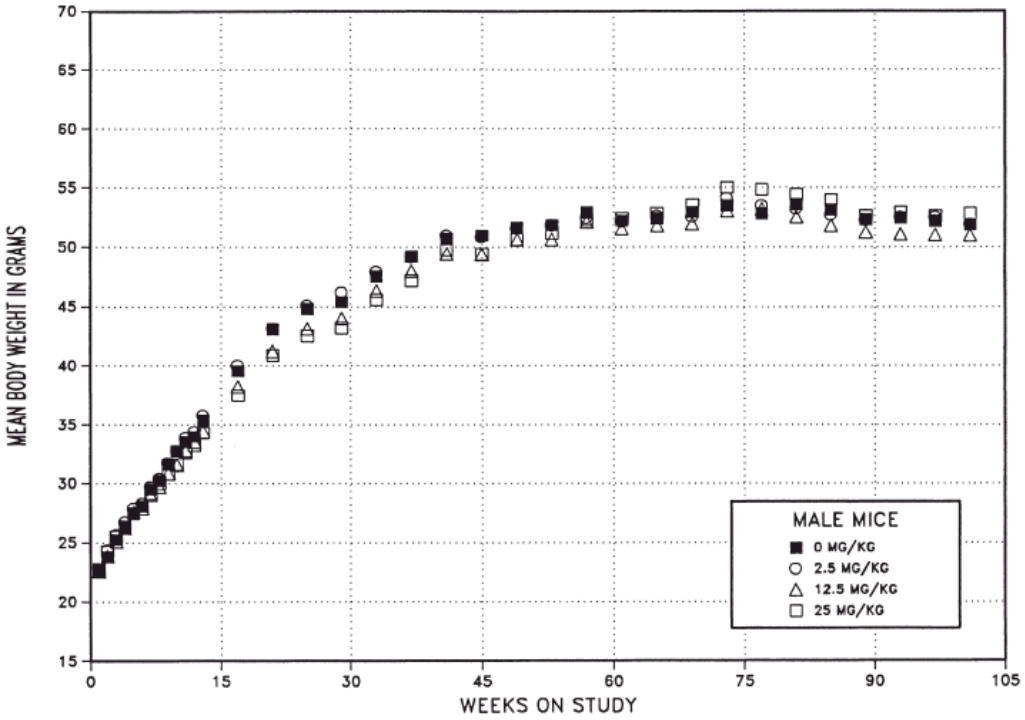


Figure 2: Mean body weight in female mice in the 2-year carcinogenicity study
(excerpted from NTP report TR 540 2008)

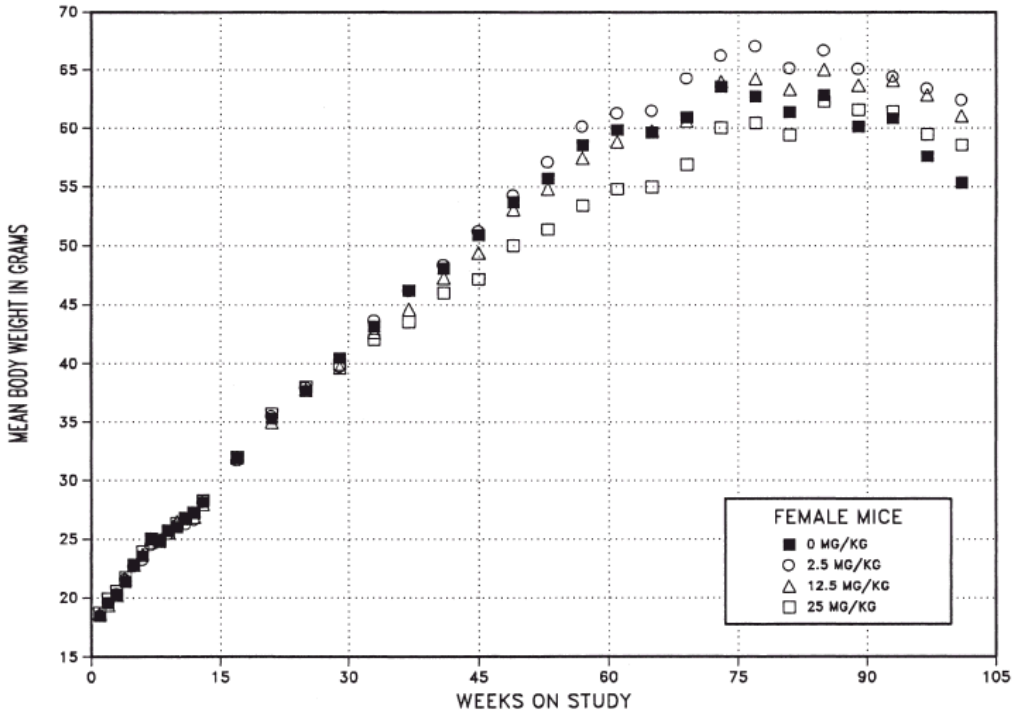


Table 3: Mean body weights for mice in the 2-year carcinogenicity study

Interval	Mean body weight (g)							
	Males				Females			
Dose (mg/kg)	0 Control	2.5	12.5	25	0 Control	2.5	12.5	25
Weeks 1-13	29.3	29.5	28.9	28.9	23.8	23.7	23.8	24.0
Weeks 14-52	47.0	47.2	45.6	45.2	43.0	43.2	42.4	41.6
Weeks 53-101	52.6	52.6	51.7	53.2	59.9	63.4	61.5	58.1

Food Consumption

Not conducted

Hematology

- Hematological responses included anemia in males at 25 mg/kg as indicated by decreases in erythrocytes, hematocrit, and hemoglobin and methemoglobinemia in females at 12.5 mg/kg and males and females at 25 mg/kg as indicated by increases in methemoglobin.
- Heinz bodies were also increased in both males and females at 12.5 and 25 mg/kg methylene blue.

Table 4: Hematology findings for mice in the 2-year carcinogenicity study

Index	Mean		Percentage deviation from Control					
	Control 0 mg/kg		2.5 mg/kg		12.5 mg/kg		25 mg/kg	
	Males	Females	Males	Females	Males	Females	Males	Females
Erythrocytes (10^6/ µL)								
Month 3	10.98	10.35	-	-	-	-	↓8**	-
Month 12	10.02	9.82	-	-	-	-	↓6*	-
Month 18	9.20	9.04	-	-	-	-	-	-
Hematocrit (auto;%)								
Month 3	50.4	48.1	-	-	-	-	↓7**	-
Month 12	46.3	47.6	-	-	-	-	↓5	-
Month 18	41.4	41.9	-	-	-	-	-	-
Hemoglobin (g/dL)								
Month 3	17.0	16.3	-	-	-	-	↓9**	-
Month 12	15.5	15.8	-	-	-	-	↓6	-
Month 18	14.0	14.2	-	-	-	-	-	-
Methemoglobin (g/dL)								
Month 3	0.33	0.41	-	-	-	-	↑58*	-
Month 12	0.38	0.34	-	-	-	-	↑34	↑85**
Month 18	0.22	0.22	-	-	-	↑55*	↑100	↑136**
Reticulocytes (10^5/µL)								
Week 2	4.13	4.26	↑12*	-	-	-	-	-
Month 3	4.37	4.53	-	↑7*	-	↑8*	↑14	↑19**
Month 12	2.75	2.79	↑12	-	↑23**	↑45**	↑54**	↑65**
Month 18	2.72	2.93	↑19	↑40	-	-	↑25	↑10

↑= increase ↓=decrease - = no test-article related changes
* P≤0.05 ** P≤0.01

Index	Mean							
	Control 0 mg/kg		2.5 mg/kg		12.5 mg/kg		25 mg/kg	
	Males	Females	Males	Females	Males	Females	Males	Females
Heinz bodies (%)								
Week 2	0.2	0.1	-	-	-	0.3*	1.2**	5.1**
Month 3	0.0	0.1	-	-	2.2**	1.5**	23.3**	18.6**
Month 12	0.0	0.1	-	-	2.3**	1.0**	13.3**	6.2**
Month 18	0.1	0.2	-	-	5.0**	4.9**	19.8**	22.0**

- = no test-article related changes
* P≤0.05 ** P≤0.01

Urinalysis

Unremarkable

Gross Pathology

Unremarkable

Histopathology

Peer Review: Yes; reviewed by an independent quality assessment laboratory and the NTP Pathology Working Group chairperson

Neoplastic

- The incidences of carcinoma and adenoma or carcinoma (combined) in the small intestine were increased in males treated with methylene blue.
- The incidences of malignant lymphoma were increased at 25 mg/kg in males and 12.5 and 25 mg/kg in females treated with methylene blue.

Table 5: Neoplastic findings for mice in the 2-year carcinogenicity study

Neoplastic findings		No. of animals affected (Percentage of animals affected)							
		Males				Females			
Dose (mg/kg)		0	2.5	12.5	25	0	2.5	12.5	25
Number of animals examined		50	50	50	50	50	50	50	50
Organ/tissue	Finding								
Small intestine	Carcinoma	0 (0%)	1 (2%)	2 (4%)	4 (8%)	1 (2%)	0 (0%)	0 (0%)	0 (0%)
	Adenoma or carcinoma	1 (2%)	2 (4%)	4 (8%)	6 (12%)	1 (2%)	1 (2%)	0 (0%)	0 (0%)
Various	Malignant lymphoma	2 (4%)	2 (4%)	2 (4%)	5 (10%)	6 (12%)	4 (8%)	9 (18%)	12 (24%)

Non Neoplastic

- The incidences of hematopoietic cell proliferation in the spleen were increased at 12.5 and 25 mg/kg methylene blue in both males and females.
- Inflammation of the nose was observed in mice treated with methylene blue.

Table 6: Non neoplastic findings for mice in the 2-year carcinogenicity study

Non-neoplastic findings		No. of animals affected							
		Males				Females			
Dose (mg/kg)		0	2.5	12.5	25	0	2.5	12.5	25
Organ	Finding								
Nose	No. examined	50	50	50	50	50	50	50	50
	Inflammation	1	3	3	6	0	3	7*	11**
Spleen	No. examined	49	50	49	48	47	47	49	50
	Hematopoietic cell proliferation	14	16	25*	29**	23	21	31	40**

* P≤0.05 ** P≤0.01

Toxicokinetics

Not conducted

Dosing Solution Analysis

Dose formulations were prepared every 4 weeks and were analyzed every 3 months. All dose formulations analyzed were within 10% of the target concentrations.

2-year carcinogenicity study in rats

Key Study Findings

- Survival in the methylene blue-treated groups was similar to that of controls.
- Body weights were lower in both males and females treated with 25 and 50 mg/kg methylene blue compared to controls.
- Methylene blue caused pancreatic islet adenomas or carcinomas (combined) in male rats.
- Non-neoplastic findings included hyperplasia in pancreatic islets and focal hyperplasia in the acinus of the pancreas at 25 and 50 mg/kg methylene blue in males and hematopoietic cell proliferation and capsule fibrosis in the spleen at all doses of methylene blue in both males and females.

Methods

Doses:	0, 5, 25, or 50 mg/kg
Frequency of dosing:	Once daily, 5 days per week for 2 years (106 weeks)
Dose volume:	5 mL/kg
Route of administration:	Oral gavage
Formulation/Vehicle:	0.5% aqueous methylcellulose
Basis of dose selection:	Results of 3-month rat study: 50 mg/kg chosen as high dose since it produced some anemia but was not expected to affect longevity in a 2-year study; lowest dose selected because it was within the range of human therapeutic use of methylene blue trihydrate

Species/Strain:	F344/N rats
Number/Sex/Group:	50/sex/group
Age:	6 weeks old at beginning of study
Animal housing:	Males: 3/cage Females: 5/cage
Paradigm for dietary restriction:	Food and water available *ad libitum*
Dual control employed:	None
Interim sacrifice:	Not conducted
Satellite groups:	Hematology: Groups of 10/sex/group were administered the same doses for up to 18 months and were evaluated at 2 weeks, 3, 12, or 18 months for hematology

Observations and times:

Mortality:	Twice daily
Clinical signs:	Observed twice daily Clinical findings recorded monthly (every 4 weeks) beginning Week 5 for main study animals
Body weights:	Day 1, Weekly for first 13 weeks, at 4-week intervals thereafter, and at necropsy for main study animals
Food consumption:	Not conducted
Hematology:	At 2 weeks and 3, 12, and 18 months in satellite groups
Urinalysis:	At 3, 12, and 18 months in 5/sex/group main study animals; placed in metabolism cages for 24-hours for urine collection
Gross pathology:	At necropsy on main study animals
Histopathology:	Conducted for all main study animals

Results

Mortality

Table 7: Survival of rats in the 2-year carcinogenicity study

	Males				Females			
Dose (mg/kg)	0 Control	5	25	50	0 Control	5	25	50
Animals initially in study	50	50	50	50	50	50	50	50
Accidental deaths[a]	0	0	1	2	0	0	0	1
Other[a]	0	0	0	0	0	1	0	0
Moribund	13	7	5	10	9	13	5	3
Natural deaths	6	10	5	7	6	4	9	11
Animals surviving to study termination	31[c]	33	39	31[d]	35	32	36[c]	35
Mean survival (days)[b]	693	694	711	674	696	697	693	658

[a]= Censored from survival analyses by NTP

[b]= Mean of all deaths (uncensored, censored, and terminal sacrifice)

[c]= Includes two animals that died last week of study

[d]= Includes one animal that died last week of study

Clinical Signs

- There was a dose-related increase in the number of animals with eye abnormalities (a combination of various clinical observations) in male rats treated with methylene blue, however, the finding was not observed in females.

Table 8: Clinical signs in rats in the 2-year carcinogenicity study

Clinical sign	No. of animals affected			
	Males			
Dose (mg/kg/day)	0 Control	5	25	50
Number of animals examined	50	50	50	50
Eye abnormalities	2	5	7	8

Body Weights

- Body weights were lower in both males and females treated with 25 and 50 mg/kg methylene blue compared to controls. The mean body weights were lower starting on Week 29 at 25 mg/kg and Week 21 at 50 mg/kg for males and Week 73 at 25 mg/kg and Week 53 at 50 mg/kg for females. At the end of the study (Week 101) the mean weights were 9-13% lower than controls.

Figure 3: Mean body weight in male rats in the 2-year carcinogenicity study
(excerpted from NTP report TR 540 2008)

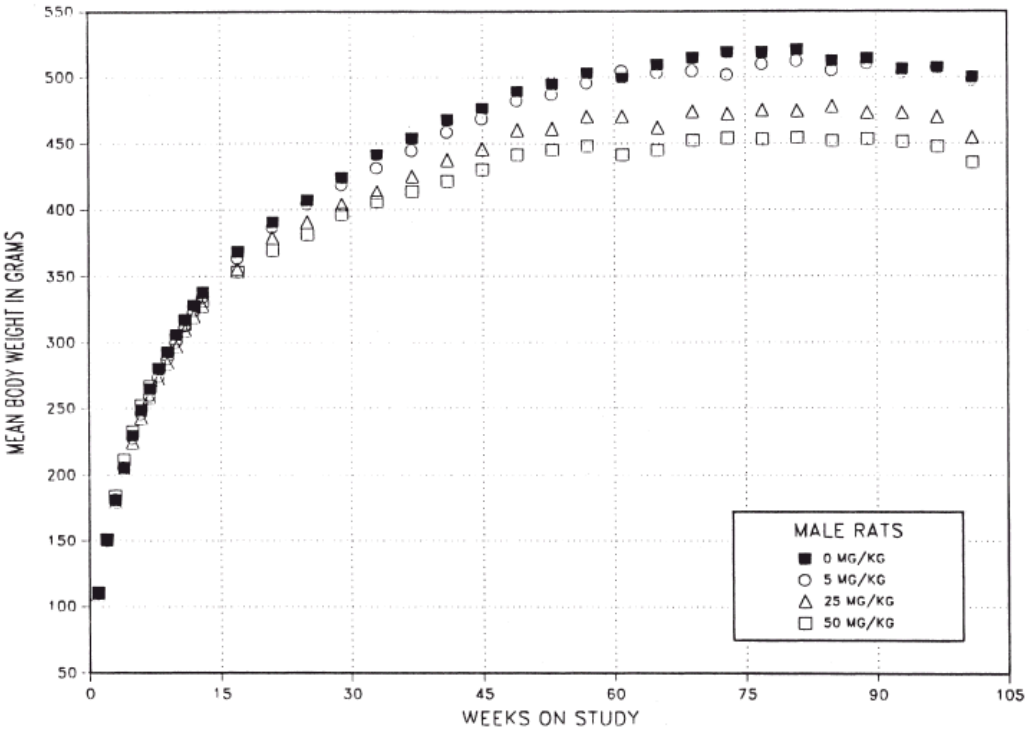


Figure 4: Mean body weight in female rats in the 2-year carcinogenicity study
(excerpted from NTP report TR 540 2008)

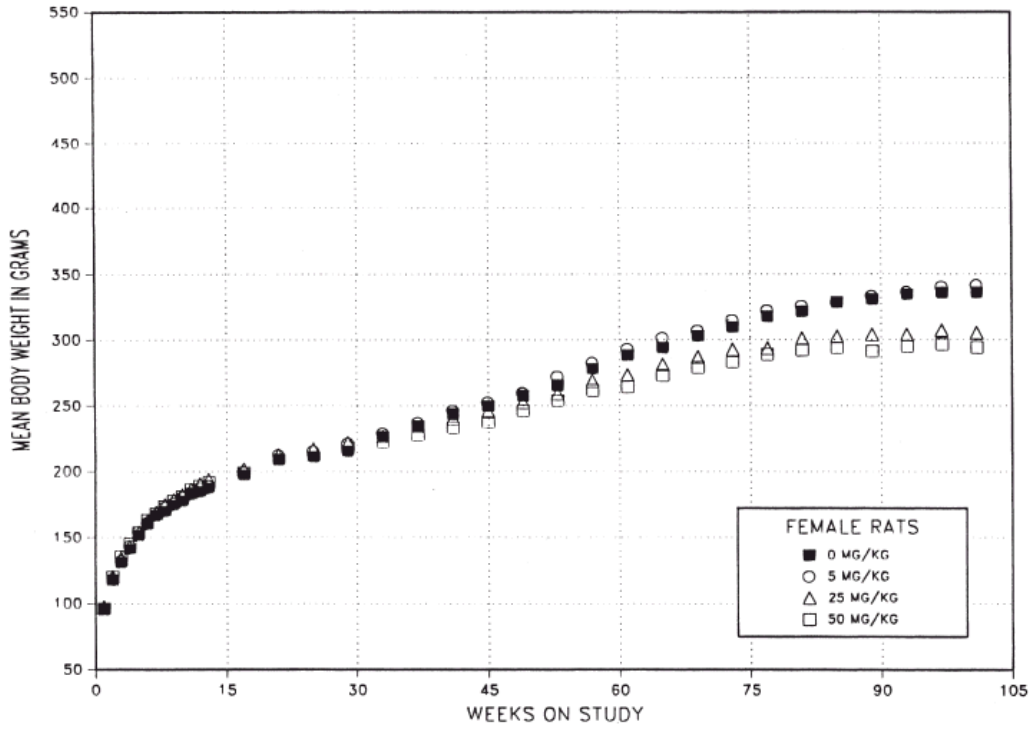


Table 9: Mean body weights for rats in the 2-year carcinogenicity study

Interval	Mean body weight (g)							
	Males				**Females**			
Dose (mg/kg)	0 Control	5	25	50	0 Control	5	25	50
Weeks 1-13	250	247	244	250	157	158	160	160
Weeks 14-52	435	429	412	401	227	230	228	223
Weeks 53-101	509	503	470	449	311	315	291	282

Food Consumption

Not conducted

Hematology

- Hematological responses included anemia as indicated by decreases in erythrocytes, hematocrit, and hemoglobin and methemoglobinemia as indicated by increases in methemoglobin.
- Heinz bodies were also increased in females at 25 and 50 mg/kg methylene blue.

Table 10: Hematology findings for rats in the 2-year carcinogenicity study

Index	Mean		Percentage deviation from Control					
	Control 0 mg/kg		5 mg/kg		25 mg/kg		50 mg/kg	
	Males	Females	Males	Females	Males	Females	Males	Females
Erythrocytes (10^6/ µL)								
Month 3	8.79	8.21	-	-	↓3*	↓3*	↓10**	↓9**
Month 12	8.82	8.27	-	-	↓6**	↓7**	↓9**	↓15**
Month 18	8.22	7.92	-	-	-	↓6**	↓7**	↓11**
Hematocrit (auto;%)								
Month 3	46.0	44.8	-	-	-	-	↓5**	↓4*
Month 12	44.9	46.0	-	-	↓5**	↓4**	↓5**	↓10**
Month 18	46.0	43.7	-	-	-	↓3*	-	↓6**
Hemoglobin (g/dL)								
Month 3	15.5	15.2	-	-	↓3*	-	↓7**	↓7**
Month 12	15.1	15.5	-	-	↓5**	↓5**	↓6**	↓11**
Month 18	15.5	14.9	-	-	-	↓5**	↓5*	↓9**
Methemoglobin (g/dL)								
Month 3	0.21	0.16	-	-	-	-	↑38**	↑69**
Month 12	0.16	0.19	-	-	↑56**	↑47**	↑100**	↑68**
Month 18	0.12	0.14	-	-	↑100**	↑107**	↑175**	↑129**
Reticulocytes (10^5/µL)								
Month 3	3.44	3.10	-	-	-	↑12*	↑41**	↑47**
Month 12	2.44	2.21	-	-	↑25**	↑42**	↑62**	↑120**
Month 18	2.84	2.64	-	-	↑33**	↑33**	↑71**	↑84**
Platelets (10^3/µL)								
Month 3	579.4	623.1	-	-	-	-	↑8	↑8**
Month 12	635.2	602.2	-	-	-	↑15**	↑10*	↑26**
Month 18	547.2	522.8	-	-	-	↑17*	↑18	↑30**

↑= increase ↓=decrease - = no test-article related changes
* P≤0.05 ** P≤0.01

Index	Mean							
	Control 0 mg/kg		5 mg/kg		25 mg/kg		50 mg/kg	
	Males	Females	Males	Females	Males	Females	Males	Females
Heinz bodies (%)								
Month 3	0.4	0.2	0.7	-	0.7	-	-	-
Month 12	0.1	0.1	-	-	-	-	-	-
Month 18	0.1	0.1	-	-	-	0.4**	-	7.1**

- = no test-article related changes
** P≤0.01

Urinalysis

Unremarkable

Gross Pathology

Unremarkable

Histopathology

Peer Review: Yes; reviewed by an independent quality assessment laboratory and the NTP Pathology Working Group chairperson

Neoplastic

- The incidences of adenoma and adenoma or carcinoma (combined) in the pancreatic islets were increased in males treated with methylene blue.

Table 11: Neoplastic findings for rats in the 2-year carcinogenicity study

Neoplastic findings		No. of animals affected (Percentage of animals affected)							
		Males				**Females**			
Dose (mg/kg)		0	5	25	50	0	5	25	50
Number of animals examined		50	50	50	50	49	48	48	49
Organ/tissue	Finding								
Pancreatic islets	Adenoma	4 (8%)	9 (18%)	12 (24%)	8 (16%)	2 (4%)	0 (0%)	1 (2%)	1 (2%)
	Adenoma or carcinoma	4 (8%)	9 (18%)	14 (28%)	8 (16%)	2 (4%)	0 (0%)	1 (2%)	1 (2%)

Non Neoplastic

- The incidences of hyperplasia in pancreatic islets and focal hyperplasia in the acinus of the pancreas were increased in males treated with 25 and 50 mg/kg methylene blue.
- The incidences of hematopoietic cell proliferation and capsule fibrosis in the spleen were increased at all doses of methylene blue in both males and females.

Table 12: Non neoplastic findings for rats in the 2-year carcinogenicity study

Non-neoplastic findings		No. of animals affected							
		Males				Females			
Dose (mg/kg)		0	5	25	50	0	5	25	50
Number of animals examined		50	50	50	50	49	48	48/49‡	49
Organ	Finding								
Pancreas	Hyperplasia in pancreatic islets	13	13	17	26**	13	15	15	15
	Hyperplasia (focal), acinus	4	6	15**	12*	3	1	2	1
Spleen	Hematopoietic cell proliferation	11	12	17	20*	3	5	7	8
	Capsule fibrosis	1	7*	12**	30**	8	17*	12	20**

‡ = 48 pancreatic islets were examined and 49 spleens were examined

* P≤0.05 ** P≤0.01

Toxicokinetics

Not conducted

Dosing Solution Analysis

- Dose formulations were prepared every 4 weeks and were analyzed every 3 months. All dose formulations analyzed were within 10% of the target concentrations.

Conclusions for 2-year mouse and rat carcinogenicity studies

In the NTP report for the 2-year carcinogenicity studies in the mouse and rat, the conclusion was made that there was some evidence of carcinogenic activity of methylene blue based on increased incidences of pancreatic islet cell adenoma and adenoma or carcinoma (combined) in male rats and increased incidences of carcinoma and adenoma or carcinoma in the small intestine in male mice. The increased incidence of malignant lymphoma in male and female mice was considered possibly related to methylene blue trihydrate administration.

A statistical review of the NTP carcinogenicity studies for methylene blue trihydrate was performed by the FDA, with a particular focus on the findings that were reported in the NTP report. A statistical review was conducted by Dr. Karl Lin of the Division of Biometrics 6 in the Office of Biostatistics. Below is a table from the review with a summary of the FDA and NTP analysis results.

Table 13: Summary of FDA and NTP analysis results of the six selected tumor types

(excerpted from statistical review for carcinogenicity studies)

Species	Gender	Tumor Type (Numbers of Tumor Bearing Animals)	One-Sided P-Value			
			NTP Survival-adjusted Poly-3 Test		FDA Survival-unadjusted Exact Permutation Test	
			Trend Test	Control-High Pairwise Test	Trend Test	Control-High Pairwise Test
Rats	Males	Pancreatic Islets Adenoma (4, 9, 12, 8)	0.201	0.155	0.212	0.178
Rats	Males	Pancreatic Islets Adenoma + Carcinoma (4, 9, 14, 8)	0.174	0.155	0.191	0.178
Mice	Males	Small Intestine Carcinoma (0, 1, 2, 4)	0.027	0.071	0.018	0.059
Mice	Males	Small Intestine Adenoma+ Carcinoma (1, 2, 4, 6)	0.029	0.071	0.019	0.056
Mice	Males	Malignant Lymphoma (2, 2, 2, 5)	0.126	0.250	0.136	0.218
Mice	Females	Malignant Lymphoma (6, 4, 9, 12)	0.025	0.126	0.027	0.096

The same type of statistical tests was used for both the NTP and FDA analyses. The NTP analyses were adjusted for survival. Since survival was comparable between the methylene blue-treated mice and rats and controls, the FDA analyses were unadjusted for survival. The values for the NTP and FDA analyses are similar. According to the percentage of animals in the current control groups with each tumor type, small intestine carcinoma in male mice was rare, while small intestine adenoma or carcinoma (combined) in male mice, malignant lymphoma in male or female mice, and pancreatic islet adenoma and adenoma or carcinoma (combined) in male rats were common tumors.

Based on these analyses and the FDA criteria for a positive carcinogenicity response presented below, FDA's conclusion is that there are no statistically significant neoplastic findings in the 2-year mouse carcinogenicity study. Additional information was taken into consideration for the finding of pancreatic islet adenoma or carcinoma (combined) in male rats. According to the historical incidence for all routes of administration provided in the NTP report, adenoma or carcinoma (combined) in pancreatic islets has been observed in 92/1,448 (6.4%) of controls, with a mean of 6.8% and a range of 0-14%. Further, the incidences of hyperplasia in pancreatic islets were also increased in males treated with 25 and 50 mg/kg methylene blue. Although the findings were not statistically significant, the FDA concluded that methylene blue caused pancreatic islet

adenomas or carcinomas (combined) in male rats based on the incidences exceeding the historical control incidence, particularly at the mid dose of 25 mg/kg.

Table 14: FDA criteria for positive carcinogenicity response

Rare Tumors <1% in control/historical		Common Tumors (>1% control/historical)	
Trend Analysis	Pairwise Analysis	Trend Analysis	Pairwise Analysis
P<0.025	P<0.05	P<0.005	P<0.01
Both should be statistically significant in order to consider it a positive		Both should be statistically significant in order to consider it a positive	

The results of the 2-year mouse and rat carcinogenicity studies were presented to the Executive CAC on March 18, 2014 and the following recommendations and conclusions were made:

Rat:

- The Committee concurred that the study was acceptable, although daily dosing would have been more representative of any future chronic usage.

- The Committee concurred that pancreatic islet adenomas or carcinomas (combined) in the males were drug related because the incidence exceeded the historical control, particularly in the mid dose group. Furthermore, it was noted that the animals were not dosed on weekends (and thus had time to recover from drug-related effects). The terminal half-life of the drug is reported to be around 5 hours.

Mouse:

- The Committee concurred that the study was acceptable.

- The Committee concurred that there were no drug-related neoplasms in the study.

Histopathology inventory for 2-year carcinogenicity studies

Study	NTP report TR 540	NTP report TR 540
Species	Mouse	Rat
Adrenals	X	X
Aorta	X	X
Bone Marrow smear	X	X
Bone	X	X
Brain	X	X
Cecum	X	X
Cervix		
Clitoral gland	X	X
Colon	X	X
Duodenum	X	X
Epididymis	X	X
Esophagus	X	X
Eye		
Fallopian tube		
Gall bladder	X	
Gross lesions	X	X
Harderian gland	X	X
Heart	X	X
Ileum	X	X
Injection site		
Jejunum	X	X
Kidneys	X	X
Lacrimal gland		
Larynx		
Liver	X	X
Lungs	X	X
Lymph nodes, cervical		
Lymph nodes mandibular	X	X
Lymph nodes, mesenteric	X	X
Mammary Gland	X	X

Study	NTP report TR 540	NTP report TR 540
Species	Mouse	Rat
Masses in tissue	X	X
Nasal cavity	X	X
Optic nerves		
Ovaries	X	X
Pancreas	X	X
Parathyroid	X	X
Peripheral nerve		
Pharynx		
Pituitary	X	X
Preputial gland	X	X
Prostate	X	X
Rectum	X	X
Salivary gland	X	X
Sciatic nerve		
Seminal vesicles	X	X
Skeletal muscle		
Skin	X	X
Spinal cord		
Spleen	X	X
Sternum		
Stomach	X	X
Teeth		
Testes	X	X
Thymus	X	X
Thyroid	X	X
Tongue		
Trachea	X	X
Urinary bladder	X	X
Uterus	X	X
Vagina		
Zymbal gland		

X, histopathology performed

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/s/

BRENDA J GEHRKE
03/25/2014

HALEH SABER
03/25/2014

PHARMACOLOGY/TOXICOLOGY FILING CHECKLIST FOR NDA

NDA/BLA Number: 204630 **Applicant: Provepharma SAS** **Stamp Date: 9/13/2013**

Drug Name: Methylene blue **NDA Type: 505 (b) (2)**

On **initial** overview of the NDA application for filing:

	Content Parameter	Yes	No	Comment
1	Is the pharmacology/toxicology section organized in accord with current regulations and guidelines for format and content in a manner to allow substantive review to begin?	X		
2	Is the pharmacology/toxicology section indexed and paginated in a manner allowing substantive review to begin?	X		
3	Is the pharmacology/toxicology section legible so that substantive review can begin?	X		
4	Are all required (*) and requested IND studies (in accord with 505 b1 and b2 including referenced literature) completed and submitted (carcinogenicity, mutagenicity, teratogenicity, effects on fertility, juvenile studies, acute and repeat dose adult animal studies, animal ADME studies, safety pharmacology, etc)?	X		
5	If the formulation to be marketed is different from the formulation used in the toxicology studies, have studies by the appropriate route been conducted with appropriate formulations? (For other than the oral route, some studies may be by routes different from the clinical route intentionally and by desire of the FDA).	X		In some animal studies, oral route of administration is used. However, (b) (4) Injection was intravenously administered in dogs in the bridging repeat dose toxicology study (by the Applicant). There are human data with IV dosing (with methylene blue of slightly different formulation than (b) (4) Injection). The acceptability of these data is a review issue.
6	Does the route of administration used in the animal studies appear to be the same as the intended human exposure route? If not, has the applicant submitted a rationale to justify the alternative route?	X		See above.
7	Has the applicant submitted a statement(s) that all of the pivotal pharm/tox studies have been performed in accordance with the GLP regulations (21 CFR 58) or an explanation for any significant deviations?	X		It is not known whether studies conducted by NTP or studies in reference literatures are GLP studies.
8	Has the applicant submitted all special studies/data requested by the Division during pre-submission discussions?	X		

File name: 5_Pharmacology_Toxicology Filing Checklist for NDA_BLA or Supplement 010908

PHARMACOLOGY/TOXICOLOGY FILING CHECKLIST FOR NDA

	Content Parameter	Yes	No	Comment
9	Are the proposed labeling sections relative to pharmacology/toxicology appropriate (including human dose multiples expressed in either mg/m2 or comparative serum/plasma levels) and in accordance with 201.57?	X		For animal-to-human comparisons, the Applicant used a direct dose-to-dose conversion. This may not be appropriate as the routes of administration are different (oral in animals vs IV in humans). An IR may be sent to the Applicant later during the NDA review.
10	Have any impurity – etc. issues been addressed? (New toxicity studies may not be needed.)	X		According to the Applicant the specifications of impurity levels in the drug substance (in particular, (b) (4) and drug product are in line with ICH guidance. The acceptance of the content of impurities will be determined during the review of the submission.
11	Has the applicant addressed any abuse potential issues in the submission?			Not applicable.
12	If this NDA/BLA is to support a Rx to OTC switch, have all relevant studies been submitted?			Not applicable.

IS THE PHARMACOLOGY/TOXICOLOGY SECTION OF THE APPLICATION FILEABLE? ____Yes____

If the NDA/BLA is not fileable from the pharmacology/toxicology perspective, state the reasons and provide comments to be sent to the Applicant.

Please identify and list any potential review issues to be forwarded to the Applicant for the 74-day letter.

Brenda J. Gehrke, Ph.D. November 12, 2013

Reviewing Pharmacologist Date

Haleh Saber, Ph.D. November 12, 2013

Team Leader/Supervisor Date

File name: 5_Pharmacology_Toxicology Filing Checklist for NDA_BLA or Supplement 010908

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/s/

BRENDA J GEHRKE
11/12/2013

HALEH SABER
11/12/2013