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FINAL REPORT

Laboratory Project ID [redacted]

Sponsor Reference No. [redacted]

A Combined *In Vivo* Micronucleus and Comet Assay of [redacted] in Sprague Dawley Rats

Test Guidelines: OECD 474 and 489

Author: Michael S. Cockburn

SPONSOR:

[redacted]

PERFORMING LABORATORY:
Charles River Laboratories
Ashland, LLC
1407 George Road
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21 November 2019

COMPLIANCE STATEMENT AND REPORT APPROVAL

The following is a detailed description of all differences between the practices used in this study and those required by the United States Code of Federal Regulations, Title 40, Parts 160 and 792: Good Laboratory Practice Standards and as accepted by Regulatory Authorities throughout the European Union (OECD Principles of Good Laboratory Practice), Japan (MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement:

- Characterization of the test substance was performed by the Sponsor according to established SOPs, controls, and approved test methods to ensure integrity and validity of the results generated; these analyses were not conducted in compliance with the GLP regulations.

Date: 21 NOV 2019

Typed Name of Signer: Michael S. Cockburn

Typed Name of Laboratory: Charles River Laboratories Ashland, LLC

[redacted] Report.pdf MD5 Checksum: E17E5771E783BDEEE01E7BC56AFA7DFA

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QUALITY ASSURANCE STATEMENT

Study Number: [redacted]

This study has been audited by Quality Assurance in accordance with the applicable Good Laboratory Practice regulations. Reports were submitted in accordance with SOPs as follows:

QA INSPECTION DATES

		Dates Findings Submitted to:	
Date(s) of Audit	**Phase(s) Audited**	**Study Director**	**Study Director Management**
21-Jan-2019	Dose Administration	21-Jan-2019	21-Jan-2019
13-Feb-2019 - 14-Feb-2019	Final Protocol	14-Feb-2019	14-Feb-2019
08-Mar-2019	Data Review - Clinical Pathology	08-Mar-2019	08-Mar-2019
08-Mar-2019	Data Review - Necropsy	08-Mar-2019	08-Mar-2019
08-Mar-2019	Data Review - Technical Operations	08-Mar-2019	08-Mar-2019
08-Mar-2019	Data Review - Formulations	08-Mar-2019	08-Mar-2019
11-Mar-2019	Data Review - Technical Operations	11-Mar-2019	11-Mar-2019
11-Mar-2019	Inhalation Technical Report	11-Mar-2019	11-Mar-2019
01-Apr-2019	Data Review - Flow Cytometry	01-Apr-2019	01-Apr-2019
10-May-2019	Protocol Amendment 01	10-May-2019	10-May-2019
10-May-2019	Protocol Amendment 02	10-May-2019	10-May-2019
09-Jul-2019	Data Review - Formulations	09-Jul-2019	09-Jul-2019
09-Jul-2019	Data Review - Necropsy	09-Jul-2019	09-Jul-2019
09-Jul-2019	Data Review - Technical Operations	09-Jul-2019	09-Jul-2019
09-Jul-2019	Data Review - Technical Operations	09-Jul-2019	09-Jul-2019
09-Jul-2019	Inhalation Technical Report	09-Jul-2019	09-Jul-2019
11-Jul-2019 - 12-Jul-2019	Report	12-Jul-2019	12-Jul-2019
18-Nov-2019	Final Report	18-Nov-2019	18-Nov-2019

In addition to the above-mentioned audits, process-based and/or routine facility inspections were also conducted during the course of this study. Inspection findings, if any, specific to this study were reported by Quality Assurance to the Study Director and Management and listed as a Phase Audit on this Quality Assurance Statement.

The Quality Assurance Statements for the work conducted at the Test Sites were reviewed and are included in the appropriate section of this report.

The Final Report has been reviewed to assure that is accurately describes the materials and methods, and that the reported results accurately reflect the raw data.

Date: 21 Nov 2019

Dustin Risner, BA
Quality Assurance Auditor

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1. RESPONSIBLE PERSONNEL

1.1. Testing Facility

Study Director	Michael S. Cockburn
Site Director	Erica L. Lashley, MBA, BS, LAT
Scientific Report Review	James M. Randazzo, PhD, DABT
Archivist and Supervisor, Archives	Brooke N. Coblentz, BS
Director, Laboratory Animal Medicine	Jessica N. Keen, MS, DVM, DACLAM
Director, Operations	Tammye L. Edwards, BS
Director, Specialty Toxicology and Scientific Services	JoAnna Bultman, PhD, DABT
Senior Director, Laboratory Sciences	Elizabeth A. Groeber, PhD, MBA

1.2. Principal Investigators (PI) at Sponsor or Sponsor-Designated Test Site(s)

Micronucleus Assay	Megan Young, PhD BioReliance Corporation, Rockville, MD
Comet Assay	Shannon Bruce, MFS BioReliance Corporation, Rockville, MD

2. SUMMARY

The objective of this study was to assess the potential of the test substance, [redacted], to induce micronuclei and/or to cause DNA damage in rat liver, lung, kidney, and nasal tissue when administered via nose-only inhalation to Sprague Dawley rats for 6 hours per day for up to 3 consecutive days.

The study design was as follows:

Text Table 1
Experimental Design – Phase 1

Group Number	Treatment	Target Exposure Concentrations (ppm)	Positive Control Concentration (mg/mL)	Positive Control Dose Volume (mL/kg)	Number of Animals[a,b]	
					Males	Females
1	Filtered Air	0	-	-	6	6
2	[redacted]	375	-	-	6	6
3	[redacted]	750	-	-	6	6
4	[redacted]	1500	-	-	6	6
5	Positive Control[c]	CP: 20 mg/kg/day	2	10	6	6
		EMS: 200 mg/kg/day	20	10		

CP = Cyclophosphamide monohydrate; EMS = Ethyl methanesulfonate; - = Not applicable.
[a] Filtered air (negative control group) and test substance were administered via nose-only inhalation for 3 consecutive days (6 hours per day) on Days 1–3.
[b] Up to 6 surviving rats/group were utilized for collection of peripheral blood between 1 and 2 hours following the final exposure/dose. Additionally, for 5 surviving rats/group, samples of bone marrow in addition to samples of the liver, lung, kidney, and nasal tissue were collected between 2 and 4 hours after completion of the final exposure (Groups 1–4) or after the second dose of EMS (Group 5), which coincided with being approximately 18–24 hours after the second dose of CP.
[c] CP was administered via oral gavage on Days 1 and 2. EMS was administered via oral gavage on Days 2 and 3.

Text Table 2
Experimental Design – Phase 2

Group Number	Treatment	Target Exposure Concentrations (ppm)	Positive Control Concentration (mg/mL)	Positive Control Dose Volume (mL/kg)	Number of Males[a,b]
1	Filtered Air	0	-	-	6
2	[redacted]	375	-	-	6
3	[redacted]	750	-	-	6
4	[redacted]	1500	-	-	6
5	Positive Control[c]	EMS: 200 mg/kg/day	20	10	6

EMS = Ethyl methanesulfonate; - = Not applicable.
[a] Filtered air (negative control group) and test substance were administered via nose-only inhalation for 2 consecutive days (6 hours per day) on Days 1–2.
[b] For 5 surviving rats/group, samples of the liver, lung, kidney, and nasal tissue were collected between 2 and 4 hours after completion of the final exposure (Groups 1–4) or after the second dose of EMS (Group 5).
[c] EMS was administered via oral gavage on Days 1 and 2.

Animals were administered filtered air or [redacted] via nose-only inhalation exposures. For Phase 1, the test substance or filtered air was administered to males and females once daily for 3 consecutive days, for 6 hours per day. For the positive control group (Group 5), cyclophosphamide monohydrate (CP) was administered via oral gavage on Days 1 and 2, and ethyl methanesulfonate (EMS) was administered via oral gavage on Days 2 and 3. Due to the lack of a positive response in the positive control group of the comet assay (resulting in an invalid assay), a second phase was conducted for only the comet endpoint. For Phase 2, the test substance or filtered air was administered to only males once daily for 2 consecutive days, for 6 hours per day. For the positive control group (Group 5), ethyl methanesulfonate (EMS) was administered via oral gavage on Days 1 and 2. Achieved mean analyzed exposure concentrations in the 375, 750, and 1500 ppm groups were 381, 753, and 1501 ppm, respectively, for Phase 1, and 375, 741, and 1494 ppm, respectively, for Phase 2.

The following parameters and end points were evaluated in this study: clinical signs, body weights, body weight gains, food consumption, micronucleus evaluation (bone marrow; Phase 1 only), and in vivo comet assay (liver, lung, kidney, and nasal tissue).

In Phase I, all animals survived to the scheduled euthanasia. There were no test substance-related clinical observations noted for males and females at any exposure concentration. Test substance-related body weight losses were noted in the 750 and 1500 ppm group males and 375, 750, and 1500 ppm group females during the exposure period (Days 1–3). In addition, test substance-related lower food consumption was noted for males and females at all exposure concentrations during the exposure period (Days 1–3).

For Phase I, there was no significant increase in the number of micronuclei in the test substance-exposed animals compared to the filtered air controls in both males and females. The filtered air control values were compatible with the expected range of percent micronucleated reticulocytes (%MnRETs). There was a statistically significant increase in MnRETs in the positive control group as compared to the concurrent control group. All criteria for a valid assay were met. Under the conditions of this study, the administration of [redacted] at exposure concentrations up to and including 1500 ppm was concluded to be negative in the micronucleus assay.

In Phase 2, all animals survived to the scheduled euthanasia. There were no clinical observations noted for males at any exposure concentration. A test substance-related body weight loss was noted in the 1500 ppm group males during the exposure period (Days 1–2). In addition, test substance-related lower food consumption was noted for males at all exposure concentrations during the exposure period (Days 1–2); however, these differences did not occur in an exposure-related manner.

During Phase 1 of the assay (both sexes), the test substance, [redacted], could be evaluated as negative (non-DNA damaging) in male liver cells only. For the remaining tissues tested, the assay did not meet all the acceptance criteria (especially as related to the positive control treatment), therefore was considered invalid. During Phase 2 (males only), the test substance has been evaluated as negative (non-DNA damaging) during the in vivo alkaline Comet assay; all valid assay criteria were met, for all tissues tested.

Based on the results of this study, exposure of male and female Crl:CD(SD) rats to [redacted] via nose-only inhalation for 6 hours per day for up to 3 consecutive days at target

exposure concentrations of 375, 750, and 1500 ppm resulted in a negative response for induction of bone marrow nuclei and induction of DNA damage in the liver, lung, kidney, and nasal cavity.

3. INTRODUCTION

The objective of this study was to assess the potential of the test substance, [redacted], to induce micronuclei and/or to cause DNA damage in rat liver, lung, kidney, and nasal tissue when administered via nose-only inhalation to Sprague Dawley rats for 6 hours per day for up to 3 consecutive days.

The micronucleus assay is a test for clastogenic agents that interfere with normal mitotic cell division.[1] Micronuclei (MN) are small chromatin bodies consisting of entire chromosomes and/or acentric chromosome fragments that lag behind at mitotic anaphase. At telophase, these chromosomes and/or fragments are not segregated to either daughter nucleus and form single or multiple MN in the cytoplasm. During the maturation of erythroblasts to erythrocytes, the nucleus is extruded. MN, if present persist in the cytoplasm of these anucleate cells. Aneugens that affect spindle fiber function or formation, as well as clastogenic agents, can be detected through MN induction.[2] In this study, bone marrow was be analyzed for the presence of micronucleated PCEs (MN-PCEs).

The in vivo alkaline comet (single cell gel electrophoresis) assay (referred to as the comet assay) is used for the detection of single and double stranded DNA breaks in cells or nuclei isolated from multiple tissues of animals that have been exposed to potentially genotoxic material(s). In this study, single cells embedded in agarose on a slide were lysed with detergent and high salt concentration. This lysis step digests the cellular and nuclear membranes and allows the release of coiled DNA loops, generally called nucleoids and DNA fragments. Electrophoresis under alkaline conditions (pH > 13) results in structures resembling comets, which, by using appropriate fluorescent stains, can be observed by fluorescence microscopy. DNA fragments migrate away from the "head" into the "tail" based on their size, and the intensity of the comet tail relative to the total intensity (head plus tail) reflects the amount of DNA breakage.

The design of this study is based on OECD Guidelines 474 and 489.

The study protocol, the last amended study protocol, and deviations are presented in Appendix 1.

Study Initiation Date:	19 Dec 2018
Experimental Start Date:	19 Jan 2019
Initiation of Dosing (Phase 1):	19 Jan 2019
Completion of In-life (Phase 1):	22 Jan 2019
Initiation of Dosing (Phase 2):	20 May 2019
Completion of In-Life (Phase 2):	21 May 2019
Experimental Termination Date:	06 Jun 2019

4. MATERIALS AND METHODS

4.1. Test Substance, Positive Control Substances, and Positive Control Substance Vehicles

4.1.1. Test Substance

Identification: [redacted]

Lot No.:	[redacted]
Receipt Date:	15 Nov 2018
Expiration Date:	March 2020
Physical Description:	Clear, colorless gas
Purity:	98.95%
Water Content:	0.0021%
Correction Factor:	None
Storage Conditions:	Kept in a room with controls set to maintain 18°C to 24°C, protected from light
Supplier:	[redacted]

4.1.2. Positive Control Substance 1

Identification:	Cyclophosphamide monohydrate (CP; CAS No. 6055-19-2) is a known clastogen
Batch No.:	MKCG5464
Receipt Date:	15 Nov 2018
Retest Date:	May 2021
Physical Description:	White powder
Purity:	99.1%
Water Content:	5.6%
Storage Conditions:	Kept in a refrigerator set to maintain a target of 5°C

4.1.3. Positive Control Substance 2

Identification:	Ethyl methanesulfonate (EMS; CAS No. 62-50-0) is a known substance that induces DNA strand breaks
Batch Nos.:	BCBS6100V and BCBW8635
Receipt Dates:	10 May 2017 and 04 Jan 2019, respectively
Retest Date:	10 May 2022 and 04 Jan 2024, respectively
Physical Description:	Clear, colorless liquid
Storage Conditions:	Kept in a room with controls set to maintain 18°C to 24°C, purged with nitrogen, with desiccant

4.1.4. Positive Control Substance Vehicle 1

Identification: Deionized water

4.1.5. Positive Control Substance Vehicle 2

Identification: 0.9% sodium chloride for injection, USP

Lot Nos.:	X49930 and AF5407
Receipt Dates:	16 Oct 2018 and 22 Apr 2019, respectively
Expiration Dates:	01 Jun 2020 and 01 Sep 2020, respectively
Physical Description:	Clear, colorless liquid
Storage Conditions:	Kept in a room with controls set to maintain 18°C to 24°C

4.2. Test Substance Characterization

The Sponsor provided to the Testing Facility documentation of the identity, strength, purity, composition, and stability for the test substance. A Certificate of Analysis was provided to the Testing Facility and is presented in Appendix 2.

4.3. Reserve Samples

Reserve samples were not collected for this study.

4.4. Test Substance Inventory and Disposition

Records of the receipt, distribution, and storage of test substance were maintained. All unused test substance was returned to the Sponsor.

4.5. Inhalation Exposure Methods

Filtered air (control group) and test substance atmospheres of [redacted] were administered as 6-hour, nose-only inhalation exposures for up to 3 consecutive days.

Exposures were conducted using (7.9-L) stainless steel, nose-only systems with grommets in exposure ports to engage animal holding tubes for Phase 1 and 0.74-L 12-port module CH technologies flow-past (directed-flow) nose-only exposure system for Phase 2.

Air supplied to the nose-only systems was provided from the Inhalation Department breathing quality, in-house compressed air source and a HEPA- and charcoal-filtered, temperature- and humidity-controlled supply air source. All nose-only system exhaust passed through the facility exhaust system, which consists of redundant exhaust blowers preceded by activated-charcoal and HEPA-filtration units. Details of inhalation exposure methods are presented in Appendix 3.

All animals were housed in an animal colony room during non-exposure hours. Prior to each exposure, the animals were placed into nose-only exposure restraint tubes in the colony room and transported to the exposure room. Animals were then placed on the nose-only exposure system, exposed for the requisite duration (up to 6 hours), and returned to their home cages in the animal colony room. Food and water were withheld during the animal exposure periods.

The mean temperature and mean relative humidity of the exposure atmospheres were 22 ± 3ºC and 50 ± 20%, respectively. Oxygen content of the exposure atmospheres was measured during the method development phase and was 20.9% for all groups for both phases of the study.

4.6. Exposure Atmosphere Generation Methods

4.6.1. Control Exposure System

The control exposure system (0 ppm) was operated as follows. Supply air was delivered to the nose-only exposure system using a rotameter-type flowmeter.

4.6.2. Test Substance Exposure Systems

Test substance atmosphere was generated by releasing the test substance gas from the original cylinder. The test substance was delivered from the cylinder to a stainless steel manifold, where it was distributed to each exposure system. The test substance was directed to the inlet of each exposure system, where it was mixed with supply air to achieve the desired atmosphere concentrations. The test substance passed through a 3-way valve prior to mixing with supply air at the nose-only system inlet. Initiation of the bypass directed test substance to the facility exhaust. This allowed for continual flow of the test substance from the original cylinder in the instance where generation was needed when one (or more) of the exposure systems generation needed to be bypassed due to staggering of the exposures. Additional details of exposure atmosphere generation methods are presented in Appendix 3.

4.7. Methods of Characterization of Exposure Atmospheres

4.7.1. Nominal Exposure Concentrations

Nominal exposure concentrations were not calculated for this study.

4.7.2. Analyzed Exposure Concentrations

Analyzed concentrations of [redacted] in the exposure atmospheres was determined using a gas chromatograph (GC) equipped with a Flame Ionization Detector (FID). Samples were collected from the approximate animal-breathing zone of the exposure system. Under the control of the WINH system, sampling and analyses were performed as follows: The program controlled an external multi-position valve that permitted sequential sampling from the exposure room and each test substance exposure system. Manual bag samples were collected from Exposure System 1 (Control Group) using a Metal Bellows sampling pump and 10-L Tedlar® gas bags. Manual bag sampling was performed due to the location of the control exposure system being located in Exposure Room 6 for Phase I, separate from Room 3 where the GC was located. The manual bag samples were analyzed on the GC during each sample round for Exposure System 1. For Phase II, all exposure systems were located in Exposure Room 1 where system the sampling was performed.

Gas sampling injection onto the chromatography column occurred via an internal gas-sampling valve with a sample loop, the chromatograph was displayed, and the area under the sample peak was calculated and stored. The WINH system acquired the stored peak area data and used an ln-quadratic equation based on the GC calibration curve to calculate the measured concentration in ppm. Additional details of the exposure atmosphere characterization methods are presented in Appendix 3 and the results are summarized in Section 8.1.

4.8. Preparation of Positive Control Substances

CP dosing formulations were prepared on each day of administration as a weight-to-volume mixture in deionized water. The dosing formulations were stirred continuously on ice during dosing. Details of the preparation and dispensing of positive control substance 1 have been retained in the Study Records.

EMS dosing formulations were prepared on each day of administration as a weight-to-volume mixture in 0.9% sodium chloride for injection, USP. The dosing formulations were maintained at room temperature (18°C to 24°C). Details of the preparation and dispensing of positive control substance 2 have been retained in the Study Records.

4.9. Test System

4.9.1. Receipt

On 08 Jan 2019 (Phase 1) and 09 May 2019 (Phase 2), Crl:CD(SD) rats were received from Charles River Laboratories, Inc., Raleigh, NC. The animals were approximately 8 weeks old and weighed between 167 and 246 g (Phase 1) and between 222 and 282 g (Phase 2) at the initiation of dosing.

4.9.2. Justification for Test System and Number of Animals

The Crl:CD(SD) rat was chosen as the animal model for this study as it is an accepted rodent species for nonclinical toxicity testing by regulatory agencies.

The total number of animals used in this study was based on OECD Guidelines 474 and 489. Group size at the initiation of the study (up to 6/sex/group) was chosen to provide a minimum of 5 analyzable samples/sex/group for each endpoint. Because no difference in systemic toxicity was noted between males and females in Phase 1, only males were used for Phase 2.

At this time, studies in laboratory animals provide the best available basis for extrapolation to humans and are required to support regulatory submissions. Acceptable models which do not use live animals currently do not exist.

4.9.3. Animal Identification

Upon receipt, each animal was identified using a subcutaneously implanted electronic identification chip (BMDS system).

4.9.4. Environmental Acclimation

After receipt at the Testing Facility, the Crl:CD(SD) rats were acclimated prior to initiation of dosing. To screen animals for poor tolerance to restraint and to limit potential effects on respiration of the novel environment/conditions of restraint, the animals were subjected to restraint in nose-only exposure tubes. Animals were acclimated to restraint tubes 4 times (1 acclimation/day) prior to their first day of exposure. Animals were acclimated to restraint in nose-only exposure restraint tubes by increasing the restraint time over the pretreatment period (first day – 1 hour, second day – 2 hours, third day – 3 hours, and fourth day – 6 hours; times were approximate). Following the restraint period, each animal was observed for clinical signs of injury or stress.

4.9.5. Selection, Assignment, and Disposition of Animals

Animals were assigned to groups by a stratified randomization scheme designed to achieve similar group mean body weights. Males and females were randomized separately. Animals in poor health were not assigned to groups.

The disposition of all animals was documented in the Study Records.

4.9.6. Husbandry

4.9.6.1. Housing

On arrival, animals were group housed (2 to 3 animals of the same sex) until randomization. Following randomization, animals were group housed (2 animals of the same sex and same dosing group together) in solid-bottom cages containing appropriate bedding equipped with an automatic watering valve.

Animals were separated during designated procedures/activities. Each cage was clearly labeled with a cage card indicating study number, group number, cage number, dosage level/exposure concentration, animal number(s), and sex. Cages were arranged on the racks in group order.

Animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals.*[3] The animal facilities at Charles River Ashland are accredited by AAALAC International.

4.9.6.2. Environmental Conditions

Target temperatures of 68°F to 78°F (20°C to 26°C) with a relative target humidity of 30% to 70% were maintained. A 12 hour light/12 hour dark cycle was maintained, except when interrupted for designated procedures. Ten or greater air changes per hour with 100% fresh air (no air recirculation) were maintained in the animal rooms.

4.9.6.3. Food

PMI Nutrition International, LLC Certified Rodent LabDiet 5CR4 meal was provided ad libitum throughout the study, except during designated procedures including acclimation to nose-only restraint and during inhalation exposure periods.

The feed was analyzed by the supplier for nutritional components and environmental contaminants. Results of the analysis are provided by the supplier and are on file at the Testing Facility.

It was considered that there were no known contaminants in the feed that would interfere with the objectives of the study.

4.9.6.4. Water

Municipal tap water after treatment by reverse osmosis was freely available to each animal via an automatic watering system, except during acclimation to nose-only restraint and inhalation exposure periods.

Periodic analysis of the water was performed, and results of these analyses are on file at the Testing Facility.

It was considered that there were no known contaminants in the water that could interfere with the outcome of the study.

4.9.6.5. Animal Enrichment

Animals were socially housed for psychological/environmental enrichment and were provided with environmental enrichment as appropriate to aid in maintaining the animals' oral health.

4.9.6.6. Veterinary Care

Veterinary care was available throughout the course of the study; however, no examinations or treatments were required.

4.10. Experimental Design

Text Table 3
Experimental Design – Phase 1

Group Number	Treatment	Target Exposure Concentrations (ppm)	Positive Control Concentration (mg/mL)	Positive Control Dose Volume (mL/kg)	Animal Numbers[a,b]	
					Males	Females
1	Filtered Air	0	-	-	1001-1006	1501-1506
2	[redacted]	375	-	-	2001-2006	2501-2506
3	[redacted]	750	-	-	3001-3006	3501-3506
4	[redacted]	1500	-	-	4001-4006	4501-4506
5	Positive Control[c]	CP: 20 mg/kg/day	2	10	5001-5006	5501-5506
		EMS: 200 mg/kg/day	20	10		

CP = Cyclophosphamide monohydrate; EMS = Ethyl methanesulfonate; - = Not applicable.

[a] Filtered air (negative control group) and test substance were administered via nose-only inhalation for 3 consecutive days (6 hours per day) on Days 1–3.

[b] Up to 6 surviving rats/group were utilized for collection of peripheral blood between 1 and 2 hours following the final exposure/dose. Additionally, for 5 surviving rats/group, samples of bone marrow in addition to samples of the liver, lung, kidney, and nasal tissue were collected between 2 and 4 hours after completion of the final exposure (Groups 1–4) or after the second dose of EMS (Group 5), which coincided with being approximately 18–24 hours after the second dose of CP.

[c] CP was administered via oral gavage on Days 1 and 2. EMS was administered via oral gavage on Days 2 and 3.

Text Table 4
Experimental Design – Phase 2

Group Number	Treatment	Target Exposure Concentrations (ppm)	Positive Control Concentration (mg/mL)	Positive Control Dose Volume (mL/kg)	Animal Numbers[a,b]
1	Filtered Air	0	-	-	1007-1012
2	[redacted]	375	-	-	2007-2012
3	[redacted]	750	-	-	3007-3012
4	[redacted]	1500	-	-	4007-4012
5	Positive Control[c]	EMS: 200 mg/kg/day	20	10	5007-5012

EMS = Ethyl methanesulfonate; - = Not applicable.

[a] Filtered air (negative control group) and test substance were administered via nose-only inhalation for 2 consecutive days (6 hours per day) on Days 1–2.

[b] For 5 surviving rats/group, samples of the liver, lung, kidney, and nasal tissue were collected between 2 and 4 hours after completion of the final exposure (Groups 1–4) or after the second dose of EMS (Group 5).

[c] EMS was administered via oral gavage on Days 1 and 2.

4.10.1. Administration of Test Materials

For Phase 1, filtered air (control) and test substance atmospheres were administered to Groups 1–4 as 6-hour, nose-only inhalation exposures once daily for 3 consecutive days. For Group 5, CP was administered via oral gavage on Days 1 and 2, and EMS was administered via oral gavage on Days 2 and 3.

For Phase 2, filtered air (control) and test substance atmospheres were administered to Groups 1–4 as 6-hour, nose-only inhalation exposures once daily for 2 consecutive days. For Group 5, EMS was administered via oral gavage on Days 1 and 2.

4.10.2. Justification of Route and Dose Levels

The route of administration for the test substance was inhalation exposure because this is the unintended route of human exposure. Nose-only exposure methods were used to reduce the potential for dermal exposure or oral exposure resulting from grooming. In order to perform nose-only exposure, it was necessary to restrain the rats in specially designed nose-only holding tubes. The period of restraint was necessary to achieve the maximum feasible exposure to the test substance.

Per OECD Guidelines 474 and 489, it was not necessary to administer the concurrent positive control substance by the same route as the test substance. The route of administration of the positive control substances (oral gavage) was chosen based on past experience by Charles River and BioReliance with both types of assays.

The target exposure concentrations were selected by the Sponsor Representative in consultation with the Study Director based, in part, on a previous inhalation study conducted using [redacted] (Sponsor-provided data). In that study, [redacted] had localized effects in the nose at concentrations of 250 and 550 ppm. Based on these findings, exposure concentrations of 375, 750, and 1500 ppm were selected for this study.

4.11. In-life Procedures, Observations, and Measurements

4.11.1. Mortality (Both Phases)

Throughout the study, animals were observed for general health/mortality and moribundity twice daily, once in the morning and once in the afternoon. Animals were not removed from the cage during observations, unless necessary for identification or confirmation of possible findings.

4.11.2. Observations (Both Phases)

4.11.2.1. Detailed Clinical Observations

The animals were removed from the cage, and a detailed clinical observation was performed within 4 days of receipt, on the day of randomization, on Day 1 (prior to exposure), and on the day of necropsy (prior to exposure) (see Appendix 1 – Study Protocol and Deviations).

4.11.2.2. Cage Side Observations

Cage side observations were performed prior to exposure and 0 to 2 hours postexposure for Groups 1–4 and at the time of dosing and 0 to 2 hours postdose for Group 5. Animals were not removed from the cage during observation, unless necessary for identification or confirmation of possible findings.

During social housing, some observations (e.g., fecal observations) may not have been attributable to an individual animal.

4.11.3. Body Weights (Both Phases)

Animals were weighed individually within 4 days of receipt, on the day of randomization, on Day 1, and on the day of necropsy (prior to exposure).

4.11.4. Food Consumption (Both Phases)

Food consumption was quantitatively measured on Day 1 and on the day of necropsy (prior to exposure).

4.12. Terminal Procedures

Terminal procedures are summarized in Text Table 5 and Text Table 6.

Text Table 5
Terminal Procedures – Phase 1

Group No.	No. of Animals		Scheduled Euthanasia Day	Necropsy Procedures			Micronucleus Evaluation	Comet Assay
	Males	Females		Necropsy	Tissue Collection	Bone Marrow Collection		
1	6	6	3	-	X[a]	X	Bone Marrow	Select Tissues[b]
2	6	6			X[a]		Bone Marrow	Select Tissues[b]
3	6	6			X[a]		Bone Marrow	Select Tissues[b]
4	6	6			X[a]		Bone Marrow	Select Tissues[b]
5	6	6			X[a]		Bone Marrow	Select Tissues[b]

X = Procedure conducted; - = not applicable.
[a] See Tissue Collection and Preservation table for listing of tissues.
[b] Lung, liver, kidney, and nasal tissue.

Text Table 6
Terminal Procedures – Phase 2

Group No.	No. of Males	Scheduled Euthanasia Day	Necropsy Procedures	Comet Assay
			Necropsy	
1	6	2	-	Select Tissues[a]
2	6			Select Tissues[a]
3	6			Select Tissues[a]
4	6			Select Tissues[a]
5	6			Select Tissues[a]

X = Procedure conducted; - = not applicable.
[a] Lung, liver, kidney, and nasal tissue.

4.12.1. Unscheduled Deaths

No animals died during the course of the study in Phases 1 and 2.

4.12.2. Scheduled Euthanasia

The first 5 animals/group/sex in Phases 1 and 2 surviving until the scheduled euthanasia were anesthetized by isoflurane inhalation and euthanized by exsanguination. Animals were not fasted prior to the scheduled euthanasia. No macroscopic examination was performed.

4.12.3. Tissue Collection and Preservation

Representative samples of the tissues identified in Text Table 7 were collected from 5 animals/group in Phases 1 and 2 and preserved in 10% neutral buffered formalin, unless otherwise indicated. Animals that were not utilized for tissue collection were euthanized and discarded.

Text Table 7
Tissue Collection and Preservation

Liver (sections of 2 lobes) Lungs (including bronchi, fixed by constant pressure inflation with fixative)	Kidney Nasal cavity[a]

[a] Also collected from the additional animal/sex in Groups 1–4 not used for micronucleus and/or comet assay evaluations.

4.12.4. Micronucleus Evaluation (Phase 1)

4.12.4.1. Peripheral Blood Collection

Blood samples were collected from all animals at approximately 1–3 hours following the last exposure or dose. Blood (approximately 0.5 mL) was collected via the jugular vein into tubes containing K_2EDTA and samples from 5 animals/sex/group were processed per Section 4.12.4.2.

4.12.4.2. Whole Blood Preparation and Micronucleus Evaluation

Whole blood samples were diluted in anticoagulant, divided into 2 aliquots of approximately 180 µL each (primary and secondary samples) into separate 15 mL conical vials containing cold fixative, and then fixed in cold methanol for approximately 72 hours. Samples were then removed from frozen storage and washed out of fixative (see Appendix 1 – Study Protocol and Deviations). The primary and secondary samples were placed into long term storage solution (LTSS) and stored frozen (at a target of -70°C) until shipped to BioReliance Corporation, Rockville, MD, for analysis.

Of the 6 samples/sex/group available, 5 samples in LTSS were washed with ice cold 1% FBS solution and maintained on wet ice. The cells were then pelleted by centrifugation, and the supernatant was poured off leaving a small amount of supernatant with the pellet. The cells were re-suspended and 20 µL of suspension were added to 80 µL of staining solution containing RNase, FITC-conjugated anti-CD 71 antibodies and PE-conjugated anti-CD 61 antibodies. The samples were incubated at 2°C to 8°C for 30 minutes, re-suspended, then incubated at room temperature for an additional 30 minutes. DNA staining solution (propidium iodide; 0.3 to 2 mL) was added and then the samples were placed on wet ice for at least 5 minutes prior to the flow cytometric analysis.

The frequency of micronucleated reticulocytes in peripheral blood was analyzed after flow cytometer calibration using Malaria infected biostandard and negative control standards provided in the Litron kit. Up to 20,000 RETs per animal, when possible, were analyzed.

A detailed description of the methods and the results of the micronucleus evaluation is presented in Appendix 5.

4.12.4.3. Bone Marrow Collection

Bone marrow was collected from the first 5 animals/sex/group at the time of euthanasia from the right femur of animals anesthetized by inhalation of isoflurane and euthanized by exsanguination. Five animals/sex/group in the negative control (Group 1) and test substance-treated groups were euthanized approximately 2–4 hours following the last exposure (Groups 2–4) or second dose of EMS (Group 5), and the nasal cavity was collected (Groups 1–4)

or discarded without tissue collection (Group 5). Bone marrow was aspirated or flushed 2 to 3 times from the right femur into a centrifuge tube using a syringe containing heat inactivated fetal bovine serum (HI FBS). The bone marrow was centrifuged and all but approximately 0.25 mL (or a volume approximately twice that of the cell pellet) of HI FBS was decanted, and the pellet was re-suspended in the remaining HI FBS. Bone marrow smears were prepared by placing approximately 1 drop of cell suspension onto a minimum of 4 appropriately labeled, clean microscope slides. Each slide was coded so that the treatment group would not be revealed during subsequent analysis. The slides were air dried, fixed in 100% methanol for approximately 20 minutes, and allowed to air dry a second time. The slides were stored for possible future analysis.

4.12.5. Comet Assay Evaluation (Both Phases)

A detailed description of the methods and the results of the comet assay evaluation is presented in Appendix 6.

4.12.5.1. Tissue Collection for Comet Assay

Five (5) animals/sex/group (as appropriate by phase) had nasal tissue, lung, kidney, and liver collected between 2 and 4 hours following their last exposure (Groups 1–4), or second dose of EMS (Group 5) following collection of the bone marrow. Charles River personnel removed the head, lung, kidney, and liver. Samples of the nasal tissue, left lung, kidney, and liver were placed in chilled mincing solution (Hanks' balanced salt solution with EDTA and DMSO). The tissue samples were then minced with fine scissors to release the cells. The cell suspension was strained into a pre-labeled conical polypropylene tube through a cell strainer and kept on wet ice during preparation of the slides.

Slides of the processed nasal tissue, lung, kidneys, and liver were prepared by BioReliance Corporation personnel at Charles River and were stored at room temperature with desiccant. The slides were shipped at ambient temperature via overnight courier to BioReliance Corporation, Rockville, MD, for analysis.

5. STATISTICAL ANALYSIS

5.1. Statistics Conducted by Charles River Ashland

All statistical tests were conducted at the 5% significance level. All pairwise comparisons were conducted using two sided tests and are reported at the 1% and 5% levels.

Numerical data collected on scheduled occasions for the listed variables were analyzed as indicated according to sex and occasion. Descriptive statistics number, mean and standard deviation were reported whenever possible. Values may also be expressed as a percentage of predose or control values or fold change of control values when deemed appropriate. Inferential statistics were performed according to the matrix below when possible, but excluded semi-quantitative data, and any group with less than 3 observations. Calculated values on Provantis tables may not be reproducible from the individual values presented because all calculations were conducted using non-rounded values.

Text Table 8
Statistical Matrix

Variables for Inferential Analysis	Statistical Method and System Parametric (Provantis)
Body Weight	X
Body Weight Gain	X
Food Consumption	X

The following pairwise comparisons were made:

Group 2 vs. Group 1

Group 3 vs. Group 1

Group 4 vs. Group 1

Levene's test[4] was used to assess the homogeneity of group variances. The groups were compared using a parametric one-way analysis of variance (ANOVA)[5] *F*-test if Levene's test was not significant or the Kruskal-Wallis nonparametric ANOVA test[6] if it was significant. If the overall *F*-test or Kruskal-Wallis test was found to be significant, then pairwise comparisons were conducted using Dunnett's test[7] or Dunn's test[8], respectively.

5.2. Statistics Conducted by BioReliance Corporation

5.2.1. Peripheral Blood Micronucleus Data

Statistical analysis was performed on the micronucleus frequency (%MnRET) and %RET using the animal as the unit. The mean and standard deviation of %MnRET and %RET was presented for each treatment group.

The use of parametric or nonparametric statistical methods in evaluation of data were based on the variation between groups. The group variances for micronucleus frequency for the filtered air control and test substance groups was compared using Levene's test (significance level of $p \leq 0.05$). If the variation between groups is found not to be significant, a parametric one-way ANOVA was performed followed by a Dunnett's post-hoc analysis to compare each dose group to the concurrent filtered air control. If Levene's test indicates heterogeneous group variances (significance level of $p \leq 0.05$), the suitability of a transformation of the original data was evaluated (e.g. using logarithm transformed values of the original data) in an attempt to meet the normality criterion. Afterwards, statistical analysis was performed using the parametric tests described above. If parametric tests are not acceptable, non-parametric statistical methods (Kruskal Wallis and/or Mann Whitney test) may be used in evaluation of data.

A linear regression analysis was conducted to assess dose responsiveness in the test substance treated groups ($p \leq 0.01$ and $R^2 \geq 70\%$).

A pair-wise comparison (Student's T-test; $p \leq 0.05$) was used to compare the positive control group to the concurrent vehicle control group. If parametric tests were not acceptable, non-parametric statistical methods (Kruskal Wallis and/or Mann Whitney test) may have been used in evaluation of data.

5.2.2. Comet Assay

In order to quantify the test substance-related effects on DNA damage, the following statistical analysis was performed:

- The use of parametric or non-parametric statistical methods in evaluation of data was based on the variation between groups. The group variances for % Tail DNA (or other parameters of DNA damage) generated for the negative control (filtered air control) and test substance-treated groups were compared using Levene's test (significant level of $p \leq 0.05$). If the differences and variations between groups were found not to be significant, a parametric one-way ANOVA followed by a Dunnett's post-hoc test was performed (significant level of $p < 0.05$). If Levene's test indicated heterogeneous group variances ($p \leq 0.05$), the suitability of a transformation of the original data was evaluated (e.g. using logarithm, or BoxCox transformed values of the original data) in an attempt to meet the normality criteria. Afterwards, statistical analyses were performed using the parametric tests described above. If parametric tests were not acceptable, non-parametric statistical methods (Kruskal Wallis or Mann Whitney test) were used in evaluation of data.
- Linear regression analysis was used to determine a dose response relationship ($p < 0.01$).
- Pair-wise comparison (Student's t-test, $p \leq 0.05$) was used to compare the data from the positive control group against the negative control group. If needed, non-parametric statistical methods (Kruskal Wallis or Mann Whitney test) were used in evaluation of data.

6. COMPUTERIZED SYSTEMS

Critical computerized systems used in the study are listed below or presented in the appropriate phase report. All computerized systems used in the conduct of this study have been validated (with the exception of Microsoft Office); when a particular system has not satisfied all requirements, appropriate administrative and procedural controls were implemented to assure the quality and integrity of data.

As Charles River Ashland transitions between various computer systems, the study number may appear as [redacted], [redacted], or [redacted] in in the data records and report.

Text Table 9
Critical Computerized Systems

Program/System	Version No.	Description
Bio Medic Data Systems (BMDS) Implantable Micro Identification™ (IMI-1000)	N/A	Animal identification.
Deviation Information Library	2.1	Deviations.
Formulations Dispense System (CR-FDS)	2.04	Program utilized the assigned barcodes created in Provantis Dispense™ to track the dosing containers throughout the storage and dosing process.
Inhalation Exposure Data Collection System (WINH)	1.26	Monitors and records inhalation chamber temperature, relative humidity, ventilation rate, and negative pressure.
Metasys DDC Electronic Environmental Control System	12.04	Controls and monitors animal room environmental conditions.

Program/System	Version No.	Description
Microsoft Office 2010 or higher	N/A	Used in conjunction with the publishing software to generate study reports.
Provantis	10.2.1	Comprehensive system (Instem LSS Limited) used for in-life and postmortem data collection and reporting.
Provantis Dispense™	10.2.1	Comprehensive system (Instem LSS Limited) to manage test materials, including receipt, formulation instructions, and accountability.
WIL Metasys	2.28	In-house developed system used to record and report animal room environmental conditions.
WIL Toxicology Data Management System™ (WTDMS™)	Various	In-house developed system used for collection and reporting of other data.

N/A = not applicable.
Note: Version numbers of WTDMS™ programs used for the study are presented in the Study Records (input programs) and Facility Records (release dates).

7. RETENTION OF RECORDS, SAMPLES, AND SPECIMENS

All study-specific raw data, electronic data, documentation, protocol, retained samples and specimens, and Final Reports will be archived by no later than the date of Final Report issue. All materials generated by Charles River or by a Test Site from this study will be transferred to a Charles River archive. At least 1 year after issue of the Draft Report, the Sponsor will be contacted.

Following finalization, the original signed Final Report (paper copy) will be transferred to the Sponsor at the following address:

[redacted]

All transferred records will be maintained in the Sponsor's archives. A full copy of the final signed report will be retained and archived at Charles River.

Any work product, including documents, specimens, and samples, that were required by this protocol, its amendments, or other written instructions of the Sponsor to be shipped by Charles River to another location will be appropriately packaged and labeled as defined by Charles River SOPs and delivered to a common carrier for shipment. Charles River will not be responsible for shipment following delivery to the common carrier.

Electronic data generated by the Testing Facility were archived as noted above, except that the data collected using Deviation Information Library, Dispense, Provantis, and reporting files stored on SDMS were archived at the Charles River Laboratories facility location in Wilmington, MA.

All records, retained samples and specimens, and reports generated from phases or segments performed by Sponsor-designated subcontractors were returned to the Testing Facility for archiving. Archival location is detailed in the applicable PI reports.

8. RESULTS

8.1. Characterization of Exposure Atmospheres

8.1.1. Analyzed Exposure Concentrations

(Appendix 3)

The overall mean analyzed concentration for each exposure chamber are presented below.

Text Table 10
Overall Mean Analyzed Exposure Concentrations – Phase 1

Exposure Chamber:	1	2	3	4
Target Concentration (ppm):	0	375	750	1500
Mean Concentration (ppm):	0	381	753	1501
Standard Deviation:	0.0	6.2	9.9	27.3
Number of Exposures:	4	4	4	4

Text Table 11
Overall Mean Analyzed Exposure Concentrations – Phase 2

Exposure Chamber:	1	2	3	4
Target Concentration (ppm):	0	375	750	1500
Mean Concentration (ppm):	0	375	741	1494
Standard Deviation:	0.0	27.6	59.4	84.1
Number of Exposures:	2	2	2	2

8.2. Phase 1

8.2.1. Mortality

(Table 1 and Appendix 4, Table 4.1)

All animals survived until scheduled necropsy.

8.2.2. Observations

(Table 2 and Appendix 4, Table 4.2)

There were no test substance-related clinical observations. All clinical observations in the test substance-treated groups were limited to single animals, were not noted in a dose-related manner, and/or were common findings for laboratory rats of this age and strain.

8.2.3. Body Weights

(Figure 1, Figure 2, Table 3, Table 4, and Appendix 4, Table 4.3, Table 4.4)

Test substance-related mean body weight losses were noted in the 750 and 1500 ppm group males and the 375, 750, and 1500 ppm group females when the entire exposure period (Days 1–3) was evaluated compared to the filtered air control group; differences were generally statistically significant. As a result, slightly lower (not statistically significant) mean body weights were noted for males at 750 and 1500 ppm (4.9% and 5.2%, respectively) and females at 375, 750, and 1500 ppm (2.2%, 3.3%, and 6.1%, respectively) compared to the filtered air control group on Day 3.

Body weights for males in the 375 ppm group were unaffected by test substance administration. Differences were slight and not statistically significant.

8.2.4. Food Consumption

(Table 5 and Appendix 4, Table 4.5)

Test substance-related lower mean food consumption was noted for males and females at 375, 750, and 1500 ppm compared to the filtered air control group when the entire exposure period (Days 1–3) was evaluated; differences were generally statistically significant.

8.2.5. Micronucleus Evaluation

(Appendix 5)

There was no significant increase in the number of micronuclei in the test substance-exposed animals compared to the filtered air control in both males and females. The filtered air control substance values in both males and females were compatible with the expected range of percent micronucleated reticulocytes (%MnRETs). There was a statistically significant increase in MnRETs in the positive control group as compared to the concurrent control group. All criteria for a valid assay were met.

Under the conditions of this study, the administration of the test substance [redacted] at exposure concentrations up to and including 1500 ppm was concluded to be negative in the micronucleus assay.

8.2.6. In Vivo Comet Assay

(Appendix 6)

During Phase 1 of the assay (both sexes), the test substance, [redacted], could be evaluated as negative (non-DNA damaging) in male liver cells only. For the remaining tissues tested, the assay did not meet all the acceptance criteria (especially as related to the positive control treatment), and therefore was considered invalid.

8.3. Phase 2

8.3.1. Mortality

(Table 6 and Appendix 4, Table 4.6)

All animals survived until scheduled necropsy.

8.3.2. Observations

(Table 7 and Appendix 4, Table 4.7)

There were no clinical observations noted for males at any exposure concentration.

8.3.3. Body Weights

(Figure 3, Table 8, Table 9, and Appendix 4, Table 4.8, Table 4.9)

A test substance-related statistically significant mean body weight loss was noted in the 1500 ppm group males and the when the entire exposure period (Days 1–2) was evaluated compared to the filtered air control group. As a result, a slightly lower (2.6%; not statistically significant) mean body weight was noted for males in this group compared to the filtered air control group on Day 2.

Body weights for males in the 375 and 750 ppm groups were unaffected by test substance administration. Differences were slight and not statistically significant.

8.3.4. Food Consumption

(Table 10 and Appendix 4, Table 4.10)

Test substance-related lower mean food consumption was noted for males at 375, 750, and 1500 ppm compared to the filtered air control group when the entire exposure period (Days 1–2) was evaluated; the differences did not occur in an exposure-related manner and was statistically significant for the 375 ppm group only.

8.3.5. In Vivo Comet Assay

(Appendix 6)

A statistically significant % Tail DNA was observed in male liver cells in the mid concentration group (750 ppm) when compared to the concurrent vehicle control; however, this increase was within historical control range and has been evaluated as not biologically relevant, and no concentration-response was observed. The group mean vehicle control % Tail DNA in male liver cells was within the historical control range. The group mean positive control % Tail DNA in male liver cells was statistically significant when compared to the concurrent group mean vehicle control and was compatible with the historical control range.

No statistically significant increases in % Tail DNA were observed in the kidney, lung, and nasal cavity of the test substance-treated groups compared to the negative control group. The negative control was within historical control range. The positive control was statistically significant when compared to the negative control.

All valid assay criteria were met.

Under the conditions of this study, the administration of [redacted] at exposure concentrations of 375, 750, and 1500 ppm did not cause a significant increase in DNA damage in the kidney, liver, lung, or nasal cavity relative to the concurrent negative control. Therefore, [redacted] was concluded to be negative (non-DNA damaging) in the in vivo comet assay.

9. CONCLUSIONS

Based on the results of this study, exposure of male and female Crl:CD(SD) rats to [redacted] via nose-only inhalation for 6 hours per day for up to 3 consecutive days at target exposure concentrations of 375, 750, and 1500 ppm resulted in a negative response for induction of bone marrow micronuclei and induction of DNA damage in the liver, lung, kidney, and nasal cavity.

10. REFERENCES

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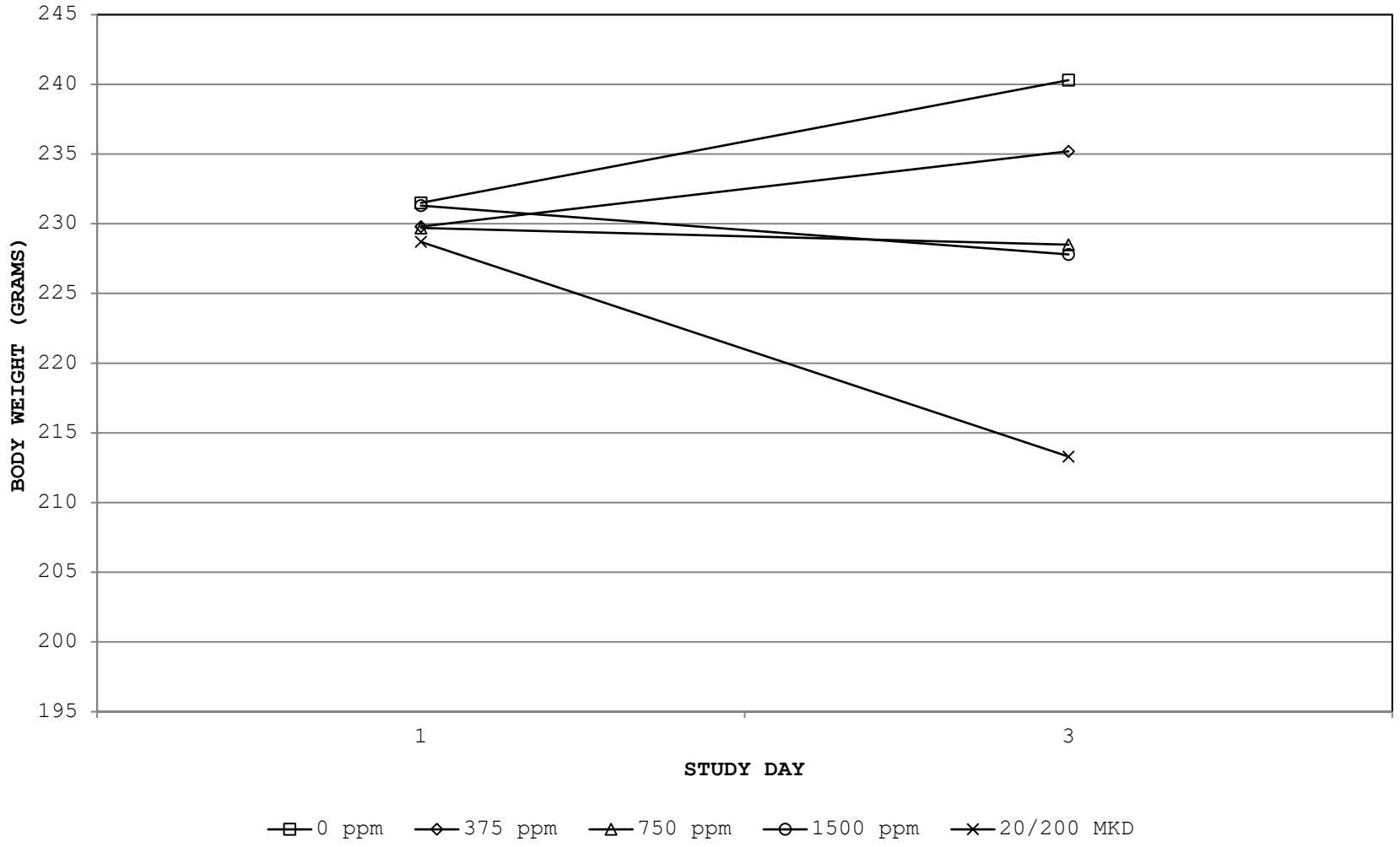
FIGURES

Study No.: [redacted]

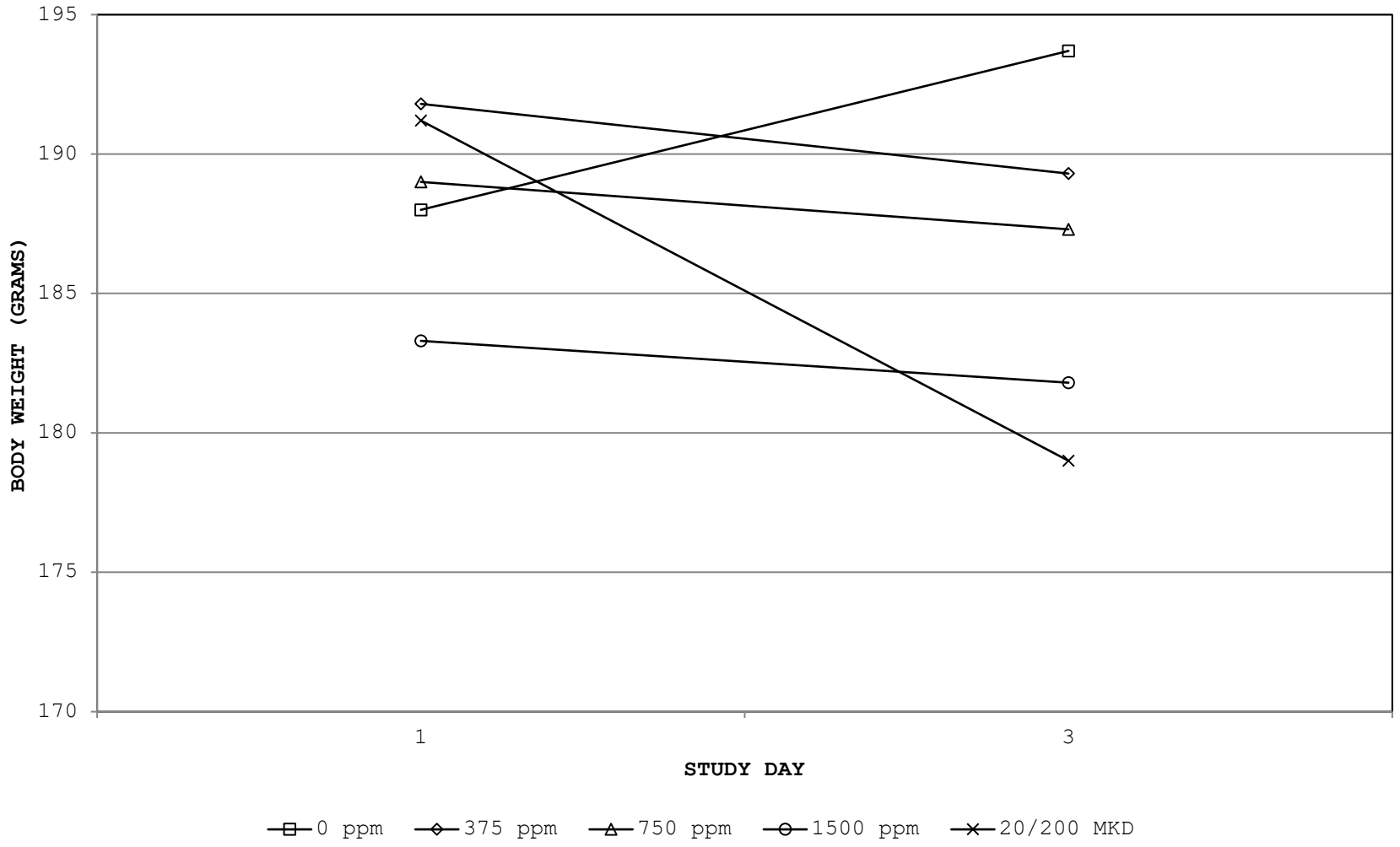
Figure 1
Summary of Body Weights: Males - Phase 1
Data Presented As Mean



GraphMacro_v1.23 13-Jun-2019

Study No.: [redacted]

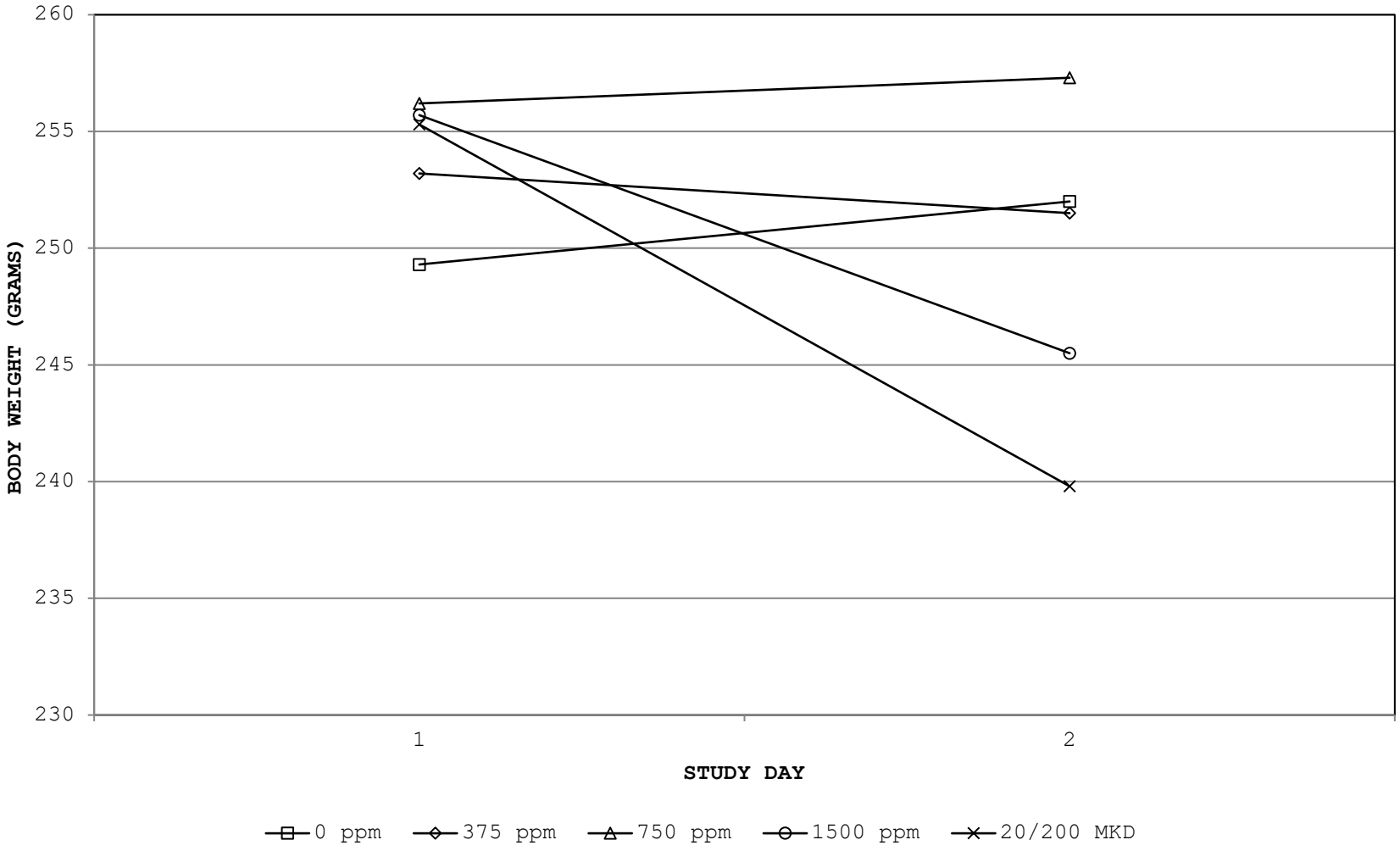
Figure 2
Summary of Body Weights: Females - Phase 1
Data Presented As Mean



GraphMacro_v1.23 13-Jun-2019

Study No.: [redacted]

Figure 3
Summary of Body Weights: Phase 2
Data Presented As Mean



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TABLES

[redacted]

TABLES EXPLANATION PAGE

All Day(s) referenced throughout the outputs generated are Study Days beginning with Study Day 1, the first day of dosing.

Abbreviations consistent throughout the Summary and Individual Tables.

Note: All of the abbreviations listed on these pages may not be applicable to this report.

Abbreviation	Description
MKD	milligrams/kilograms/day
mg/kg	milligrams/kilograms
ppm	parts per million
% Diff	% Difference from Group 1
tCtrl	Times control (fold change)
1F, 2F, 3F, 4F, 5F	Group 1 Female, Group 2 Female, Group 3 Female, Group 4 Female, Group 5 Female
1M, 2M, 3M, 4M, 5M	Group 1 Male, Group 2 Male, Group 3 Male, Group 4 Male, Group 5 Male
g	grams
kg	kilograms
mg	milligrams
N	Number of values included in analysis
M, F	Male, Female
<, >	Out of range
--	Not scheduled to be performed/dead
-	Not applicable

Phases 1 and 2
Group 1 - 0 ppm
Group 2 - 375 ppm
Group 3 - 750 ppm
Group 4 - 1500 ppm
Group 5 - 20/200 MKD

Abbreviation	Description	Abbreviation	Description
ANIC	Animal not in cage or incorrect cage during measurement	OA	Omitted activity
AVS	Suspected aberrant value	REHO	Animal rehoused during measurement interval
COME	See comment value excluded	REPL	Animal replaced during measurement interval
COMI	See comment value included	Sup	Suppress
COMM	Comment added	TARE	Balance tared
Exc	Exclude	Temp	Temperature
Int.	Interval	TERR	Technical error
NA	Not applicable	UPTD	Unable to perform due to technical difficulty
NC	Not calculable	UTD	Unable to determine
No.	Animal number	Wt	Weight
NSCH	Not scheduled to be performed		

CLINICAL OBSERVATIONS

Abbreviation	Description	Abbreviation	Description
0	White	A	Slight group housed
1	Slight	B	Moderate group housed
2	Moderate	C	Severe group housed
3	Severe	D	Scab ended
4	Black	DE	Detailed examination
5	Blue	G	Lesion ended
6	Brown	L	Lesion present
7	Clear	M	Mass present
8	Green	N	Severity not applicable
9	Red	S	Scab present
-	Severity not recorded	X	Present
!	Comment present	Y	Yellow

BODY WEIGHTS

Abbreviation	Description	Abbreviation	Description
AVS	Suspected aberrant value	TERR	Technical Error
E	Excluded	UPTD	Unable to perform due to technical difficulty
OA	Omitted Activity	X	Excluded from mean
%UD	%		

FOOD CONSUMPTION

Abbreviation	Description	Abbreviation	Description
AFE	Animal found with no food during measurement interval – exclude	Nr	Not reported due to animal replacement
AFNF	Animal found with no food during measurement interval – include	ONEG	Original value negative, animal did not eat
ANH	Animal found with no hopper during measurement interval	Pf	Powdered food
ANW	Animal found with no water during measurement interval	Sf	Supplemental food
Ar	Animals rehoused	SPIL	Spilled food by animal
AVS	Aberrant value suppressed/excluded	UTD	Unable to determine
Bf	Bowl on floor	W	Wet or contaminated food
Fd	Food deprived	Wa	Water added
FSNC	Food supplementation given during interval, value not calculable	WETF	Wet or contaminated food
NC	Not calculable	X	Excluded from mean
Np	Not scheduled to be performed		

MORTALITY

Abbreviation	Description	Abbreviation	Description
0–2 Ho	Removal symptom entered 0–2 hours postdosing	PostRx	Observation postdosing
ACCD	Accidental death	PreRx	Observation predosing
AD	Accidental death	REC	Recovery euthanasia
AM SIRT	Mortality/moribundity check in the morning	REL	Released
CSO	Cage side observation	TE	Terminal euthanasia
DE	Detailed examination	TERM	Terminal euthanasia
FD	Found dead	UE	Unscheduled euthanasia
INTM	Interim euthanasia	UNSC	Unscheduled euthanasia
PM SIRT	Mortality/moribundity check in the afternoon		

Table 1 Summary of Survival: Phase 1

[redacted]

Observation Type: All Types	Male				Female			
From Day 1 (Start Date) to 3 (Start Date)	0 ppm	375 ppm	750 ppm	1500 ppm	0 ppm	375 ppm	750 ppm	1500 ppm
Terminal Euthanasia								
Number of Animals Affected	6	6	6	6	6	6	6	6
Number of Times Recorded	6	6	6	6	6	6	6	6

Table 1 Summary of Survival: Phase 1

[redacted]

Observation Type: All Types From Day 1 (Start Date) to 3 (Start Date)	Male 20/200 MKD	Female 20/200 MKD
Terminal Euthanasia		
Number of Animals Affected	6	6
Number of Times Recorded	6	6

Table 2 Summary of Clinical Observations: Phase 1

[redacted]

Observation Type: Toxicology Observations	Male				Female			
From Day 1 (Start Date) to 3 (Start Date)	0 ppm	375 ppm	750 ppm	1500 ppm	0 ppm	375 ppm	750 ppm	1500 ppm
Fur, Staining, Urogenital, Yellow								
Number of Animals Affected	0	0	0	0	0	0	0	1
Number of Times Recorded	0	0	0	0	0	0	0	1
% of Affected Animals	0	0	0	0	0	0	0	17
First to Last seen	-	-	-	-	-	-	-	3 - 3
Skin, Scab, Periorbital, Left								
Number of Animals Affected	0	0	1	0	0	0	0	0
Number of Times Recorded	0	0	1	0	0	0	0	0
% of Affected Animals	0	0	17	0	0	0	0	0
First to Last seen	-	-	3 - 3	-	-	-	-	-
Teeth, Broken								
Number of Animals Affected	0	0	0	0	1	0	0	0
Number of Times Recorded	0	0	0	0	2	0	0	0
% of Affected Animals	0	0	0	0	17	0	0	0
First to Last seen	-	-	-	-	2 - 3	-	-	-

Table 2 Summary of Clinical Observations: Phase 1

[redacted]

Observation Type: Toxicology Observations From Day 1 (Start Date) to 3 (Start Date)	Male 20/200 MKD	Female 20/200 MKD
Fur, Staining, Urogenital, Yellow		
Number of Animals Affected	0	0
Number of Times Recorded	0	0
% of Affected Animals	0	0
First to Last seen	-	-
Skin, Scab, Periorbital, Left		
Number of Animals Affected	0	0
Number of Times Recorded	0	0
% of Affected Animals	0	0
First to Last seen	-	-
Teeth, Broken		
Number of Animals Affected	0	0
Number of Times Recorded	0	0
% of Affected Animals	0	0
First to Last seen	-	-

Table 3 Summary of Body Weights: Phase 1

[redacted]

Bodyweight (g)

Sex: Male		Day(s) Relative to Start Date	
		1	3
0 ppm	Mean	231.5	240.3
	SD	8.7	8.7
	N	6	6
375 ppm	Mean	229.8	235.2
	SD	9.1	10.5
	N	6	6
	%Diff	-0.7	-2.1
750 ppm	Mean	229.7	228.5
	SD	8.6	8.4
	N	6	6
	%Diff	-0.8	-4.9
1500 ppm	Mean	231.3	227.8
	SD	10.7	14.8
	N	6	6
	%Diff	-0.1	-5.2
20/200 MKD	Mean	228.7	213.3
	SD	11.1	8.5
	N	6	6
	%Diff	-1.2	-11.2

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Table 3 Summary of Body Weights: Phase 1

[redacted]

Bodyweight (g)

Sex: Female		Day(s) Relative to Start Date	
		1	3
0 ppm	Mean	188.0	193.7
	SD	6.9	8.8
	N	6	6
375 ppm	Mean	191.8	189.3
	SD	9.9	13.6
	N	6	6
	%Diff	2.0	-2.2
750 ppm	Mean	189.0	187.3
	SD	11.4	12.7
	N	6	6
	%Diff	0.5	-3.3
1500 ppm	Mean	183.3	181.8
	SD	10.4	9.5
	N	6	6
	%Diff	-2.5	-6.1
20/200 MKD	Mean	191.2	179.0
	SD	7.0	9.9
	N	6	6
	%Diff	1.7	-7.6

Anova & Dunnett

Table 4 Summary of Body Weight Gains (g): Phase 1

[redacted]

Bodyweight Gain (Interval)

Sex: Male		Day(s) Relative to Start Date
		1 → 3
0 ppm	Mean	8.8
	SD	3.0
	N	6
375 ppm	Mean	5.3
	SD	3.1
	N	6
750 ppm	Mean	-1.2**
	SD	3.2
	N	6
1500 ppm	Mean	-3.5**
	SD	5.2
	N	6
20/200 MKD	Mean	-15.3
	SD	4.3
	N	6

Anova & Dunnett: ** = $p \leq 0.01$

Table 4 Summary of Body Weight Gains (g): Phase 1

[redacted]

Bodyweight Gain (Interval)

Sex: Female		Day(s) Relative to Start Date
		1 → 3
0 ppm	Mean	5.7
	SD	6.7
	N	6
375 ppm	Mean	-2.5*
	SD	5.6
	N	6
750 ppm	Mean	-1.7*
	SD	2.7
	N	6
1500 ppm	Mean	-1.5
	SD	3.9
	N	6
20/200 MKD	Mean	-12.2
	SD	7.3
	N	6

Anova & Dunnett: * = $p \leq 0.05$

Table 5 Summary of Food Consumption: Phase 1

[redacted]

Food Mean Daily Consumption (g/animal/day)

Sex: Male		Day(s) Relative to Start Date
		1 → 3
0 ppm	Mean	19.58
	SD	0.79
	N	6
375 ppm	Mean	16.83**
	SD	1.10
	N	6
	%Diff	-14.04
750 ppm	Mean	15.00**
	SD	1.18
	N	6
	%Diff	-23.40
1500 ppm	Mean	11.33**
	SD	1.15
	N	6
	%Diff	-42.13
20/200 MKD	Mean	9.75
	SD	0.59
	N	6
	%Diff	-50.21

Anova & Dunnett: ** = $p \leq 0.01$

Table 5 Summary of Food Consumption: Phase 1

[redacted]

Food Mean Daily Consumption (g/animal/day)

Sex: Female		Day(s) Relative to Start Date
		1 → 3
0 ppm	Mean	15.08
	SD	0.56
	N	6
375 ppm	Mean	13.33
	SD	0.34
	N	6
	%Diff	-11.60
750 ppm	Mean	10.50**
	SD	0.45
	N	6
	%Diff	-30.39
1500 ppm	Mean	8.42**
	SD	0.85
	N	6
	%Diff	-44.20
20/200 MKD	Mean	8.00
	SD	0.77
	N	6
	%Diff	-46.96

Kruskal-Wallis & Dunn: ** = $p \leq 0.01$

Table 6 Summary of Survival: Phase 2

[redacted]

Observation Type: All Types	Male				
From Day 1 (Start Date) to 2 (Start Date)	0 ppm	375 ppm	750 ppm	1500 ppm	200 MKD
Terminal Euthanasia					
Number of Animals Affected	6	6	6	6	6
Number of Times Recorded	6	6	6	6	6

Table 7 Summary of Clinical Observations: Phase 2

[redacted]

Observation Type: All Types From Day 1 (Start Date) to 2 (Start Date)	Male				
	0 ppm	375 ppm	750 ppm	1500 ppm	200 MKD
	No Abnormalities Detected for All Animals				

Table 8 Summary of Body Weights: Phase 2

[redacted]

Bodyweight (g)

Sex: Male		Day(s) Relative to Start Date	
		1	2
0 ppm	Mean	249.3	252.0
	SD	21.5	20.3
	N	6	6
375 ppm	Mean	253.2	251.5
	SD	12.4	8.9
	N	6	6
	%Diff	1.5	-0.2
750 ppm	Mean	256.2	257.3
	SD	13.3	12.5
	N	6	6
	%Diff	2.7	2.1
1500 ppm	Mean	255.7	245.5
	SD	11.0	9.4
	N	6	6
	%Diff	2.5	-2.6
200 MKD	Mean	255.3	239.8
	SD	11.9	10.1
	N	6	6
	%Diff	2.4	-4.8

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Table 9 Summary of Body Weight Gains (g): Phase 2

[redacted]

Bodyweight Gain (Interval)

Sex: Male		Day(s) Relative to Start Date
		1 → 2
0 ppm	Mean	2.7
	SD	3.1
	N	6
375 ppm	Mean	-1.7
	SD	3.9
	N	6
750 ppm	Mean	1.2
	SD	4.4
	N	6
1500 ppm	Mean	-10.2**
	SD	8.7
	N	6
200 MKD	Mean	-15.5
	SD	7.5
	N	6

Anova & Dunnett: ** = $p \leq 0.01$

Table 10 Summary of Food Consumption: Phase 2

[redacted]

Food Mean Daily Consumption (g/animal/day)

Sex: Male		Day(s) Relative to Start Date
		1 → 2
0 ppm	Mean	18.83
	SD	1.37
	N	6
375 ppm	Mean	8.33**
	SD	6.85
	N	6
	%Diff	-55.75
750 ppm	Mean	13.17
	SD	7.81
	N	6
	%Diff	-30.09
1500 ppm	Mean	10.17
	SD	5.68
	N	6
	%Diff	-46.02
200 MKD	Mean	6.67
	SD	1.57
	N	6
	%Diff	-64.60

Kruskal-Wallis & Dunn: ** = $p \leq 0.01$

APPENDIX 1

Study Protocol and Deviations

DEVIATIONS

All deviations that occurred during the study have been authorized/acknowledged by the Study Director, assessed for impact, and documented in the study records. All study protocol deviations and those SOP deviations that could have impacted the quality or integrity of the study are listed below. Minor SOP deviations that did not impact the quality or integrity of the study have been included at the discretion of the Study Director.

None of the deviations were considered to have impacted the overall integrity of the study or the interpretation of the study results and conclusions.

Formulations and Dosing

- **Protocol Section 9.1.2.** states that the ethyl methanesulfonate formulation will be prepared on the second and third day of positive control dosing (Days 1 and 2 for the positive control animals). Due to the addition of Phase 2 only being 2 days of exposure, ethyl methanesulfonate preparation occurred on Days 1 and 2 instead of Days 2 and 3.

  Impact Assessment: This deviation did not negatively impact the quality or integrity of the data or the outcome of the study because ethyl methanesulfonate formulations for each phase were prepared on the day prior to necropsy and the day of necropsy, as required for the comet assay.

In-life Observations, Measurements, and Evaluations

- **Protocol Section 13.3.3.** states that detailed clinical observations will be collected from all animals within 4 days of receipt, on the day of randomization, and on Days 1 and 3 (prior to exposure). On Day 2, an additional detailed clinical observation was collected from all Phase 1 animals.

  Impact Assessment: The collection of additional data did not negatively impact the quality or integrity of the data or the outcome of the study.

Laboratory Evaluations

- **Protocol Section 17.1.** states that whole blood samples will be fixed in cold methanol for at least 72 hours and after at least 72 hours of fixation, both sets of samples will be removed from frozen storage and washed out of fixative. Nine, 7, 6, 6, and 6 whole blood samples from the 0 ppm, 375 ppm, 750 ppm, 1500 ppm, and positive control groups, respectively, in Phase 2 were removed from frozen storage and washed out of fixative following only 70 hours 8 minutes to 71 hours 59 minutes of fixation. In addition, the time that the whole blood samples for Male No. 1006 in the 0 ppm group and Male No. 5001 in the positive control group was not recorded; therefore, it was not able to be determined how long these 2 samples remained in fixative prior to being removed from frozen storage and washed out of fixative.

  Impact Assessment: After consultation with the Principal Investigator, washing the samples that were fixed in methanol approximately 0–2 hours prior to at least 72 hours of fixation did not have an effect on the sample quality. Fixation stabilizes the proteins on the cell surfaces

and the specified 72-hour period is more than the time required to perform this task. Furthermore, the values of the positive control animals, some of which were affected, were within the BioReliance historical control data range. Therefore, this deviation did not impact the quality of the integrity of the study data.

PROTOCOL AMENDMENT NO. 2

Testing Facility Study No. [redacted]

Sponsor Reference No. [redacted]

A Combined *In Vivo* Micronucleus and Comet Assay of [redacted] in Sprague Dawley Rats

SPONSOR:

[redacted]

TESTING FACILITY:
Charles River Laboratories Ashland, LLC
1407 George Road
Ashland, OH 44805
United States

SUMMARY OF CHANGES AND JUSTIFICATIONS

Study Protocol effective date: 19 December 2018

Note: When applicable, additions are indicated in bold underlined text and deletions are indicated in bold strikethrough text in the affected sections of the document.

Item or Section(s)	Justification/Reason for Change
Amendment 1	**Date: 19 February 2019**
1.1 Study Classification	Corrected name of Primary Treatment Ingredient ID.
2. Proposed Study Schedule	Added experimental initiation and termination dates.
14.1 Micronucleus Blood Collection (Groups 1-5)	Corrected whole blood process section reference.
Amendment 2	**Date: 02 May 2019**
1. Objective	Due to a lack of positive response in the Comet assay from the positive controls, a second phase will be added to this study. Added wording for inclusion of phase 2.
2. Proposed Study Schedule	Defined phase 1 and 2.
9.1.1 Cyclophosphamide	Added phase clarification due to addition of phase 2.
9.1.2 Ethyl Methanesulfonate	Added phase 1 heading and wording for phase 2.
10. Test System	Defined phase 1 and 2.
10.2 Justification for Selection	Added justification for addition of phase 2.
12.4.1 Organization of Test Groups	Defined phase 1 and added phase 2.
12.4.3 Treatment Regimen	Defined phase 1 and added phase 2.
12.5.2 Nose-Only Inhalation	To generalize section to cover the use of both types of nose-only system.
12.6.2 Actual Concentration	Changed heading from Actual to Analyzed (correction of typographical error)
13.3.3 Detailed Clinical Observations (All Animals)	To generalize this section to cover both phases, removed Day 3 and added “On the day of necropsy”.
13.3.6 Individual Body Weights	To generalize this section to cover both phases, removed Day 3 and added “On the day of necropsy”.
13.3.7 Food Weights	To generalize this section to cover both phases, removed Day 3 and added “On the day of necropsy”.
14.1 Micronucleus Blood Collection (Groups 1-5)	Defined as being for phase 1 only.
15.2 Tissue Collection for Comet Assay	Added wording to include appropriate phases and the retention of cell suspension.
15.3 Bone Marrow Collection	Defined as being for phase 1 only.
16.1 Preparation of Comet Slides	Added wording for retention of cell suspension.
17. Micronucleus Test	Defined as being for phase 1 only.
19.3 Peripheral Blood Micronucleus Data	Defined as being for phase 1 only.

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1. OBJECTIVE

The objective of this study is to assess the potential of [redacted] to induce micronuclei and/**or** to cause DNA damage in rat liver, lung, kidney, and nasal tissue when administered via nose-only inhalation to Sprague Dawley rats for 6 hours per day for **up to** 3 consecutive days.

1.1. Study Classification

Study Category:	Genetic Toxicology
Study Type:	Genotoxicity In Vivo
Study Design:	Parallel
Primary Treatment CAS Registry Number:	Not Available
Primary Treatment Unique Ingredient ID:	[redacted]
Class of Compound:	Not Available

2. PROPOSED STUDY SCHEDULE

Proposed study dates are listed below. Actual applicable dates will be included in the Final Report.

Phase 1

Animal Arrival:	08 Jan 2019
Animal Randomization:	17 Jan 2019
Initiation of Dosing – Males (Experimental Start Date):	19 Jan 2019
Initiation of Dosing – Females:	20 Jan 2019
End of dosing (Terminal Necropsy):	21-22 Jan 2019

Phase 2

Animal Arrival:	**09 May 2019**
Initiation of Dosing – Males Only:	**20 May 2019**
End of dosing (Terminal Necropsy):	**21 May 2019**
Experimental Termination Date:	~~**19 Mar**~~ **03 Jul** 2019
Draft Report:	~~**23 Apr**~~ **23 Jul** 2019

3. GUIDELINES FOR STUDY DESIGN

The design of this study was based on the study objective and the following study design guidelines:

- OECD Guideline 474. *Mammalian Erythrocyte Micronucleus Test*.

- OECD Guideline 489. *In Vivo Mammalian Alkaline Comet Assay*.

4. REGULATORY COMPLIANCE

The study will be performed in accordance with the United States Code of Federal Regulations, Title 40, Parts 160 and 792: Good Laboratory Practice Standards and as accepted by Regulatory Authorities throughout the European Union (OECD Principles of Good Laboratory Practice), Japan (MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

Exceptions to GLPs include the following study elements:

- Characterization of the test substance was performed by the Sponsor according to established SOPs, controls, and approved test methods to ensure integrity and validity of the results generated; these analyses were not conducted in compliance with the GLP regulations.

5. QUALITY ASSURANCE

5.1. Testing Facility

The Testing Facility Quality Assurance Unit (QAU) will monitor the study to assure that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with Good Laboratory Practice regulations. The QAU will review the protocol, conduct inspections at intervals adequate to assure the integrity of the study, and audit the Final Report to assure that it accurately describes the methods and standard operating procedures and that the reported results accurately reflect the raw data of the study.

The Testing Facility QAU contact for this study is indicated below:

R. Kelvin Mentzer, BS, RQAP-GLP
Charles River
1407 George Rd.
Ashland, OH 44805
Tel: 419.282.2111
Fax: 419.289.3650
E-mail: kelvin.mentzer@crl.com

5.2. Sponsor-Designated Subcontractor

The following study phases will be audited by the Sponsor-designated Subcontractor QAU:

- Micronucleus Assay
- Comet Assay

For all study phases inspected by Sponsor or Sponsor-designated subcontractor QAU(s), copies of each periodic inspection report will be made available to the Study Director, Testing Facility Management, and the Testing Facility QAU.

The Micronucleus assay and Comet Test Site QAU contact for this study is indicated below:

Luleayenwa Aberra-Degu
BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850
Tel: 301.610.2667
E-mail: luleayenwa.aberra-degu@milliporesigma.com

6. SPONSOR

Sponsor Representative

[redacted]

7. RESPONSIBLE PERSONNEL

Study Director

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Management Contact

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Principal Investigators (PI) - Sponsor-designated

Micronucleus Assay	Megan Young BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850 Tel: 301.610.2152 E-mail: megan.young@milliporesigma.com
Comet Assay	Shannon Bruce BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850 Tel: 301.610.2741 E-mail: shannon.bruce@milliporesigma.com

Each PI is required to report any deviations or other circumstances that could affect the quality or integrity of the study to the Study Director in a timely manner. Each PI will provide a report addressing their assigned phase of the study, which will be included as an appendix to the Final Report. The phase report will include the following:

- A Statement of Compliance
- A QA Statement
- The archive site for all records, samples, specimens and reports generated from the phase or segment (alternatively, details regarding the retention of the materials may be provided to the Study Director for inclusion in the Final Report)
- A listing of critical computerized systems used in the conduct and/or interpretation of the assigned study phase

8. TEST SUBSTANCE AND VEHICLE INFORMATION

8.1. Test Substance

8.1.1. Identification

[redacted]

8.1.2. Lot Number

To be documented in the study records.

8.1.3. Purity

Purity information is the responsibility of the Sponsor. A Certificate of Analysis or appropriate documentation will be provided by the Sponsor.

8.1.4. Stability

The test substance is considered to be stable under the storage conditions provided by the Sponsor.

8.1.5. Physical Description

To be documented by Charles River.

8.1.6. Storage Conditions

In a room with controls set to maintain 18°C to 24°C.

8.1.7. Administration Dose Form

Gas

8.1.8. Reserve Samples

Reserve samples of the test substance will not be taken due to the duration of this study.

8.1.9. Personnel Safety Data

A Safety Data Sheet (SDS), or equivalent documentation, will be provided by the Sponsor (if available). It is the responsibility of the Sponsor to notify the test facility of any special handling requirements of the test substance. Otherwise routine safety precautions will be followed. Appropriate gloves, safety glasses and arm covers will be worn by individuals working with neat test material or formulations.

8.1.10. Test Substance Disposition

All neat test substance remaining at study completion will be returned to the Sponsor at the address provided below.

Test substance will be shipped at ambient temperature on a non-holiday Monday, Tuesday, or Wednesday by overnight courier to:

[redacted]

8.2. Positive Control Article 1

8.2.1. Identification

Cyclophosphamide monohydrate (CP, CAS number 6055-19-2) is a known clastogen.

8.2.2. Lot Number

To be documented in the study records.

8.2.3. Purity

To be provided in the Certificate of Analysis (if applicable).

8.2.4. Stability

The positive control article is considered to be stable under the storage conditions provided by the manufacturer.

8.2.5. Physical Description

To be documented by Charles River in the study records.

8.2.6. Administration Dose Form

Suspension

8.2.7. Positive Control Article Disposition

Any remaining positive control article will be retained under the storage conditions provided by the manufacturer.

8.2.8. Personnel Safety

A Safety Data Sheet (SDS), or equivalent, will be provided by the manufacturer. Double nitrile gloves and a half-face negative respirator with cartridge #60923 are to be worn while preparing and administering doses. If the preparation is done in a Ventilated Balance Safety Enclosure (VBSE), then a respirator is not needed. Otherwise, routine safety precautions will be followed.

8.3. Positive Control Article 2

8.3.1. Identification

Ethyl methanesulfonate (EMS, CAS No. 62-50-0), is a known substance that induces DNA strand breaks.

8.3.2. Lot Number

To be documented in the study records (if applicable).

8.3.3. Purity

To be provided in the Certificate of Analysis (if applicable).

8.3.4. Stability

The positive control article is considered to be stable under the storage conditions provided by the manufacturer.

8.3.5. Physical Description

To be documented by Charles River in the study records.

8.3.6. Storage Conditions

To be stored as per the conditions provided by the manufacturer.

8.3.7. Administration Dose Form

Solution

8.3.8. Positive Control Article Disposition

Any remaining positive control article will be retained under the storage conditions provided by the manufacturer.

8.3.9. Personnel Safety

An SDS, or equivalent, will be provided by the manufacturer. Double nitrile gloves and a half-face negative respirator with cartridge #60923 are to be worn while preparing and administering doses. If the preparation is done in a Ventilated Balance Safety Enclosure (VBSE) then a respirator is not needed. Otherwise, routine safety precautions will be followed.

9. PREPARATION AND ANALYSES OF DOSING FORMULATIONS

9.1. Method and Frequency of Preparation

9.1.1. Cyclophosphamide <u>(Phase 1 Only)</u>

The cyclophosphamide formulation will be prepared for dosing as a weight-to-volume mixture in deionized water. A complete description of the method of preparation for the cyclophosphamide formulation will be documented in the study records and described in the final report. The cyclophosphamide formulation will be prepared on the first and second day of positive control dosing (Days 1 and 2 for the positive control animals). The cyclophosphamide formulation will be stirred continuously, on ice, during preparation and dosing.

9.1.2. Ethyl Methanesulfonate

Phase 1

The ethyl methanesulfonate formulation will be prepared for dosing as a weight-to-volume mixture in 0.9% saline. A complete description of the method of preparation for the ethyl methanesulfonate formulation will be documented in the study records and described in the final report. The ethyl methanesulfonate formulation will be prepared on the second and third day of positive control dosing (Days 2 and 3 for the positive control animals). The ethyl methanesulfonate formulation does not need to be stirred continuously and may be stored at room temperature during preparation and dosing.

Phase 2

The ethyl methanesulfonate formulation will be prepared for dosing as a weight-to-volume mixture in 0.9% saline. A complete description of the method of preparation for the ethyl methanesulfonate formulation will be documented in the study records and described in the final report. The ethyl methanesulfonate formulation will be prepared on the second and third day of positive control dosing (Days 1 and 2 for the positive control animals). The ethyl methanesulfonate formulation does not need to be stirred continuously and may be stored at room temperature during preparation and dosing.

9.2. Analysis of Positive Control Article Formulations

No analysis to confirm either cyclophosphamide or ethyl methanesulfonate in the dosing formulations will be performed as part of this study.

10. TEST SYSTEM

Species:	Rat
Strain:	Crl:CD(SD) Sprague Dawley rat
Source:	Charles River Laboratories. Facility to be documented in the raw data.
Number of Males Ordered **(Phase 1)**:	35
Number of Females Ordered **(Phase 1)**:	35
Number of Males Ordered (Phase 2):	**33**
Target Age at the Initiation of Dosing:	7-8 weeks. Animals not utilized on study will be assigned to the Charles River colony or euthanized by CO_2 inhalation and discarded.
Target Weight at the Initiation of Dosing:	200 to 320 g (males)/150 to 250 g (females)

The actual age and weight of animals received will be listed in the Final Report. Females will be nulliparous and nonpregnant.

10.1. Identification System

A permanent animal number will be assigned to each individual animal. Each animal will be identified using a subcutaneously implanted electronic identification microchip (BMDS system). The microchip will be the primary means to uniquely identify animals assigned to study. Individual cage cards will be affixed to each cage and will display at least the animal number, cage number, group number, dosage level, study number, and sex of the animal.

Replacement microchips may be implanted as necessary throughout the course of the study. An ear tag may be used as the alternate unique identifier.

10.2. Justification for Selection

The Sprague Dawley rat was chosen as the animal model for this study as it is an accepted rodent species for nonclinical toxicity testing by regulatory agencies.

The number of animals selected is based on OECD Guidelines 474 and 489. Group size at the initiation of the study (up to 6/sex/group) was chosen to provide a minimum of 5 analyzable samples/sex/group for each endpoint.

For phase 2, since no difference in systemic toxicity was noted between males and females in phase 1, only males will be used.

At this time, studies in laboratory animals provide the best available basis for extrapolation to humans and are required to support regulatory submissions. Acceptable models which do not use live animals currently do not exist.

11. SPECIFIC ANIMAL MAINTENANCE SCHEDULE

11.1. Animal Receipt and Acclimation

Each animal will be inspected by qualified personnel upon receipt. Animals judged to be in good health will be placed in acclimation for at least 7 days. See respective sections for parameters to be evaluated.

11.2. Animal Housing

All animals will be housed in groups of 2 to 3 per cage following receipt in clean, solid bottom cages with bedding material (Bed-O-Cobs® or other suitable material) in an environmentally controlled room. Animals may be temporarily separated for protocol-specified activities and this will be documented in the study records. Any animal whose cage mate(s) are removed from study (morbidity or unscheduled death) may remain individually housed for the remainder of the study. In addition, animals may be individually housed due to aggressive behavior toward a cage mate. The cages will be cleaned and changed routinely at a frequency consistent with maintaining good animal health.

All animals will be maintained in accordance with the *Guide for the Care and Use of Laboratory Animals*.[1] The facilities at Charles River Ashland are fully accredited by the Association for

Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

11.3. Environmental Conditions

Environmental controls in the animal room will be set to maintain a temperature of 68°F to 78°F (20°C to 26°C) and relative humidity at 30% to 70%. Temperature and relative humidity will be monitored continuously. Data for these 2 parameters will be scheduled for automatic collection on an hourly basis. Fluorescent lighting will provide illumination for a 12-hour light/dark photoperiod. Temporary interruptions to the light/dark cycles may be made to accommodate protocol-specified activities. The ventilation rate will be set at a minimum of 10 room air changes per hour, 100% fresh air.

11.4. Drinking Water

Reverse osmosis-treated water will be available ad libitum, except during exposure periods and acclimation to nose-only restraint tubes. The municipal water supplying the laboratory will be analyzed for contaminants according to SOPs. No contaminants are reasonably expected to be present that would interfere with the objectives of the study, therefore, no testing will be conducted as part of the study.

11.5. Basal Diet

PMI Nutrition International, LLC Certified Rodent LabDiet® 5CR4 meal will be offered ad libitum during the study, except during exposure periods, and acclimation to nose-only restraint tubes. SOPs provide specifications for acceptable levels of heavy metals and pesticides that are reasonably expected to be present in the diet without interfering with the purpose or conduct of the study. No contaminants are reasonably expected to be present that would interfere with the objectives of the study; therefore, no testing will be conducted as part of the study.

11.6. Environmental Enrichment

Enrichment devices will be provided to each animal for environmental enrichment and to aid in maintaining the animals' oral health, beginning during acclimation and continuing throughout the course of the study.

12. EXPERIMENTAL DESIGN

12.1. Acclimation to Restraint in Nose-Only Exposure Holding Tubes

To screen animals for poor tolerance of restraint and to limit the potential effects on respiration of the novel environment/conditions of restraint, the animals will be subjected to restraint in nose-only exposure tubes.

Animals will be acclimated to restraint tubes four times (1 acclimation/day) prior to their first day of exposure. Animals will be acclimated to restraint in nose-only exposure restraint tubes by

increasing the restraint time over the acclimation period (1st day-1 hour, 2nd day-2 hours, 3rd day-4 hours and 4th day-6 hours; times are approximate). Following the restraint period, each animal will be observed for clinical signs of injury or stress.

12.2. Animal Selection and Randomization

During the acclimation period, animals judged to be suitable for testing will be assigned to groups at random based on body weight stratification into a block design using a computer program. Animals may be arbitrarily assigned to the positive control group. Animals will then be arranged into the appropriately assigned groups and housed in social groups of 2 to 3 per cage within the treatment group.

Individual body weights at randomization will be within ± 20% of the mean. Following randomization, it may be necessary to replace individual animal(s), prior to initiation of dosing. Individual replacement animals will be selected from the remaining unassigned animals and assigned arbitrarily. The reason(s) for replacement will be documented in the study records. Animals may not be replaced after Day 1.

12.3. Route and Rationale of Test Substance Administration

The route of administration will be inhalation exposure since this is the unintended route of human exposure. Nose-only exposure methods will be used to reduce the potential for dermal exposure or oral exposure resulting from grooming. In order to perform nose-only exposure, it is necessary to restrain the rats in specially designed nose-only holding tubes. The period of restraint is necessary to achieve the maximum feasible exposure to the test substance.

Per OECD Guidelines 474 and 489, it is not necessary to administer the concurrent positive control article by the same route as the test substance. The route of administration of the positive control articles (oral gavage) was chosen based on past experience by Charles River and BioReliance with both types of assays.

12.4. Organization of Test Groups, Dosage Levels, and Treatment Regimen

12.4.1. Organization of Test Groups

The following tables presents the study group arrangement.

Study Design **Phase 1**

Group Number	Treatment	Target Exposure Concentration (ppm)	Positive Control Concentration (mg/mL)	Positive Control Dose Volume (mL/kg)	Number of Animals[a,c]	
					Males	Females
1	Filtered Air	0	NA	NA	6	6
2	[redacted]	375	NA	NA	6	6
3	[redacted]	750	NA	NA	6	6
4	[redacted]	1500	NA	NA	6	6
5	Positive Control[b]	CP: 20 mg/kg/day	2	10	6	6
		EMS: 200 mg/kg/day	20	10		

[a] Filtered air (negative control group) and vaporized test substance will be administered via nose-only inhalation for 3 consecutive days (6 hrs/day), Days 1-3.

[b] The positive control substance, cyclophosphamide (CP), will be administered via oral gavage to rats in Group 5 at a dosage of 20 mg/kg/day on Days 1 and 2. The positive control article, ethyl methanesulfonate (EMS), will be administered via oral gavage to rats in Group 5 at a dosage of 200 mg/kg/day on Days 2 and 3.

[c] Up to 6 surviving rats/group will be utilized for collection of peripheral blood between 1 and 2 hours following the final exposure/dose. Additionally, for 5 surviving rats/group, samples of bone marrow in addition to samples of the liver, lung, kidney, and nasal tissue will be collected between 2 and 4 hours after completion of the final exposure (Groups 1-4) or after the second dose of EMS (Group 5), which should coincide with being approximately 18-24 hours after the second dose of cyclophosphamide.

NA Not applicable

Study Design Phase 2

Group Number	Treatment	Target Exposure Concentration (ppm)	Positive Control Concentration (mg/mL)	Positive Control Dose Volume (mL/kg)	Number of Animals[a,c] Males
1	Filtered Air	0	NA	NA	6
2	[redacted]	375	NA	NA	6
3	[redacted]	750	NA	NA	6
4	[redacted]	1500	NA	NA	6
5	Positive Control[b]	EMS: 200 mg/kg/day	20	10	6

[a] Filtered air (negative control group) and vaporized test substance will be administered via nose-only inhalation for 2 consecutive days (6 hrs/day), Days 1-2.

[b] The positive control article, ethyl methanesulfonate (EMS), will be administered via oral gavage to rats in Group 5 at a dosage of 200 mg/kg/day on Days 1 and 2.

[c] Tissue samples of the liver, lung, kidney, and nasal tissue from 5 surviving rats/group will be collected between 2 and 4 hours after completion of the final exposure (Groups 1-4) or after the second dose of EMS (Group 5).

NA Not applicable

12.4.2. Justification of Dosage Levels

The target exposure concentrations were selected by the Sponsor Representative in consultation with the Study Director based, in part, on a previous inhalation study conducted using [redacted].

In that study, [redacted] had localized effects in the nose at concentrations of 250 and 550 ppm. Based on these findings, exposure concentrations of 375, 750, and 1500 ppm were selected for this study.

12.4.3. Treatment Regimen

Phase 1

Filtered air (control) and test substance atmospheres will be administered as 6-hour, nose-only inhalation exposures for three consecutive days, as outlined below.

Cyclophosphamide will be administered via oral gavage to rats in Group 5 at a dosage of 20 mg/kg/day on Days 1 and 2. Ethyl methanesulfonate will be administered via oral gavage to rats in Group 5 at a dose of 200 mg/kg/day on Days 2 and 3.

The first day of dosing will be Day 1; the week of dosing will be Week 1.

On the day of each animal's final exposure/dose, surviving animals from each study group will be euthanized and samples will be collected between 2 and 4 hours post-exposure. To accommodate post-exposure activities, initiation of initial and final exposures will be staggered (by group and or sex).

All animals will be housed in an animal colony room during non-exposure hours. Prior to each exposure, the animals selected for exposure will be transferred to nose-only restraint tubes and transported to the exposure room(s). Animals will then be exposed for the requisite duration and returned to their home cages.

Phase 2

Filtered air (control) and test substance atmospheres will be administered as 6-hour, nose-only inhalation exposures for two consecutive days, as outlined below.

Ethyl methanesulfonate will be administered via oral gavage to rats in Group 5 at a dose of 200 mg/kg/day on Days 1 and 2.

The first day of dosing will be Day 1; the week of dosing will be Week 1.

On the day of the final exposure/dose, surviving animals from each study group will be euthanized and samples will be collected between 2 and 4 hours post-exposure. To accommodate post-exposure activities, initiation of the final exposures will be staggered (by group).

All animals will be housed in an animal colony room during non-exposure hours. Prior to each exposure, the animals selected for exposure will be transferred to nose-only restraint tubes and transported to the exposure room(s). Animals will then be exposed for the requisite duration and returned to their home cages.

12.5. Methods of Administration

12.5.1. Oral Gavage

The positive control articles, CP and EMS, will be administered orally by gavage. Each dose will be administered via a syringe equipped with an Instech feeding tube. The dose volume will be 10 mL/kg.

The CP dosing formulation will be stirred continuously on ice during dosing.

The EMS dosing formulation will be maintained at room temperature during dosing.

Individual doses will be calculated based upon the most recent individual body weights to provide the proper dose.

12.5.2. Nose-Only Inhalation

Nose-only exposures will be conducted using stainless steel, ~~**conventional**~~ nose-only exposure systems ~~**(CNOS)**~~, with grommets in exposure ports to engage animal holding tubes. Dedicated exposure systems will be used: one for the filtered-air control group and one for the test substance-treated groups.

The exposure systems will be operated under dynamic conditions. Air supplied to the exposure systems will be provided from a HEPA and charcoal-filtered air source and/or breathing quality, in-house compressed air source. Exposure system airflow rates will be based on the

requirements for vapor generation and dilution and will provide a sufficient volume for the number of animals exposed and for exposure atmosphere sampling.

Exposure system airflow rates will be recorded at least hourly during each exposure. The airflow rates for each nose-only system may be monitored by measuring the pressure drop between the ports of a venturi tube using a Dwyer Magnehelic® Indicating Transmitter pressure gauge. Each gauge will be calibrated for conversion from pressure to airflow in standard liters per minute. If venturi-based methods for measuring system airflow rate cannot be used, recorded values will be calculated from calibration curves for the vapor generation device and flowmeters.

Average temperature and relative humidity of the exposure atmospheres will be 22 ± 3ºC and 50 ± 20%, respectively. Temperature and relative humidity will be monitored with a temperature and humidity transmitter probe for each nose-only exposure system.

Temperature and relative humidity will be monitored and recorded at least hourly during each exposure. If possible, temperature, relative humidity, and airflow rates will be monitored and recorded through the use of the Inhalation Exposure Data Collection System (WINH) and a personal computer. In the event of a failure of the automated data collection system (e.g., malfunction of a component or of a hardware connection), manual recording will be used.

Oxygen content of the exposure atmosphere at each exposure concentration will be determined during method development and will be at least 19%.

12.5.3. Control and Test Substance Exposure Atmosphere Generation Methods

For the filtered-air control group (Group 1), HEPA- and charcoal-filtered humidified air will be mixed with breathing-quality in-house compressed air as needed to provide a comparable airflow rate and relative humidity to that used for the test substance-treated groups.

Final details of methods for generation of exposure atmospheres, equipment specifications, and operating conditions will be defined during the method development and included in a system description, which will be approved by the Study Director.

12.6. Methods of Characterization of Exposure Atmospheres

12.6.1. Nominal Concentration

If possible based on the generation methods used, nominal exposure concentrations will be calculated. At a minimum, test substance usage will be determined and documented in the study records.

12.6.2. ~~Actual~~Analyzed Concentrations

Analyzed concentrations of [redacted] in the exposure atmospheres will be determined using a gas chromatograph (GC) equipped with a Flame Ionization Detector (FID).

Concentration will be recorded approximately every 60 minutes throughout the exposure period. Additional samples may be collected for diagnostic purposes and to assist the laboratory technical staff in maintaining stable exposure concentrations.

Samples may be collected from the nose-only exposure systems using tubing connected to a vacuum pump or by a computer controlled multiposition valve and a sample loop. If applicable, the WINH Inhalation Exposure Data Collection System will control the GC sampling and acquire peak area values from the GC for the concentration analyses and the program will calculate exposure concentrations from the peak area values using a quadratic equation representing the standard curve for the GC calibration. Alternatively, the control of the GC sampling and calculation of exposure concentrations may be performed manually.

The calibration of the gas chromatograph will be considered acceptable if the R^2 value is ≥ 0.98 and the individual points of the calibration are within 10% of their target concentrations.

Final details of methods for determination of exposure concentrations, including sampling conditions, equipment specifications and operating conditions, will be defined during method development and included in a system description, which will be approved by the Study Director.

13. IN-LIFE PROCEDURES, OBSERVATIONS, AND MEASUREMENTS

13.1. Viability Observations

All animals will be observed for mortality, abnormalities, and signs of pain and distress twice daily, once in the morning and once in the afternoon. Moribund animals will be euthanized and necropsied as soon as possible. Animals found dead will be necropsied as soon as possible to ensure that tissues will not be lost due to autolysis.

13.2. Animals to Be Euthanized in Extremis

Animals that experience severe or chronic pain or distress that cannot be relieved will be euthanized. All main study animals to be euthanized in extremis will undergo a final detailed clinical observation and a body weight will be collected prior to release for euthanasia and subsequent necropsy. Animals will be anesthetized by isoflurane inhalation followed by exsanguination, which completes the euthanasia.

13.3. Observations

13.3.1. Cage Side Observations (Groups 1-4)

- Prior to exposure
- 0 to 2 hours post exposure

The absence or presence of findings will be recorded for individual animals.

The prior to exposure observations need not be conducted on the days of detailed clinical observations, provided that the detailed clinical observations are conducted prior to exposure.

13.3.2. Cage Side Observations (Group 5)

- At the time of dosing
- 0 to 2 hours postdose

The absence or presence of findings will be recorded for individual animals.

13.3.3. Detailed Clinical Observation (All Animals)

- Within 4 days of receipt
- On the day of randomization
- On Day 1 (prior to exposure)
- ~~**On Day 3 (prior to exposure)**~~
- **On the day of necropsy (prior to exposure)**

The absence or presence of findings will be recorded for individual animals.

13.3.4. Social Housing Observations

If there are any cage findings for a social group that need to be recorded, the findings will be attributed to all animals within each socially housed group.

13.3.5. Unscheduled Observations

Findings noted outside the above-specified observation periods will also be recorded. Only the presence of unscheduled observations will be recorded; the absence of findings will thus not be recorded.

13.3.6. Individual Body Weights

- Within 4 days of receipt
- On the day of randomization
- On Day 1
- ~~**On Day 3**~~
- **<u>On the day of necropsy (prior to exposure)</u>**

13.3.7. Food Weight Data

- On Day 1
- ~~**On Day 3**~~
- **<u>On the day of necropsy (prior to exposure)</u>**

Food consumption will be measured on a per cage basis. Food consumption will be normalized to the number of animals/cage and will be reported in grams/animal/day. Food spillage within the cage will not be accounted for due to the use of bedding.

14. LABORATORY EVALUATIONS

14.1. Micronucleus Blood Collection (Groups 1-5<u>; Phase 1 only</u>)

- All surviving rats/group (Groups 1-4) will be utilized for collection of peripheral blood approximately 1-3 hours following the last exposure.
- All surviving rats/group (Group 5) will be utilized for collection of peripheral blood approximately 1-3 hours following the second dose.

The animals will not be fasted prior to blood collection. Approximately 0.5 mL of blood will be collected into K_2EDTA tubes (2.0 mL tube) from the jugular vein (blood will be collected from the retro-orbital sinus of animals anesthetized with isoflurane, if necessary). Blood samples will be checked for clots. Samples will be redrawn, as necessary, to provide adequate blood samples.

Whole blood will be transferred to the Immunotoxicology group and processed as per Section 17.1.

15. TERMINAL PROCEDURES – ANATOMIC PATHOLOGY

15.1. Macroscopic Examination

<u>Animals Euthanized in Extremis or Dying Spontaneously:</u>

A gross necropsy will be conducted on animals dying spontaneously or euthanized in extremis.

Animals will be anesthetized by isoflurane inhalation followed by exsanguination, which completes the euthanasia. Necropsy will include examination of the external surface, all orifices

and the cranial, thoracic, abdominal, and pelvic cavities, including viscera. Carcasses will be discarded without tissue collection.

Scheduled Necropsy:

Two to four hours following the final exposure, the animals will be anesthetized by isoflurane inhalation followed by exsanguination, which completes the euthanasia. A gross macroscopic examination will not be performed on rats surviving to the scheduled euthanasia.

Immediately following euthanasia, for 5 animals/sex/group, bone marrow from the right femur will be collected and processed as described in Section 15.3. It may be necessary to use the left femur instead of the right femur. The reason(s) for using the left femur will be appropriately documented in the study records.

Additionally, samples of the lung, liver, kidney, and nasal tissue will be collected and processed as described in Section 15.2. At the time of necropsy, the following table notes the tissues and organs that will be collected and placed in 10% neutral-buffered formalin. The carcasses and remaining tissues will be discarded.

Tissue Collection and Preservation

Liver (sections of 2 lobes) Lungs (including bronchi, fixed by constant pressure inflation with fixative)	Kidney

Additionally, for Groups 1-4 in which the extra animal/sex survives, the nasal cavity will be collected, flushed with fixative, and placed in 10% neutral-buffered formalin. For these animals, samples for the Comet assay (liver, lung, kidney, and nasal tissue), or bone marrow, or other tissues will not be collected.

15.2. Tissue Collection for Comet Assay

Five (5) surviving animals**/sex**/group **(as appropriate by phase)** will have nasal tissue, lung, kidney, and liver collected between 2 and 4 hours following their last exposure (Groups 1-4) or second dose of EMS (Group 5). Charles River personnel will remove the head, lung, kidney, and liver. If not needed, the extra animal/sex will be anesthetized by isoflurane inhalation followed by exsanguination, which completes the euthanasia, and the nasal cavity will be collected (as previously described) (Groups 1-4) or discarded without tissue collection (Group 5).

Samples of the nasal tissue, left lung, kidney, and liver will be collected by BioReliance staff and placed in chilled mincing solution (Hanks' balanced salt solution with EDTA and DMSO). The tissue samples will then be minced with fine scissors to release the cells. The cell suspension will be strained into a pre-labeled conical polypropylene tube through a Cell Strainer and may be kept on wet ice during preparation of the slides.

Preparation of the cell suspensions will be performed by BioReliance staff while at the Testing Facility according to applicable BioReliance SOPs.

Any remaining cell suspensions from phase 2 will be frozen at -20ºc for possible future analysis.

Slides will be processed as described in Section 16.

15.3. Bone Marrow Collection (Phase 1 Only)

Five (5) surviving animals/group will have bone marrow collected between 2 and 4 hours following their last exposure (Groups 1-4) or second dose of EMS (Group 5) as described below. If not needed, the extra animal/sex will be anesthetized by isoflurane inhalation followed by exsanguination, which completes the euthanasia, and the nasal cavity will be collected (as previously described) (Groups 1-4) or discarded without tissue collection (Group 5).

Using a syringe containing fetal bovine serum, the bone marrow will be aspirated or flushed from the right femur into a centrifuge tube. Cells will be aspirated at least 2-3 times to ensure all cells are collected and there are no cell clumps. The suspension will be centrifuged and all but approximately 0.25 mL of the supernatant will be removed. The pellet will be resuspended in the remaining fetal bovine serum. Using a 23-gauge needle with syringe, approximately one drop of the cell suspension will be used to prepare a bone marrow smear on an appropriately labeled, clean microscope slide. A minimum of four slides per animal will be prepared. Slides will be allowed to air dry and will then be fixed in 100% methanol for approximately 20 minutes and allowed to air dry again. The slides will be stored for possible future analysis. If not stained and scored, the slides will be archived with the study.

15.4. Histology

To be added by amendment, if warranted based on the results of the Comet assay.

15.5. Histopathology

To be added by amendment, if warranted based on the results of the Comet assay.

16. COMET ASSAY

Section **16** provides a brief description of the activities that will be performed by BioReliance for this study (BioReliance Reference No. AF56EM.151.BTL). Work will be conducted in accordance to their onsite SOPs.

16.1. Preparation of Comet Slides

Preparation of Comet slides will be performed by BioReliance staff while at the Testing Facility according to applicable BioReliance SOPs.

At least four slides or wells per animal will be prepared per organ/tissue. An aliquot of 1.25-7.5 µL of each cell suspension per slide will be mixed with 0.5% low melting agarose. The cell/agarose suspension will be applied to microscopic slides, commercially available, pre-treated multi-well or previously coated with 1% normal melting agarose. The slides will be placed at 2-8ºC for at least 15 minutes to allow the gels to solidify.

Any remaining cell suspension remaining following slide preparation during phase 2 will be will be stored frozen at -20°c for possible future analysis.

Slides will be identified with a random code that reflects the study number, group, animal number, and organ/tissue. Three slides or wells will be used for scoring and the remaining slides or wells will be backup. These slides or wells may be used in additional scoring, if deemed necessary.

Each slide will be submerged in a lysis solution at least overnight at 2-8ºC. The lysis solution will be composed of 100mM EDTA (disodium), 2.5 M sodium chloride, 10 mM tris hydroxymethyl aminomethane in purified water; pH10; 1% triton-X100 and 10% DMSO will be added on the day of use or commercially available lysis solution will be used after the addition of 10% DMSO on the day of use.

After cell lysis, slides will be washed with neutralization buffer (0.4 M tris hydroxymethyl aminomethane in purified water, approximately pH 7.5) and placed in an electrophoresis chamber. The chamber reservoirs will be filled with alkaline buffer (300 mM sodium hydroxide and 1 mM EDTA (disodium) in purified water, pH > 13) for approximately 20 minutes at 2-10ºC, protected from light for the unwinding of DNA. Electrophoresis will be conducted in the same buffer following DNA unwinding for 30 minutes at 0.7 volts/cm.

The slides will be removed from the electrophoresis chamber and washed with neutralization buffer for at least 10 minutes. The slides will be dehydrated with 200-proof ethanol for at least 5 minutes, then air-dried for at least 2 hours and then stored at room temperature with desiccant.

16.2. Slide Shipment

Slides of the processed nasal tissue, lung, kidney, and liver prepared by BioReliance at the Test Facility will be stored at room temperature with desiccant. Slides will be shipped on the first available Monday, Tuesday, or Wednesday at ambient temperature to BioReliance by overnight shipment to the following address:

BioReliance
Toxicology Testing Facility (Building 5)
9630 Medical Center Drive
Rockville, MD 20850

Attention: Albert Brew-Hagan
(Phone: 301-610-2146, Fax: 301-610-2560,
E-mail: albert.brew@ milliporesigma.com, marilena.lekavicius@ milliporesigma.com, faith.rider@external.milliporesigma.com, and lana.mcdowell@ milliporesigma.com.

16.3. Slide Staining

Staining of Comet slides will be performed by BioReliance staff at their Test Site according to applicable BioReliance SOPs.

Slides designated for staining will be stained with Sybrgold™ prior to scoring.

16.4. Scoring of Comet Slides

Scoring of Comet slides will be performed by BioReliance staff at their Test Site according to applicable BioReliance SOPs.

Three slides or wells per animal per treatment will be used. Fifty randomly selected cells will be scored per slide, resulting in a total of 150 cells evaluated per animal. If one of the three slides or wells does not have 50 scorable cells, additional cells may be scored using the backup slides or wells. If 150 cells are not available, then the calculations will be performed using the number of scorable cells.

The following endpoints of DNA damage will be assessed and measured:

- Comet Tail Migration; defined as the distance from the perimeter of the Comet head to the last visible point in the tail.
- % Tail DNA (also known as % tail intensity or % DNA in tail); defined as the percentage of DNA fragments present in the tail.
- Tail Moment (also known as Olive Tail Moment); defined as the product of the amount of DNA in the tail and the tail length [(% Tail DNA x Tail Length)/ 100].[2]

Each slide will also be examined for indications of cytotoxicity. The rough estimate of the percentage of "clouds" will be determined by scanning 150 cells per animal, when possible, (percentage of "clouds" is calculated by adding the total number of clouds for all slides scored, dividing by the total number of cells scored, and multiplying by 100). Every effort will be made to score at least 150 cells; otherwise, the total number of scorable cells will be used for calculations. The "clouds," also known as "hedgehogs," are a morphological indication of highly damaged cells often associated with severe genotoxicity, necrosis, or apoptosis. A "cloud" is produced when almost the entire cell DNA is in the tail of the comet and the head is reduced in size, almost nonexistent.[3] "Clouds" with visible gaps between the nuclei and the comet tail will be excluded from comet image analysis.

Slides will be discarded prior to report finalization.

16.5. Criteria for Determination of a Valid Test

The DNA damage data (% tail DNA) in the negative control group (filtered air control) is expected to be within the historical vehicle control (negative control) range, and the positive control group must be significantly increased relative to the concurrent negative control group ($p \leq 0.05$). Additionally, concurrent positive controls should induce responses that are compatible with those generated in the historical positive control database.

16.6. Evaluation of Test Results

Once the criteria for a valid assay have been met, the results will be evaluated as follows:

Means of 150 counts of % tail DNA, Tail moment, and Tail migration will be presented for each animal and each organ. The mean and standard deviation of the mean values for % tail DNA will be presented for each treatment group.

Statistical analysis will be performed only for % tail DNA. If deemed necessary, other parameters of DNA damage (e.g., Tail moment) may be analyzed statistically and used in the overall assessment of DNA damage.

All conclusions will be based on sound scientific judgment. As a guide to interpretation of the data, the following will be considered:

- The test substance will be considered to induce a positive response in a particular tissue if the mean % tail DNA (or other parameters of DNA damage) in one or more test substance groups (doses) is significantly elevated relative to the concurrent negative control group.
- The test substance will be judged negative for induction of DNA damage if no statistically significant increase in the mean % DNA damage (or other parameters) in the test substance groups relative to the concurrent negative control group is observed.

However, the results of the statistical analysis may not be the only criterion in determination of the test substance potential to induce DNA damage. The following may be taken in consideration:

- The historical vehicle control (negative control) data; a statistically significant increase in the mean % DNA (or other parameters) may not be considered biologically relevant if the values do not exceed the range of historical vehicle control (negative control).
- Because cells undergoing necrosis or degeneration are prone to DNA degradation, independent of direct genotoxic effects of the test substance, doses that are found to be cytotoxic, by histopathology evaluation, may not be considered as relevant doses and may not be taken in consideration during the generation of the study conclusions. Accordingly, any statistically significant increase in DNA damage occurring at a cytotoxic dose may not be considered as a positive finding.
- A dose-dependent increase in the mean % tail DNA (or other parameters) across the dose levels tested; if a dose-response is evident with no statistically significant increase, additional testing, including histopathology evaluation of the tissue, may be considered.
- If criteria for either a positive or negative response are not met, the results may be judged as equivocal.

The Comet Assay report will include, but will not be limited to, information about the test substance results, discussion of comet assay results, conclusion of comet assay, historical control data, statement of compliance, QA statement, and location of archived material.

The Comet Assay report will be included as an appendix to the Charles River final report and appropriate interpretation and data will be incorporated into the text of the Charles River final report.

16.7. Electronic Data Collection Systems

Electronic systems used for the collection or analysis of data will include but not be limited to the following (version numbers are maintained in the system documentation):

Electronic Systems

Program/System	Description
LIMS Labware System	Study tracking
Excel (Microsoft Corporation)	Calculations
Minitab	Statistics
BRIQS	Deviation and audit reporting
Comet Assay IV	Scoring of Slides

17. MICRONUCLEUS TEST (PHASE 1 ONLY)

Section 17 provides a brief description of the micronucleus analysis activities that will be performed by BioReliance for this study (BioReliance Reference No. AF56EM.129FLOWPBGLP.BTL). Work will be conducted in accordance to their onsite SOPs.

17.1. Whole Blood Preparation

All whole blood samples will be diluted in anticoagulant. Two aliquots of approximately 180 µL each (primary and secondary samples) of the diluted whole blood samples will be placed into separate 15 mL conical vials containing cold fixative. The samples will then be fixed in cold methanol for at least 72 hours. After at least 72 hours of fixation, both sets of samples will be removed from frozen storage and washed out of fixative.

The primary and secondary samples will be placed into Long Term Storage Solution (LTSS) and stored in a freezer set to maintain a target of -70°C until shipped to the BioReliance, the Test Site for micronucleus evaluation.

The samples will be shipped in two separate shipments to BioReliance for evaluation. Shipments will be on different days, on the first available non-holiday Monday, Tuesday or Wednesday, the first set of samples will be shipped, with the second shipment on the following day. Samples will be shipped on dry ice to the following:

BioReliance
Toxicology Testing Facility (Building 5)
9630 Medical Center Drive
Rockville, MD 20850

Attention: Albert Brew-Hagan
(Phone: 301-610-2146, Fax: 301-610-2560,
E-mail: albert.brew@ milliporesigma.com, marilena.lekavicius@ milliporesigma.com,
faith.rider@external.milliporesigma.com, and lana.mcdowell@ milliporesigma.com.

Receipt of Blood Samples

Upon receipt, the fixed blood samples in LTSS will be delivered to the laboratory for storage at -80±5°C until flow cytometric processing.

Detection of Micronucleated Reticulocytes with Flow Cytometry

Of the up to 6 samples/sex/group available, 5 samples in LTSS will be washed with ice cold 1% FBS solution and maintained on wet ice. The cells will then be pelleted by centrifugation, and the supernatant will be poured off leaving a small amount of supernatant with the pellet. The cells will be re-suspended and 20 µL of suspension will be added to 80 µL of staining solution containing RNase, FITC-conjugated anti-CD 71 antibodies and PE-conjugated anti-CD 61 antibodies. The samples will be incubated at 2 to 8ºC for 30 minutes, re-suspended, then incubated at room temperature for an additional 30 minutes. 0.3 – 2 mL of DNA staining solution (propidium iodide) will be added then the samples will be placed on wet ice for at least 5 minutes prior to the flow cytometric analysis.

The frequency of micronucleated reticulocytes in peripheral blood will be analyzed after flow cytometer calibration using Malaria infected biostandard and negative control standards provided in the Litron kit. Up to 20,000 RETs per animal, when possible, will be analyzed.

Remaining samples will be discarded prior to report finalization.

Calculation of Flow Cytometric Analysis

The proportion of reticulocytes to total number of cells scored (%RETs) will be determined for each animal and treatment group. This calculation will be carried out as indicated below:

$$\% \text{ RET} = \frac{(UL + UR) \times 100}{(UL + UR + LL + LR)}$$

UL: The number of events in the upper-left quadrant

UR: The number of events in the upper-right quadrant

LL: The number of events in the lower-left quadrant

LR: The number of events in the lower-right quadrant

The %RETs will serve as a parameter of the test substance cytotoxicity in peripheral blood. A decrease in this ratio in the test substance groups, as compared to the Filtered Air control, would indicate a toxic effect of the test substance while an increase would represent a sign of recovery from earlier toxic insult.

The quantization of the MnRETs in peripheral blood will be expressed as percentage of MnRETs per total number of reticulocytes evaluated. The %MnRETs will be presented for each animal and the mean ± standard deviation will be calculated and presented for each treatment group as follows:

$$\%\text{MnRET} = \frac{(\text{UR}) \times 100}{(\text{UL} + \text{UR})}$$

17.2. Criteria for Determination of a Valid Test

Cell Analysis

A target of 20,000 RETs/animal will be analyzed for the presence of micronuclei (MnRETs) whenever possible. The proportion of reticulocytes to total number of cells scored (%RETs) will be determined for each animal and treatment group. The %RETs will serve as a parameter of the test article cytotoxicity in peripheral blood. A reduction in the RET proportions to less than 5% of the Filtered Air control value will be considered excessively cytotoxic and the animal data will be excluded from evaluation. Animals with fewer than 4000 RETs may be excluded from the analysis. Other quality indicators (e.g., flow plots) may be used to exclude animals with poor quality data.

Negative Controls

The group mean frequency of MnRETs should ideally be within the 95% control limits of the distribution of the historical negative control database. If the concurrent Filtered Air control data fall outside the 95% control limits, they may be acceptable as long as these data are not extreme outliers (indicative of experimental or human error).

Positive Controls

The positive control must induce a statistically significant positive response ($p \leq 0.05$).

17.3. Evaluation of Test Results

A test substance will be considered to have induced a positive response if:

a) at least one of the test substance doses exhibits a statistically significant increase when compared with the concurrent Filtered Air control ($p \leq 0.05$), and

b) when multiple doses are examined at a particular sampling time, the increase is dose-related ($p \leq 0.01$ and $R^2 \geq 70\%$), and

c) results of the group mean or of the individual animals in at least one group are outside the 95% control limit of the historical negative control data.

A test substance will be considered to have induced a clear negative response if none of the criteria for a positive response were met.

If the response is neither clearly positive nor clearly negative, or in order to assist in establishing the biological relevance of a result, the data will be evaluated by expert judgment and/or further investigations. Possible additional work may include scoring additional cells (where appropriate) or performing an additional experiment that could employ the use of modified experimental conditions. Such additional work will only be carried out following consultation with, and at the request of, the Sponsor.

In some cases, even after further investigations, the data set will preclude making a conclusion of positive or negative, at which time the response will be concluded to be equivocal. In such cases, the Principal Investigator will use sound scientific judgment and report and describe all considerations.

The Micronucleus Assay report will include, but will not be limited to, information about the test substance results, discussion of micronucleus assay results, conclusion of micronucleus assay, historical control data, statement of compliance, QA statement, and location of archived material.

The Micronucleus Assay report will be included as an appendix to the Charles River final report and appropriate interpretation and data will be incorporated into the text of the Charles River final report.

17.4. Electronic Data Collection Systems

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

Electronic Systems

Program/System	Description
LIMS Labware System	Study tracking
Excel (Microsoft Corporation)	Calculations
Minitab	Statistics
FACSDiva (BD Biosciences)	Sample Analysis
BRIQS	Deviation and audit reporting

18. COMPUTERIZED SYSTEMS

The following critical computerized systems may be used in the study. The actual critical computerized systems used will be specified in the Final Report.

As Charles River Ashland transitions between various computer systems, the study number may appear as [redacted], [redacted] or [redacted] in the data records and report.

Data for parameters not required by protocol, which are automatically generated by analytical devices used will be retained on file but not reported. Statistical analysis results that are generated by the program but are not required by protocol and/or are not scientifically relevant will be retained on file but will not be included in the tabulations.

All computerized systems used for data collection during the conduct of this study have been validated (with the exception of Microsoft Office and GraphPad Prism® 2008); when a particular system has not satisfied all requirements, appropriate administration and procedural controls

were implemented to assure the quality and integrity of the data. The actual version number will be specified in the report.

Critical Computerized Systems

Program/System	Description
Bio Medic Data Systems (BMDS) Implantable Micro Identification™ (IMI-1000)	Animal identification
Inhalation Exposure Data Collection System (WINH)	Monitors and records inhalation chamber temperature, relative humidity, ventilation rate, and negative pressure.
Logbook™ ELN	System (Instem) used to document study events.
Metasys DDC Electronic Environmental Control System	Controls and monitors animal room environmental conditions.
Microsoft Office 2010 or higher; GraphPad Prism® 2008	Used in conjunction with the publishing software to generate study reports.
Provantis	Comprehensive system (Instem LSS Limited) used for in-life and postmortem data collection and reporting.
In-house reporting software Nevis 2012 (using SAS)	Reporting of in-life and postmortem data
Provantis Dispense™	Comprehensive system (Instem LSS Limited) to manage test materials, including receipt, formulation instructions, and accountability.
SAS®	Statistical (non-WTDMS™) analyses
Watson LIMS™	Laboratory Information Management System used for sample tracking, run planning, quantitation, and reporting results.
WIL Formulations Dispense System (WFDS)	In-house developed system for use in conjunction with Provantis Dispense™ to ensure proper storage and use of formulations.
WIL Metasys	In-house developed system used to record and report animal room environmental conditions.
WIL Toxicology Data Management System™ (WTDMS™)	In-house developed system used for collection and reporting of other data.

Note: Version numbers of WTDMS™ programs used for the study are presented on the report data tables (reporting programs), study records (input programs), and facility records (release dates).

19. STATISTICAL METHODS

19.1. In-life Parameters

Any data collected during the predose period will not be tabulated, summarized or statistically analyzed. All statistical analyses will be performed within the respective study phase, unless otherwise noted. Numerical data collected on scheduled occasions will be summarized and statistically analyzed as indicated below according to sex and occasion.

19.1.1. Constructed Variables

Body weight changes	Calculated between each scheduled interval.
Food Consumption	Calculated between each scheduled interval.

19.1.2. Descriptive Statistical Analyses

Means, standard deviations (or % coefficient of variation or standard error, when deemed appropriate), percentages, numbers, and/or incidences will be reported as appropriate by dataset.

19.1.3. Inferential Statistical Methods

All statistical tests will be conducted at the 5% significance level. All pairwise comparisons will be conducted using two sided tests and will be reported at the 1% and 5% levels, unless otherwise noted.

The pairwise comparisons of interest are listed below:

Group 2 vs. Group 1

Group 3 vs. Group 1

Group 4 vs. Group 1

Analyses will be performed according to the matrix below when possible, but will exclude any group with less than 3 observations.

Statistical Matrix

Variables for Inferential Analysis	Statistical Method Parametric/Non-parametric
Body Weight	X
Body Weight Gains	X
Food Consumption	X

19.1.4. Parametric/Non-parametric

All statistical tests will be conducted at the 5% significance level. All pairwise comparisons will be conducted using two sided tests and will be reported at the 1% and 5% levels, unless otherwise noted.

Levene's test will be used to assess the homogeneity of group variances.

The groups will be compared using an overall one-way ANOVA F-test if Levene's test is not significant or the Kruskal-Wallis test if it is significant. If the overall F-test or Kruskal-Wallis test is found to be significant, then pairwise comparisons will be conducted using Dunnett's or Dunn's test, respectively.

19.2. Comet Assay

In order to quantify the effects on DNA damage, the following statistical analysis will be performed:

- The use of parametric or non-parametric statistical methods in evaluation of data will be based on the variation between groups. The group variances for % tail DNA (or other parameters of DNA damage) generated for the negative control (filtered air control) and test substance-treated groups will be compared using Levene's test ($p \leq 0.05$). If the differences and variations between groups are found not to be significant, a parametric one-way ANOVA followed by a Dunnett post-hoc test will be performed ($p < 0.05$). If Levene's test indicates heterogeneous group variances ($p \leq 0.05$), the suitability of a transformation of the original data will be evaluated (e.g. using logarithm transformed values of the original data) in an attempt to meet the normality criteria. Afterwards, statistical analysis will be performed using the parametric tests described above. If parametric tests are not acceptable, non-parametric statistical methods (Kruskal Wallis or Mann Whitney test) may be used in evaluation of data.
- Linear regression analysis will be used to determine a dose response relationship ($p < 0.01$).
- Pair-wise comparison (Student's t-test, $p \leq 0.05$) will be used to compare the data from the positive control group against the negative control group. If needed, non-parametric statistical methods (Kruskal Wallis or Mann Whitney test) may be used in evaluation of data.

19.3. Peripheral Blood Micronucleus Data <u>(Phase 1 Only)</u>

Statistical analysis will be performed on the micronucleus frequency (%MnRET) and %RET using the animal as the unit. The mean and standard deviation of %MnRET and %RET will be presented for each treatment group.

The use of parametric or non-parametric statistical methods in evaluation of data will be based on the variation between groups. The group variances for micronucleus frequency for the Filtered Air control and test substance groups will be compared using Levene's test (significance level of $p \leq 0.05$). If the variation between groups is found not to be significant, a parametric one-way ANOVA will be performed followed by a Dunnett's post-hoc analysis to compare each dose group to the concurrent Filtered Air control. If Levene's test indicates heterogeneous group variances (significance level of $p \leq 0.05$), the suitability of a transformation of the original data will be evaluated (e.g. using logarithm transformed values of the original data) in an attempt to meet the normality criterion. Afterwards, statistical analysis will be performed using the parametric tests described above. If parametric tests are not acceptable, non-parametric statistical methods (Kruskal Wallis and/or Mann Whitney test) may be used in evaluation of data.

A linear regression analysis will be conducted to assess dose responsiveness in the test substance treated groups ($p \leq 0.01$ and $R^2 \geq 70\%$).

A pair-wise comparison (Student's T-test; $p \leq 0.05$) will be used to compare the positive control group to the concurrent vehicle control group. If parametric tests are not acceptable, non-

parametric statistical methods (Kruskal Wallis and/or Mann Whitney test) may be used in evaluation of data.

20. AMENDMENTS AND DEVIATIONS

Changes to the approved protocol shall be made in the form of an amendment, which will be signed and dated by the Study Director. Every reasonable effort will be made to discuss any necessary protocol changes in advance with the Sponsor.

All protocol and SOP deviations will be documented in the study records. Deviations from the protocol and/or SOP related to the phase(s) of the study conducted at a Test Site shall be documented, acknowledged by the PI/IS, and reported to the Study Director for authorization/acknowledgement. The Study Director will notify the Sponsor of deviations that may result in a significant impact on the study as soon as possible.

21. RETENTION OF RECORDS, SAMPLES, AND SPECIMENS

All study-specific raw data, electronic data, documentation, protocol, retained samples and specimens, and final reports will be archived by no later than the date of final report issue. All materials generated by Charles River or by a Test Site from this study will be transferred to a Charles River archive. At least 1 year after issue of the Draft Report, the Sponsor will be contacted.

Following finalization, the original signed final report (paper copy) will be transferred to the Sponsor at the following address:

[redacted]

All transferred records will be maintained in the Sponsor's archives. A full copy of the final signed report will be retained and archived at Charles River.

Any work product, including documents, specimens, and samples, that are required by this protocol, its amendments, or other written instructions of the Sponsor to be shipped by Charles River to another location will be appropriately packaged and labeled as defined by Charles River SOPs and delivered to a common carrier for shipment. Charles River will not be responsible for shipment following delivery to the common carrier.

22. REPORTING

A Draft Report will be prepared following completion of the study and will be finalized following consultation with the Sponsor. The report will include all information necessary to

provide a complete and accurate description of the experimental methods and results and any circumstances that may have affected the quality or integrity of the study.

The Sponsor will receive an electronic version of the Draft and Final Report provided in Adobe Acrobat PDF format (hyperlinked and searchable at final) along with a Microsoft Word version of the text. The PDF document will be created from native electronic files to the extent possible, including text and tables generated by the Testing Facility. Report components not available in native electronic files and/or original signature pages will be scanned and converted to PDF image files for incorporation. Additionally, as noted previously, a paper copy of the final report (with original signature page[s]) will be provided to the Sponsor.

Reports should be finalized within 6 months of issue of the Draft Report. If the Sponsor has not provided comments to the report within 6 months of draft issue, the report will be finalized by the Testing Facility unless other arrangements are made by the Sponsor.

23. ANIMAL WELFARE

This study will comply with all applicable sections of the Final Rules of the Animal Welfare Act regulations (Code of Federal Regulations, Title 9), the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* from the Office of Laboratory Animal Welfare,[4] and the *Guide for the Care and Use of Laboratory Animals* from the National Research Council.[1] The protocol and any amendments or procedures involving the care or use of animals in this study will be reviewed and approved by the Testing Facility Institutional Animal Care and Use Committee before the initiation of such procedures.

If an animal is determined to be in overt pain/distress, or appears moribund and is beyond the point where recovery appears reasonable, the animal will be euthanized for humane reasons in accordance with the *American Veterinary Medical Association (AVMA) Guidelines on Euthanasia* and with the procedures outlined in the protocol.[5]

By approving this protocol, the Sponsor affirms that there are no acceptable non-animal alternatives for this study, that this study is required by a relevant government regulatory agency and that it does not unnecessarily duplicate any previous experiments.

24. REFERENCES

1 National Research Council. Guide for the Care and Use of Laboratory Animals, Committee for the Update of the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, Division on Earth and Life Sciences; The National Academies Press: Washington, DC, 2011.

2 Olive PL., Banath JP., Durand RE. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cell using the "comet" assay. Radiat. Res., 122(1), 86-94, 1990.

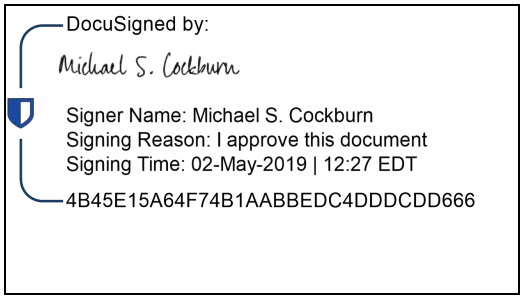
3 Collins AR., The Comet Assay for DNA Damage and Repair; Principles, Applications, and Limitations. Molecular Biotechnology, 26, 249-261, 2004.

4 Office of Laboratory Animal Welfare. *Public Health Services Policy on Humane Care and Use of Laboratory Animals*. Bethesda, MD: National Institutes of Health. March 2015.

5 American Veterinary Medical Association. *AVMA Guidelines on Euthanasia*. March 2013.

AMENDMENT APPROVAL

The signature below indicates that the Study Director approves the protocol amendment.



Michael S. Cockburn
Associate Research Scientist, Inhalation Toxicology
Study Director

SPONSOR PROTOCOL AMENDMENT APPROVAL

The protocol amendment was approved by the Sponsor by e-mail on 05 MAY 2019 ①. The signature below confirms the approval of the protocol amendment by the Sponsor Representative.

[redacted] Date: 2019-05-09

Sponsor Representative

① Added by: MSC2
MSC2
10 MAY 2019

FINAL PROTOCOL

Testing Facility Study No. [redacted]

Sponsor Reference No. [redacted]

A Combined *In Vivo* Micronucleus and Comet Assay of [redacted] in Sprague Dawley Rats

SPONSOR:

[redacted]

TESTING FACILITY:
Charles River Laboratories Ashland, LLC
1407 George Road
Ashland, OH 44805
United States

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1. OBJECTIVE

The objective of this study is to assess the potential of [redacted] to induce micronuclei and to cause DNA damage in rat liver, lung, kidney, and nasal tissue when administered via nose-only inhalation to Sprague Dawley rats for 6 hours per day for 3 consecutive days.

1.1. Study Classification

Study Category:	Genetic Toxicology
Study Type:	Genotoxicity In Vivo
Study Design:	Parallel
Primary Treatment CAS Registry Number:	Not Available
Primary Treatment Unique Ingredient ID:	[redacted]
Class of Compound:	Not Available

2. PROPOSED STUDY SCHEDULE

Proposed study dates are listed below. Actual applicable dates will be included in the Final Report.

Animal Arrival:	08 Jan 2019
Animal Randomization:	17 Jan 2019
Initiation of Dosing – Males:	19 Jan 2019
Initiation of Dosing – Females:	20 Jan 2019
End of dosing (Terminal Necropsy):	21-22 Jan 2019
Draft Report:	23 Apr 2019

3. GUIDELINES FOR STUDY DESIGN

The design of this study was based on the study objective and the following study design guidelines:

- OECD Guideline 474. *Mammalian Erythrocyte Micronucleus Test*.
- OECD Guideline 489. *In Vivo Mammalian Alkaline Comet Assay*.

4. REGULATORY COMPLIANCE

The study will be performed in accordance with the United States Code of Federal Regulations, Title 40, Parts 160 and 792: Good Laboratory Practice Standards and as accepted by Regulatory Authorities throughout the European Union (OECD Principles of Good Laboratory Practice),

Japan (MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

Exceptions to GLPs include the following study elements:

- Characterization of the test substance was performed by the Sponsor according to established SOPs, controls, and approved test methods to ensure integrity and validity of the results generated; these analyses were not conducted in compliance with the GLP regulations.

5. QUALITY ASSURANCE

5.1. Testing Facility

The Testing Facility Quality Assurance Unit (QAU) will monitor the study to assure that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with Good Laboratory Practice regulations. The QAU will review the protocol, conduct inspections at intervals adequate to assure the integrity of the study, and audit the Final Report to assure that it accurately describes the methods and standard operating procedures and that the reported results accurately reflect the raw data of the study.

The Testing Facility QAU contact for this study is indicated below:

R. Kelvin Mentzer, BS, RQAP-GLP
Charles River
1407 George Rd.
Ashland, OH 44805
Tel: 419.282.2111
Fax: 419.289.3650
E-mail: kelvin.mentzer@crl.com

5.2. Sponsor-Designated Subcontractor

The following study phases will be audited by the Sponsor-designated Subcontractor QAU:

- Micronucleus Assay
- Comet Assay

For all study phases inspected by Sponsor or Sponsor-designated subcontractor QAU(s), copies of each periodic inspection report will be made available to the Study Director, Testing Facility Management, and the Testing Facility QAU.

The Micronucleus assay and Comet Test Site QAU contact for this study is indicated below:

Luleayenwa Aberra-Degu
BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850
Tel: 301.610.2667
E-mail: luleayenwa.aberra-degu@milliporesigma.com

6. SPONSOR

Sponsor Representative

[redacted]

7. RESPONSIBLE PERSONNEL

Study Director

Michael S. Cockburn
Address as cited for Testing Facility
Tel: 419.282.6925
Fax: 419.289.3650
E-mail: michael.cockburn@crl.com

Alternate Contact

Jeffrey T. Weinberg, BS
Address as cited for Testing Facility
Tel: 419.282.6890
Fax: 419.289.3650
E-mail: jeffrey.weinberg@crl.com

Management Contact

James M. Randazzo, PhD., DABT
Address as cited for Testing Facility
Tel: 419.282.6883
Fax: 419.289.3650
E-mail: james.randazzo@crl.com

Principal Investigators (PI) - Sponsor-designated

Micronucleus Assay	Megan Young BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850 Tel: 301.610.2152 E-mail: megan.young@milliporesigma.com
Comet Assay	Shannon Bruce BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850 Tel: 301.610.2741 E-mail: shannon.bruce@milliporesigma.com

Each PI is required to report any deviations or other circumstances that could affect the quality or integrity of the study to the Study Director in a timely manner. Each PI will provide a report addressing their assigned phase of the study, which will be included as an appendix to the Final Report. The phase report will include the following:

- A Statement of Compliance
- A QA Statement
- The archive site for all records, samples, specimens and reports generated from the phase or segment (alternatively, details regarding the retention of the materials may be provided to the Study Director for inclusion in the Final Report)
- A listing of critical computerized systems used in the conduct and/or interpretation of the assigned study phase

8. TEST SUBSTANCE AND VEHICLE INFORMATION

8.1. Test Substance

8.1.1. Identification

[redacted]

8.1.2. Lot Number

To be documented in the study records.

8.1.3. Purity

Purity information is the responsibility of the Sponsor. A Certificate of Analysis or appropriate documentation will be provided by the Sponsor.

8.1.4. Stability

The test substance is considered to be stable under the storage conditions provided by the Sponsor.

8.1.5. Physical Description

To be documented by Charles River.

8.1.6. Storage Conditions

In a room with controls set to maintain 18°C to 24°C.

8.1.7. Administration Dose Form

Gas

8.1.8. Reserve Samples

Reserve samples of the test substance will not be taken due to the duration of this study.

8.1.9. Personnel Safety Data

A Safety Data Sheet (SDS), or equivalent documentation, will be provided by the Sponsor (if available). It is the responsibility of the Sponsor to notify the test facility of any special handling requirements of the test substance. Otherwise routine safety precautions will be followed. Appropriate gloves, safety glasses and arm covers will be worn by individuals working with neat test material or formulations.

8.1.10. Test Substance Disposition

All neat test substance remaining at study completion will be returned to the Sponsor at the address provided below.

Test substance will be shipped at ambient temperature on a non-holiday Monday, Tuesday, or Wednesday by overnight courier to:

[redacted]

8.2. Positive Control Article 1

8.2.1. Identification

Cyclophosphamide monohydrate (CP, CAS number 6055-19-2) is a known clastogen.

8.2.2. Lot Number

To be documented in the study records.

8.2.3. Purity

To be provided in the Certificate of Analysis (if applicable).

8.2.4. Stability

The positive control article is considered to be stable under the storage conditions provided by the manufacturer.

8.2.5. Physical Description

To be documented by Charles River in the study records.

8.2.6. Administration Dose Form

Suspension

8.2.7. Positive Control Article Disposition

Any remaining positive control article will be retained under the storage conditions provided by the manufacturer.

8.2.8. Personnel Safety

A Safety Data Sheet (SDS), or equivalent, will be provided by the manufacturer. Double nitrile gloves and a half-face negative respirator with cartridge #60923 are to be worn while preparing and administering doses. If the preparation is done in a Ventilated Balance Safety Enclosure (VBSE), then a respirator is not needed. Otherwise, routine safety precautions will be followed.

8.3. Positive Control Article 2

8.3.1. Identification

Ethyl methanesulfonate (EMS, CAS No. 62-50-0), is a known substance that induces DNA strand breaks.

8.3.2. Lot Number

To be documented in the study records (if applicable).

8.3.3. Purity

To be provided in the Certificate of Analysis (if applicable).

8.3.4. Stability

The positive control article is considered to be stable under the storage conditions provided by the manufacturer.

8.3.5. Physical Description

To be documented by Charles River in the study records.

8.3.6. Storage Conditions

To be stored as per the conditions provided by the manufacturer.

8.3.7. Administration Dose Form

Solution

8.3.8. Positive Control Article Disposition

Any remaining positive control article will be retained under the storage conditions provided by the manufacturer.

8.3.9. Personnel Safety

An SDS, or equivalent, will be provided by the manufacturer. Double nitrile gloves and a half-face negative respirator with cartridge #60923 are to be worn while preparing and administering doses. If the preparation is done in a Ventilated Balance Safety Enclosure (VBSE) then a respirator is not needed. Otherwise, routine safety precautions will be followed.

9. PREPARATION AND ANALYSES OF DOSING FORMULATIONS

9.1. Method and Frequency of Preparation

9.1.1. Cyclophosphamide

The cyclophosphamide formulation will be prepared for dosing as a weight-to-volume mixture in deionized water. A complete description of the method of preparation for the cyclophosphamide formulation will be documented in the study records and described in the final report. The cyclophosphamide formulation will be prepared on the first and second day of positive control dosing (Days 1 and 2 for the positive control animals). The cyclophosphamide formulation will be stirred continuously, on ice, during preparation and dosing.

9.1.2. Ethyl Methanesulfonate

The ethyl methanesulfonate formulation will be prepared for dosing as a weight-to-volume mixture in 0.9% saline. A complete description of the method of preparation for the ethyl methanesulfonate formulation will be documented in the study records and described in the final report. The ethyl methanesulfonate formulation will be prepared on the second and third day of positive control dosing (Days 2 and 3 for the positive control animals). The ethyl methanesulfonate formulation does not need to be stirred continuously and may be stored at room temperature during preparation and dosing.

9.2. Analysis of Positive Control Article Formulations

No analysis to confirm either cyclophosphamide or ethyl methanesulfonate in the dosing formulations will be performed as part of this study.

10. TEST SYSTEM

Species:	Rat
Strain:	Crl:CD(SD) Sprague Dawley rat
Source:	Charles River Laboratories. Facility to be documented in the raw data.
Number of Males Ordered:	35
Number of Females Ordered:	35
Target Age at the Initiation of Dosing:	7-8 weeks. Animals not utilized on study will be assigned to the Charles River colony or euthanized by CO_2 inhalation and discarded.
Target Weight at the Initiation of Dosing:	200 to 320 g (males)/150 to 250 g (females)

The actual age and weight of animals received will be listed in the Final Report. Females will be nulliparous and nonpregnant.

10.1. Identification System

A permanent animal number will be assigned to each individual animal. Each animal will be identified using a subcutaneously implanted electronic identification microchip (BMDS system). The microchip will be the primary means to uniquely identify animals assigned to study. Individual cage cards will be affixed to each cage and will display at least the animal number, cage number, group number, dosage level, study number, and sex of the animal.

Replacement microchips may be implanted as necessary throughout the course of the study. An ear tag may be used as the alternate unique identifier.

10.2. Justification for Selection

The Sprague Dawley rat was chosen as the animal model for this study as it is an accepted rodent species for nonclinical toxicity testing by regulatory agencies.

The number of animals selected is based on OECD Guidelines 474 and 489. Group size at the initiation of the study (up to 6/sex/group) was chosen to provide a minimum of 5 analyzable samples/sex/group for each endpoint.

At this time, studies in laboratory animals provide the best available basis for extrapolation to humans and are required to support regulatory submissions. Acceptable models which do not use live animals currently do not exist.

11. SPECIFIC ANIMAL MAINTENANCE SCHEDULE

11.1. Animal Receipt and Acclimation

Each animal will be inspected by qualified personnel upon receipt. Animals judged to be in good health will be placed in acclimation for at least 7 days. See respective sections for parameters to be evaluated.

11.2. Animal Housing

All animals will be housed in groups of 2 to 3 per cage following receipt in clean, solid bottom cages with bedding material (Bed-O-Cobs® or other suitable material) in an environmentally controlled room. Animals may be temporarily separated for protocol-specified activities and this will be documented in the study records. Any animal whose cage mate(s) are removed from study (morbidity or unscheduled death) may remain individually housed for the remainder of the study. In addition, animals may be individually housed due to aggressive behavior toward a cage mate. The cages will be cleaned and changed routinely at a frequency consistent with maintaining good animal health.

All animals will be maintained in accordance with the *Guide for the Care and Use of Laboratory Animals*.[1] The facilities at Charles River Ashland are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

11.3. Environmental Conditions

Environmental controls in the animal room will be set to maintain a temperature of 68°F to 78°F (20°C to 26°C) and relative humidity at 30% to 70%. Temperature and relative humidity will be monitored continuously. Data for these 2 parameters will be scheduled for automatic collection on an hourly basis. Fluorescent lighting will provide illumination for a 12-hour light/dark photoperiod. Temporary interruptions to the light/dark cycles may be made to accommodate protocol-specified activities. The ventilation rate will be set at a minimum of 10 room air changes per hour, 100% fresh air.

11.4. Drinking Water

Reverse osmosis-treated water will be available ad libitum, except during exposure periods and acclimation to nose-only restraint tubes. The municipal water supplying the laboratory will be analyzed for contaminants according to SOPs. No contaminants are reasonably expected to be present that would interfere with the objectives of the study, therefore, no testing will be conducted as part of the study.

11.5. Basal Diet

PMI Nutrition International, LLC Certified Rodent LabDiet® 5CR4 meal will be offered ad libitum during the study, except during exposure periods, and acclimation to nose-only restraint tubes. SOPs provide specifications for acceptable levels of heavy metals and pesticides that are reasonably expected to be present in the diet without interfering with the purpose or conduct of the study. No contaminants are reasonably expected to be present that would interfere with the objectives of the study; therefore, no testing will be conducted as part of the study.

11.6. Environmental Enrichment

Enrichment devices will be provided to each animal for environmental enrichment and to aid in maintaining the animals' oral health, beginning during acclimation and continuing throughout the course of the study.

12. EXPERIMENTAL DESIGN

12.1. Acclimation to Restraint in Nose-Only Exposure Holding Tubes

To screen animals for poor tolerance of restraint and to limit the potential effects on respiration of the novel environment/conditions of restraint, the animals will be subjected to restraint in nose-only exposure tubes.

Animals will be acclimated to restraint tubes four times (1 acclimation/day) prior to their first day of exposure. Animals will be acclimated to restraint in nose-only exposure restraint tubes by increasing the restraint time over the acclimation period (1st day-1 hour, 2nd day-2 hours, 3rd day-4 hours and 4th day-6 hours; times are approximate). Following the restraint period, each animal will be observed for clinical signs of injury or stress.

12.2. Animal Selection and Randomization

During the acclimation period, animals judged to be suitable for testing will be assigned to groups at random based on body weight stratification into a block design using a computer program. Animals may be arbitrarily assigned to the positive control group. Animals will then be arranged into the appropriately assigned groups and housed in social groups of 2 to 3 per cage within the treatment group.

Individual body weights at randomization will be within ± 20% of the mean. Following randomization, it may be necessary to replace individual animal(s), prior to initiation of dosing. Individual replacement animals will be selected from the remaining unassigned animals and assigned arbitrarily. The reason(s) for replacement will be documented in the study records. Animals may not be replaced after Day 1.

12.3. Route and Rationale of Test Substance Administration

The route of administration will be inhalation exposure since this is the unintended route of human exposure. Nose-only exposure methods will be used to reduce the potential for dermal exposure or oral exposure resulting from grooming. In order to perform nose-only exposure, it is necessary to restrain the rats in specially designed nose-only holding tubes. The period of restraint is necessary to achieve the maximum feasible exposure to the test substance.

Per OECD Guidelines 474 and 489, it is not necessary to administer the concurrent positive control article by the same route as the test substance. The route of administration of the positive control articles (oral gavage) was chosen based on past experience by Charles River and BioReliance with both types of assays.

12.4. Organization of Test Groups, Dosage Levels, and Treatment Regimen

12.4.1. Organization of Test Groups

The following table presents the study group arrangement.

Study Design

Group Number	Treatment	Target Exposure Concentration (ppm)	Positive Control Concentration (mg/mL)	Positive Control Dose Volume (mL/kg)	Number of Animals[a,c]	
					Males	Females
1	Filtered Air	0	NA	NA	6	6
2	[redacted]	375	NA	NA	6	6
3	[redacted]	750	NA	NA	6	6
4	[redacted]	1500	NA	NA	6	6
5	Positive Control[b]	CP: 20 mg/kg/day	2	10	6	6
		EMS: 200 mg/kg/day	20	10		

[a] Filtered air (negative control group) and vaporized test substance will be administered via nose-only inhalation for 3 consecutive days (6 hrs/day), Days 1-3.

[b] The positive control substance, cyclophosphamide (CP), will be administered via oral gavage to rats in Group 5 at a dosage of 20 mg/kg/day on Days 1 and 2. The positive control article, ethyl methanesulfonate (EMS), will be administered via oral gavage to rats in Group 5 at a dosage of 200 mg/kg/day on Days 2 and 3.

[c] Up to 6 surviving rats/group will be utilized for collection of peripheral blood between 1 and 2 hours following the final exposure/dose. Additionally, for 5 surviving rats/group, samples of bone marrow in addition to samples of the liver, lung, kidney, and nasal tissue will be collected between 2 and 4 hours after completion of the final exposure (Groups 1-4) or after the second dose of EMS (Group 5), which should coincide with being approximately 18-24 hours after the second dose of cyclophosphamide.

NA Not applicable

12.4.2. Justification of Dosage Levels

The target exposure concentrations were selected by the Sponsor Representative in consultation with the Study Director based, in part, on a previous inhalation study conducted using [redacted].

In that study, [redacted] had localized effects in the nose at concentrations of 250 and 550 ppm. Based on these findings, exposure concentrations of 375, 750, and 1500 ppm were selected for this study.

12.4.3. Treatment Regimen

Filtered air (control) and test substance atmospheres will be administered as 6-hour, nose-only inhalation exposures for three consecutive days, as outlined below.

Cyclophosphamide will be administered via oral gavage to rats in Group 5 at a dosage of 20 mg/kg/day on Days 1 and 2. Ethyl methanesulfonate will be administered via oral gavage to rats in Group 5 at a dose of 200 mg/kg/day on Days 2 and 3.

The first day of dosing will be Day 1; the week of dosing will be Week 1.

On the day of each animal's final exposure/dose, surviving animals from each study group will be euthanized and samples will be collected between 2 and 4 hours post-exposure. To accommodate post-exposure activities, initiation of initial and final exposures will be staggered (by group and or sex).

All animals will be housed in an animal colony room during non-exposure hours. Prior to each exposure, the animals selected for exposure will be transferred to nose-only restraint tubes and transported to the exposure room(s). Animals will then be exposed for the requisite duration and returned to their home cages.

12.5. Methods of Administration

12.5.1. Oral Gavage

The positive control articles, CP and EMS, will be administered orally by gavage. Each dose will be administered via a syringe equipped with an Instech feeding tube. The dose volume will be 10 mL/kg.

The CP dosing formulation will be stirred continuously on ice during dosing.

The EMS dosing formulation will be maintained at room temperature during dosing.

Individual doses will be calculated based upon the most recent individual body weights to provide the proper dose.

12.5.2. Nose-Only Inhalation

Nose-only exposures will be conducted using stainless steel, conventional nose-only exposure systems (CNOS), with grommets in exposure ports to engage animal holding tubes. Dedicated

exposure systems will be used: one for the filtered-air control group and one for the test substance-treated groups.

The exposure systems will be operated under dynamic conditions. Air supplied to the exposure systems will be provided from a HEPA and charcoal-filtered air source and/or breathing quality, in-house compressed air source. Exposure system airflow rates will be based on the requirements for vapor generation and dilution and will provide a sufficient volume for the number of animals exposed and for exposure atmosphere sampling.

Exposure system airflow rates will be recorded at least hourly during each exposure. The airflow rates for each nose-only system may be monitored by measuring the pressure drop between the ports of a venturi tube using a Dwyer Magnehelic® Indicating Transmitter pressure gauge. Each gauge will be calibrated for conversion from pressure to airflow in standard liters per minute. If venturi-based methods for measuring system airflow rate cannot be used, recorded values will be calculated from calibration curves for the vapor generation device and flowmeters.

Average temperature and relative humidity of the exposure atmospheres will be 22 ± 3ºC and 50 ± 20%, respectively. Temperature and relative humidity will be monitored with a temperature and humidity transmitter probe for each nose-only exposure system.

Temperature and relative humidity will be monitored and recorded at least hourly during each exposure. If possible, temperature, relative humidity, and airflow rates will be monitored and recorded through the use of the Inhalation Exposure Data Collection System (WINH) and a personal computer. In the event of a failure of the automated data collection system (e.g., malfunction of a component or of a hardware connection), manual recording will be used.

Oxygen content of the exposure atmosphere at each exposure concentration will be determined during method development and will be at least 19%.

12.5.3. Control and Test Substance Exposure Atmosphere Generation Methods

For the filtered-air control group (Group 1), HEPA- and charcoal-filtered humidified air will be mixed with breathing-quality in-house compressed air as needed to provide a comparable airflow rate and relative humidity to that used for the test substance-treated groups.

Final details of methods for generation of exposure atmospheres, equipment specifications, and operating conditions will be defined during the method development and included in a system description, which will be approved by the Study Director.

12.6. Methods of Characterization of Exposure Atmospheres

12.6.1. Nominal Concentration

If possible based on the generation methods used, nominal exposure concentrations will be calculated. At a minimum, test substance usage will be determined and documented in the study records.

12.6.2. Actual Concentrations

Analyzed concentrations of [redacted] in the exposure atmospheres will be determined using a gas chromatograph (GC) equipped with a Flame Ionization Detector (FID).

Concentration will be recorded approximately every 60 minutes throughout the exposure period. Additional samples may be collected for diagnostic purposes and to assist the laboratory technical staff in maintaining stable exposure concentrations.

Samples may be collected from the nose-only exposure systems using tubing connected to a vacuum pump or by a computer controlled multiposition valve and a sample loop. If applicable, the WINH Inhalation Exposure Data Collection System will control the GC sampling and acquire peak area values from the GC for the concentration analyses and the program will calculate exposure concentrations from the peak area values using a quadratic equation representing the standard curve for the GC calibration. Alternatively, the control of the GC sampling and calculation of exposure concentrations may be performed manually.

The calibration of the gas chromatograph will be considered acceptable if the R^2 value is ≥ 0.98 and the individual points of the calibration are within 10% of their target concentrations.

Final details of methods for determination of exposure concentrations, including sampling conditions, equipment specifications and operating conditions, will be defined during method development and included in a system description, which will be approved by the Study Director.

13. IN-LIFE PROCEDURES, OBSERVATIONS, AND MEASUREMENTS

13.1. Viability Observations

All animals will be observed for mortality, abnormalities, and signs of pain and distress twice daily, once in the morning and once in the afternoon. Moribund animals will be euthanized and necropsied as soon as possible. Animals found dead will be necropsied as soon as possible to ensure that tissues will not be lost due to autolysis.

13.2. Animals to Be Euthanized in Extremis

Animals that experience severe or chronic pain or distress that cannot be relieved will be euthanized. All main study animals to be euthanized in extremis will undergo a final detailed clinical observation and a body weight will be collected prior to release for euthanasia and subsequent necropsy. Animals will be anesthetized by isoflurane inhalation followed by exsanguination, which completes the euthanasia.

13.3. Observations

13.3.1. Cage Side Observations (Groups 1-4)

- Prior to exposure

- 0 to 2 hours post exposure

The absence or presence of findings will be recorded for individual animals.

The prior to exposure observations need not be conducted on the days of detailed clinical observations, provided that the detailed clinical observations are conducted prior to exposure.

13.3.2. Cage Side Observations (Group 5)

- At the time of dosing
- 0 to 2 hours postdose

The absence or presence of findings will be recorded for individual animals.

13.3.3. Detailed Clinical Observation (All Animals)

- Within 4 days of receipt
- On the day of randomization
- On Day 1 (prior to exposure)
- On Day 3 (prior to exposure)

The absence or presence of findings will be recorded for individual animals.

13.3.4. Social Housing Observations

If there are any cage findings for a social group that need to be recorded, the findings will be attributed to all animals within each socially housed group.

13.3.5. Unscheduled Observations

Findings noted outside the above-specified observation periods will also be recorded. Only the presence of unscheduled observations will be recorded; the absence of findings will thus not be recorded.

13.3.6. Individual Body Weights

- Within 4 days of receipt
- On the day of randomization
- On Day 1
- On Day 3

13.3.7. Food Weight Data

- On Day 1
- On Day 3

Food consumption will be measured on a per cage basis. Food consumption will be normalized to the number of animals/cage and will be reported in grams/animal/day. Food spillage within the cage will not be accounted for due to the use of bedding.

14. LABORATORY EVALUATIONS

14.1. Micronucleus Blood Collection (Groups 1-5)

- All surviving rats/group (Groups 1-4) will be utilized for collection of peripheral blood approximately 1-3 hours following the last exposure.
- All surviving rats/group (Group 5) will be utilized for collection of peripheral blood approximately 1-3 hours following the second dose.

The animals will not be fasted prior to blood collection. Approximately 0.5 mL of blood will be collected into K_2EDTA tubes (2.0 mL tube) from the jugular vein (blood will be collected from the retro-orbital sinus of animals anesthetized with isoflurane, if necessary). Blood samples will be checked for clots. Samples will be redrawn, as necessary, to provide adequate blood samples.

Whole blood will be transferred to the Immunotoxicology group and processed as per Section 17.1.1.

15. TERMINAL PROCEDURES – ANATOMIC PATHOLOGY

15.1. Macroscopic Examination

Animals Euthanized in Extremis or Dying Spontaneously:

A gross necropsy will be conducted on animals dying spontaneously or euthanized in extremis.

Animals will be anesthetized by isoflurane inhalation followed by exsanguination, which completes the euthanasia. Necropsy will include examination of the external surface, all orifices and the cranial, thoracic, abdominal, and pelvic cavities, including viscera. Carcasses will be discarded without tissue collection.

Scheduled Necropsy:

Two to four hours following the final exposure, the animals will be anesthetized by isoflurane inhalation followed by exsanguination, which completes the euthanasia. A gross macroscopic examination will not be performed on rats surviving to the scheduled euthanasia.

Immediately following euthanasia, for 5 animals/sex/group, bone marrow from the right femur will be collected and processed as described in Section 15.3. It may be necessary to use the left

femur instead of the right femur. The reason(s) for using the left femur will be appropriately documented in the study records.

Additionally, samples of the lung, liver, kidney, and nasal tissue will be collected and processed as described in Section 15.2. At the time of necropsy, the following table notes the tissues and organs that will be collected and placed in 10% neutral-buffered formalin. The carcasses and remaining tissues will be discarded.

Tissue Collection and Preservation

Liver (sections of 2 lobes) Lungs (including bronchi, fixed by constant pressure inflation with fixative)	Kidney

Additionally, for Groups 1-4 in which the extra animal/sex survives, the nasal cavity will be collected, flushed with fixative, and placed in 10% neutral-buffered formalin. For these animals, samples for the Comet assay (liver, lung, kidney, and nasal tissue), or bone marrow, or other tissues will not be collected.

15.2. Tissue Collection for Comet Assay

Five (5) surviving animals/group will have nasal tissue, lung, kidney, and liver collected between 2 and 4 hours following their last exposure (Groups 1-4) or second dose of EMS (Group 5). Charles River personnel will remove the head, lung, kidney, and liver. If not needed, the extra animal/sex will be anesthetized by isoflurane inhalation followed by exsanguination, which completes the euthanasia, and the nasal cavity will be collected (as previously described) (Groups 1-4) or discarded without tissue collection (Group 5).

Samples of the nasal tissue, left lung, kidney, and liver will be collected by BioReliance staff and placed in chilled mincing solution (Hanks' balanced salt solution with EDTA and DMSO). The tissue samples will then be minced with fine scissors to release the cells. The cell suspension will be strained into a pre-labeled conical polypropylene tube through a Cell Strainer and may be kept on wet ice during preparation of the slides.

Preparation of the cell suspensions will be performed by BioReliance staff while at the Testing Facility according to applicable BioReliance SOPs.

Slides will be processed as described in Section 16.

15.3. Bone Marrow Collection

Five (5) surviving animals/group will have bone marrow collected between 2 and 4 hours following their last exposure (Groups 1-4) or second dose of EMS (Group 5) as described below. If not needed, the extra animal/sex will be anesthetized by isoflurane inhalation followed by exsanguination, which completes the euthanasia, and the nasal cavity will be collected (as previously described) (Groups 1-4) or discarded without tissue collection (Group 5).

Using a syringe containing fetal bovine serum, the bone marrow will be aspirated or flushed from the right femur into a centrifuge tube. Cells will be aspirated at least 2-3 times to ensure all

cells are collected and there are no cell clumps. The suspension will be centrifuged and all but approximately 0.25 mL of the supernatant will be removed. The pellet will be resuspended in the remaining fetal bovine serum. Using a 23-gauge needle with syringe, approximately one drop of the cell suspension will be used to prepare a bone marrow smear on an appropriately labeled, clean microscope slide. A minimum of four slides per animal will be prepared. Slides will be allowed to air dry and will then be fixed in 100% methanol for approximately 20 minutes and allowed to air dry again. The slides will be stored for possible future analysis. If not stained and scored, the slides will be archived with the study.

15.4. Histology

To be added by amendment, if warranted based on the results of the Comet assay.

15.5. Histopathology

To be added by amendment, if warranted based on the results of the Comet assay.

16. COMET ASSAY

Section 15 provides a brief description of the activities that will be performed by BioReliance for this study (BioReliance Reference No. AF56EM.151.BTL). Work will be conducted in accordance to their onsite SOPs.

16.1. Preparation of Comet Slides

Preparation of Comet slides will be performed by BioReliance staff while at the Testing Facility according to applicable BioReliance SOPs.

At least four slides or wells per animal will be prepared per organ/tissue. An aliquot of 1.25-7.5 µL of each cell suspension per slide will be mixed with 0.5% low melting agarose. The cell/agarose suspension will be applied to microscopic slides, commercially available, pre-treated multi-well or previously coated with 1% normal melting agarose. The slides will be placed at 2-8ºC for at least 15 minutes to allow the gels to solidify.

Slides will be identified with a random code that reflects the study number, group, animal number, and organ/tissue. Three slides or wells will be used for scoring and the remaining slides or wells will be backup. These slides or wells may be used in additional scoring, if deemed necessary.

Each slide will be submerged in a lysis solution at least overnight at 2-8ºC. The lysis solution will be composed of 100mM EDTA (disodium), 2.5 M sodium chloride, 10 mM tris hydroxymethyl aminomethane in purified water; pH10; 1% triton-X100 and 10% DMSO will be added on the day of use or commercially available lysis solution will be used after the addition of 10% DMSO on the day of use.

After cell lysis, slides will be washed with neutralization buffer (0.4 M tris hydroxymethyl aminomethane in purified water, approximately pH 7.5) and placed in an electrophoresis chamber. The chamber reservoirs will be filled with alkaline buffer (300 mM sodium hydroxide

and 1 mM EDTA (disodium) in purified water, pH > 13) for approximately 20 minutes at 2-10ºC, protected from light for the unwinding of DNA. Electrophoresis will be conducted in the same buffer following DNA unwinding for 30 minutes at 0.7 volts/cm.

The slides will be removed from the electrophoresis chamber and washed with neutralization buffer for at least 10 minutes. The slides will be dehydrated with 200-proof ethanol for at least 5 minutes, then air-dried for at least 2 hours and then stored at room temperature with desiccant.

16.2. Slide Shipment

Slides of the processed nasal tissue, lung, kidney, and liver prepared by BioReliance at the Test Facility will be stored at room temperature with desiccant. Slides will be shipped on the first available Monday, Tuesday, or Wednesday at ambient temperature to BioReliance by overnight shipment to the following address:

BioReliance
Toxicology Testing Facility (Building 5)
9630 Medical Center Drive
Rockville, MD 20850

Attention: Albert Brew-Hagan
(Phone: 301-610-2146, Fax: 301-610-2560,
E-mail: albert.brew@ milliporesigma.com, marilena.lekavicius@ milliporesigma.com, faith.rider@external.milliporesigma.com, and lana.mcdowell@ milliporesigma.com.

16.3. Slide Staining

Staining of Comet slides will be performed by BioReliance staff at their Test Site according to applicable BioReliance SOPs.

Slides designated for staining will be stained with Sybrgold™ prior to scoring.

16.4. Scoring of Comet Slides

Scoring of Comet slides will be performed by BioReliance staff at their Test Site according to applicable BioReliance SOPs.

Three slides or wells per animal per treatment will be used. Fifty randomly selected cells will be scored per slide, resulting in a total of 150 cells evaluated per animal. If one of the three slides or wells does not have 50 scorable cells, additional cells may be scored using the backup slides or wells. If 150 cells are not available, then the calculations will be performed using the number of scorable cells.

The following endpoints of DNA damage will be assessed and measured:

- Comet Tail Migration; defined as the distance from the perimeter of the Comet head to the last visible point in the tail.

- % Tail DNA (also known as % tail intensity or % DNA in tail); defined as the percentage of DNA fragments present in the tail.
- Tail Moment (also known as Olive Tail Moment); defined as the product of the amount of DNA in the tail and the tail length [(% Tail DNA x Tail Length)/ 100].[2]

Each slide will also be examined for indications of cytotoxicity. The rough estimate of the percentage of "clouds" will be determined by scanning 150 cells per animal, when possible, (percentage of "clouds" is calculated by adding the total number of clouds for all slides scored, dividing by the total number of cells scored, and multiplying by 100). Every effort will be made to score at least 150 cells; otherwise, the total number of scorable cells will be used for calculations. The "clouds," also known as "hedgehogs," are a morphological indication of highly damaged cells often associated with severe genotoxicity, necrosis, or apoptosis. A "cloud" is produced when almost the entire cell DNA is in the tail of the comet and the head is reduced in size, almost nonexistent.[3] "Clouds" with visible gaps between the nuclei and the comet tail will be excluded from comet image analysis.

Slides will be discarded prior to report finalization.

16.5. Criteria for Determination of a Valid Test

The DNA damage data (% tail DNA) in the negative control group (filtered air control) is expected to be within the historical vehicle control (negative control) range, and the positive control group must be significantly increased relative to the concurrent negative control group ($p \leq 0.05$). Additionally, concurrent positive controls should induce responses that are compatible with those generated in the historical positive control database.

16.6. Evaluation of Test Results

Once the criteria for a valid assay have been met, the results will be evaluated as follows:

Means of 150 counts of % tail DNA, Tail moment, and Tail migration will be presented for each animal and each organ. The mean and standard deviation of the mean values for % tail DNA will be presented for each treatment group.

Statistical analysis will be performed only for % tail DNA. If deemed necessary, other parameters of DNA damage (e.g., Tail moment) may be analyzed statistically and used in the overall assessment of DNA damage.

All conclusions will be based on sound scientific judgment. As a guide to interpretation of the data, the following will be considered:

- The test substance will be considered to induce a positive response in a particular tissue if the mean % tail DNA (or other parameters of DNA damage) in one or more test substance groups (doses) is significantly elevated relative to the concurrent negative control group.

- The test substance will be judged negative for induction of DNA damage if no statistically significant increase in the mean % DNA damage (or other parameters) in the test substance groups relative to the concurrent negative control group is observed.

However, the results of the statistical analysis may not be the only criterion in determination of the test substance potential to induce DNA damage. The following may be taken in consideration:

- The historical vehicle control (negative control) data; a statistically significant increase in the mean % DNA (or other parameters) may not be considered biologically relevant if the values do not exceed the range of historical vehicle control (negative control).
- Because cells undergoing necrosis or degeneration are prone to DNA degradation, independent of direct genotoxic effects of the test substance, doses that are found to be cytotoxic, by histopathology evaluation, may not be considered as relevant doses and may not be taken in consideration during the generation of the study conclusions. Accordingly, any statistically significant increase in DNA damage occurring at a cytotoxic dose may not be considered as a positive finding.
- A dose-dependent increase in the mean % tail DNA (or other parameters) across the dose levels tested; if a dose-response is evident with no statistically significant increase, additional testing, including histopathology evaluation of the tissue, may be considered.
- If criteria for either a positive or negative response are not met, the results may be judged as equivocal.

The Comet Assay report will include, but will not be limited to, information about the test substance results, discussion of comet assay results, conclusion of comet assay, historical control data, statement of compliance, QA statement, and location of archived material.

The Comet Assay report will be included as an appendix to the Charles River final report and appropriate interpretation and data will be incorporated into the text of the Charles River final report.

16.7. Electronic Data Collection Systems

Electronic systems used for the collection or analysis of data will include but not be limited to the following (version numbers are maintained in the system documentation):

Electronic Systems

Program/System	Description
LIMS Labware System	Study tracking
Excel (Microsoft Corporation)	Calculations
Minitab	Statistics
BRIQS	Deviation and audit reporting
Comet Assay IV	Scoring of Slides

17. MICRONUCLEUS TEST

Section 17 provides a brief description of the micronucleus analysis activities that will be performed by BioReliance for this study (BioReliance Reference No. AF56EM.129FLOWPBGLP.BTL). Work will be conducted in accordance to their onsite SOPs.

17.1. Whole Blood Preparation

All whole blood samples will be diluted in anticoagulant. Two aliquots of approximately 180 µL each (primary and secondary samples) of the diluted whole blood samples will be placed into separate 15 mL conical vials containing cold fixative. The samples will then be fixed in cold methanol for at least 72 hours. After at least 72 hours of fixation, both sets of samples will be removed from frozen storage and washed out of fixative.

The primary and secondary samples will be placed into Long Term Storage Solution (LTSS) and stored in a freezer set to maintain a target of -70°C until shipped to the BioReliance, the Test Site for micronucleus evaluation.

The samples will be shipped in two separate shipments to BioReliance for evaluation. Shipments will be on different days, on the first available non-holiday Monday, Tuesday or Wednesday, the first set of samples will be shipped, with the second shipment on the following day. Samples will be shipped on dry ice to the following:

BioReliance
Toxicology Testing Facility (Building 5)
9630 Medical Center Drive
Rockville, MD 20850

Attention: Albert Brew-Hagan
(Phone: 301-610-2146, Fax: 301-610-2560,
E-mail: albert.brew@ milliporesigma.com, marilena.lekavicius@ milliporesigma.com, faith.rider@external.milliporesigma.com, and lana.mcdowell@ milliporesigma.com.

Receipt of Blood Samples

Upon receipt, the fixed blood samples in LTSS will be delivered to the laboratory for storage at -80±5°C until flow cytometric processing.

Detection of Micronucleated Reticulocytes with Flow Cytometry

Of the up to 6 samples/sex/group available, 5 samples in LTSS will be washed with ice cold 1% FBS solution and maintained on wet ice. The cells will then be pelleted by centrifugation, and the supernatant will be poured off leaving a small amount of supernatant with the pellet. The cells will be re-suspended and 20 µL of suspension will be added to 80 µL of staining solution containing RNase, FITC-conjugated anti-CD 71 antibodies and PE-conjugated anti-CD 61 antibodies. The samples will be incubated at 2 to 8ºC for 30 minutes, re-suspended, then incubated at room temperature for an additional 30 minutes. 0.3 – 2 mL of DNA staining

solution (propidium iodide) will be added then the samples will be placed on wet ice for at least 5 minutes prior to the flow cytometric analysis.

The frequency of micronucleated reticulocytes in peripheral blood will be analyzed after flow cytometer calibration using Malaria infected biostandard and negative control standards provided in the Litron kit. Up to 20,000 RETs per animal, when possible, will be analyzed.

Remaining samples will be discarded prior to report finalization.

Calculation of Flow Cytometric Analysis

The proportion of reticulocytes to total number of cells scored (%RETs) will be determined for each animal and treatment group. This calculation will be carried out as indicated below:

$$\% \text{ RET} = \frac{(UL + UR) \times 100}{(UL + UR + LL + LR)}$$

UL: The number of events in the upper-left quadrant

UR: The number of events in the upper-right quadrant

LL: The number of events in the lower-left quadrant

LR: The number of events in the lower-right quadrant

The %RETs will serve as a parameter of the test substance cytotoxicity in peripheral blood. A decrease in this ratio in the test substance groups, as compared to the Filtered Air control, would indicate a toxic effect of the test substance while an increase would represent a sign of recovery from earlier toxic insult.

The quantization of the MnRETs in peripheral blood will be expressed as percentage of MnRETs per total number of reticulocytes evaluated. The %MnRETs will be presented for each animal and the mean ± standard deviation will be calculated and presented for each treatment group as follows:

$$\%\text{MnRET} = \frac{(UR) \times 100}{(UL + UR)}$$

17.2. Criteria for Determination of a Valid Test

Cell Analysis

A target of 20,000 RETs/animal will be analyzed for the presence of micronuclei (MnRETs) whenever possible. The proportion of reticulocytes to total number of cells scored (%RETs) will be determined for each animal and treatment group. The %RETs will serve as a parameter of the test article cytotoxicity in peripheral blood. A reduction in the RET proportions to less than 5% of the Filtered Air control value will be considered excessively cytotoxic and the animal data will be excluded from evaluation. Animals with fewer than 4000 RETs may be excluded from the analysis. Other quality indicators (e.g., flow plots) may be used to exclude animals with poor quality data.

Negative Controls

The group mean frequency of MnRETs should ideally be within the 95% control limits of the distribution of the historical negative control database. If the concurrent Filtered Air control data fall outside the 95% control limits, they may be acceptable as long as these data are not extreme outliers (indicative of experimental or human error).

Positive Controls

The positive control must induce a statistically significant positive response ($p \leq 0.05$).

17.3. Evaluation of Test Results

A test substance will be considered to have induced a positive response if:

a) at least one of the test substance doses exhibits a statistically significant increase when compared with the concurrent Filtered Air control ($p \leq 0.05$), and

b) when multiple doses are examined at a particular sampling time, the increase is dose-related ($p \leq 0.01$ and $R^2 \geq 70\%$), and

c) results of the group mean or of the individual animals in at least one group are outside the 95% control limit of the historical negative control data.

A test substance will be considered to have induced a clear negative response if none of the criteria for a positive response were met.

If the response is neither clearly positive nor clearly negative, or in order to assist in establishing the biological relevance of a result, the data will be evaluated by expert judgment and/or further investigations. Possible additional work may include scoring additional cells (where appropriate) or performing an additional experiment that could employ the use of modified experimental conditions. Such additional work will only be carried out following consultation with, and at the request of, the Sponsor.

In some cases, even after further investigations, the data set will preclude making a conclusion of positive or negative, at which time the response will be concluded to be equivocal. In such cases, the Principal Investigator will use sound scientific judgment and report and describe all considerations.

The Micronucleus Assay report will include, but will not be limited to, information about the test substance results, discussion of micronucleus assay results, conclusion of micronucleus assay, historical control data, statement of compliance, QA statement, and location of archived material.

The Micronucleus Assay report will be included as an appendix to the Charles River final report and appropriate interpretation and data will be incorporated into the text of the Charles River final report.

17.4. Electronic Data Collection Systems

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

Electronic Systems

Program/System	Description
LIMS Labware System	Study tracking
Excel (Microsoft Corporation)	Calculations
Minitab	Statistics
FACSDiva (BD Biosciences)	Sample Analysis
BRIQS	Deviation and audit reporting

18. COMPUTERIZED SYSTEMS

The following critical computerized systems may be used in the study. The actual critical computerized systems used will be specified in the Final Report.

As Charles River Ashland transitions between various computer systems, the study number may appear as [redacted], [redacted] or [redacted] in the data records and report.

Data for parameters not required by protocol, which are automatically generated by analytical devices used will be retained on file but not reported. Statistical analysis results that are generated by the program but are not required by protocol and/or are not scientifically relevant will be retained on file but will not be included in the tabulations.

All computerized systems used for data collection during the conduct of this study have been validated (with the exception of Microsoft Office and GraphPad Prism® 2008); when a particular system has not satisfied all requirements, appropriate administration and procedural controls were implemented to assure the quality and integrity of the data. The actual version number will be specified in the report.

Critical Computerized Systems

Program/System	Description
Bio Medic Data Systems (BMDS) Implantable Micro Identification™ (IMI-1000)	Animal identification
Inhalation Exposure Data Collection System (WINH)	Monitors and records inhalation chamber temperature, relative humidity, ventilation rate, and negative pressure.
Logbook™ ELN	System (Instem) used to document study events.
Metasys DDC Electronic Environmental Control System	Controls and monitors animal room environmental conditions.
Microsoft Office 2010 or higher; GraphPad Prism® 2008	Used in conjunction with the publishing software to generate study reports.
Provantis	Comprehensive system (Instem LSS Limited) used for in-life and postmortem data collection and reporting.
In-house reporting software Nevis 2012 (using SAS)	Reporting of in-life and postmortem data
Provantis Dispense™	Comprehensive system (Instem LSS Limited) to manage test materials, including receipt, formulation instructions, and accountability.
SAS®	Statistical (non-WTDMS™) analyses
Watson LIMS™	Laboratory Information Management System used for sample tracking, run planning, quantitation, and reporting results.
WIL Formulations Dispense System (WFDS)	In-house developed system for use in conjunction with Provantis Dispense™ to ensure proper storage and use of formulations.
WIL Metasys	In-house developed system used to record and report animal room environmental conditions.
WIL Toxicology Data Management System™ (WTDMS™)	In-house developed system used for collection and reporting of other data.

Note: Version numbers of WTDMS™ programs used for the study are presented on the report data tables (reporting programs), study records (input programs), and facility records (release dates).

19. STATISTICAL METHODS

19.1. In-life Parameters

Any data collected during the predose period will not be tabulated, summarized or statistically analyzed. All statistical analyses will be performed within the respective study phase, unless otherwise noted. Numerical data collected on scheduled occasions will be summarized and statistically analyzed as indicated below according to sex and occasion.

19.1.1. Constructed Variables

Body weight changes	Calculated between each scheduled interval.
Food Consumption	Calculated between each scheduled interval.

19.1.2. Descriptive Statistical Analyses

Means, standard deviations (or % coefficient of variation or standard error, when deemed appropriate), percentages, numbers, and/or incidences will be reported as appropriate by dataset.

19.1.3. Inferential Statistical Methods

All statistical tests will be conducted at the 5% significance level. All pairwise comparisons will be conducted using two sided tests and will be reported at the 1% and 5% levels, unless otherwise noted.

The pairwise comparisons of interest are listed below:

Group 2 vs. Group 1

Group 3 vs. Group 1

Group 4 vs. Group 1

Analyses will be performed according to the matrix below when possible, but will exclude any group with less than 3 observations.

Statistical Matrix

	Statistical Method
Variables for Inferential Analysis	**Parametric/Non-parametric**
Body Weight	X
Body Weight Gains	X
Food Consumption	X

19.1.4. Parametric/Non-parametric

All statistical tests will be conducted at the 5% significance level. All pairwise comparisons will be conducted using two sided tests and will be reported at the 1% and 5% levels, unless otherwise noted.

Levene's test will be used to assess the homogeneity of group variances.

The groups will be compared using an overall one-way ANOVA F-test if Levene's test is not significant or the Kruskal-Wallis test if it is significant. If the overall F-test or Kruskal-Wallis test is found to be significant, then pairwise comparisons will be conducted using Dunnett's or Dunn's test, respectively.

19.2. Comet Assay

In order to quantify the effects on DNA damage, the following statistical analysis will be performed:

- The use of parametric or non-parametric statistical methods in evaluation of data will be based on the variation between groups. The group variances for % tail DNA (or other parameters of DNA damage) generated for the negative control (filtered air control) and test substance-treated groups will be compared using Levene's test ($p \leq 0.05$). If the differences and variations between groups are found not to be significant, a parametric one-way ANOVA followed by a Dunnett post-hoc test will be performed ($p < 0.05$). If Levene's test indicates heterogeneous group variances ($p \leq 0.05$), the suitability of a transformation of the original data will be evaluated (e.g. using logarithm transformed values of the original data) in an attempt to meet the normality criteria. Afterwards, statistical analysis will be performed using the parametric tests described above. If parametric tests are not acceptable, non-parametric statistical methods (Kruskal Wallis or Mann Whitney test) may be used in evaluation of data.
- Linear regression analysis will be used to determine a dose response relationship ($p < 0.01$).
- Pair-wise comparison (Student's t-test, $p \leq 0.05$) will be used to compare the data from the positive control group against the negative control group. If needed, non-parametric statistical methods (Kruskal Wallis or Mann Whitney test) may be used in evaluation of data.

19.3. Peripheral Blood Micronucleus Data

Statistical analysis will be performed on the micronucleus frequency (%MnRET) and %RET using the animal as the unit. The mean and standard deviation of %MnRET and %RET will be presented for each treatment group.

The use of parametric or non-parametric statistical methods in evaluation of data will be based on the variation between groups. The group variances for micronucleus frequency for the Filtered Air control and test substance groups will be compared using Levene's test (significance level of $p \leq 0.05$). If the variation between groups is found not to be significant, a parametric one-way ANOVA will be performed followed by a Dunnett's post-hoc analysis to compare each dose group to the concurrent Filtered Air control. If Levene's test indicates heterogeneous group variances (significance level of $p \leq 0.05$), the suitability of a transformation of the original data will be evaluated (e.g. using logarithm transformed values of the original data) in an attempt to meet the normality criterion. Afterwards, statistical analysis will be performed using the parametric tests described above. If parametric tests are not acceptable, non-parametric statistical methods (Kruskal Wallis and/or Mann Whitney test) may be used in evaluation of data.

A linear regression analysis will be conducted to assess dose responsiveness in the test substance treated groups ($p \leq 0.01$ and $R^2 \geq 70\%$).

A pair-wise comparison (Student's T-test; $p \leq 0.05$) will be used to compare the positive control group to the concurrent vehicle control group. If parametric tests are not acceptable, non-parametric statistical methods (Kruskal Wallis and/or Mann Whitney test) may be used in evaluation of data.

20. AMENDMENTS AND DEVIATIONS

Changes to the approved protocol shall be made in the form of an amendment, which will be signed and dated by the Study Director. Every reasonable effort will be made to discuss any necessary protocol changes in advance with the Sponsor.

All protocol and SOP deviations will be documented in the study records. Deviations from the protocol and/or SOP related to the phase(s) of the study conducted at a Test Site shall be documented, acknowledged by the PI/IS, and reported to the Study Director for authorization/acknowledgement. The Study Director will notify the Sponsor of deviations that may result in a significant impact on the study as soon as possible.

21. RETENTION OF RECORDS, SAMPLES, AND SPECIMENS

All study-specific raw data, electronic data, documentation, protocol, retained samples and specimens, and final reports will be archived by no later than the date of final report issue. All materials generated by Charles River or by a Test Site from this study will be transferred to a Charles River archive. At least 1 year after issue of the Draft Report, the Sponsor will be contacted.

Following finalization, the original signed final report (paper copy) will be transferred to the Sponsor at the following address:

[redacted]

All transferred records will be maintained in the Sponsor's archives. A full copy of the final signed report will be retained and archived at Charles River.

Any work product, including documents, specimens, and samples, that are required by this protocol, its amendments, or other written instructions of the Sponsor to be shipped by Charles River to another location will be appropriately packaged and labeled as defined by Charles River SOPs and delivered to a common carrier for shipment. Charles River will not be responsible for shipment following delivery to the common carrier.

22. REPORTING

A Draft Report will be prepared following completion of the study and will be finalized following consultation with the Sponsor. The report will include all information necessary to provide a complete and accurate description of the experimental methods and results and any circumstances that may have affected the quality or integrity of the study.

The Sponsor will receive an electronic version of the Draft and Final Report provided in Adobe Acrobat PDF format (hyperlinked and searchable at final) along with a Microsoft Word version

of the text. The PDF document will be created from native electronic files to the extent possible, including text and tables generated by the Testing Facility. Report components not available in native electronic files and/or original signature pages will be scanned and converted to PDF image files for incorporation. Additionally, as noted previously, a paper copy of the final report (with original signature page[s]) will be provided to the Sponsor.

Reports should be finalized within 6 months of issue of the Draft Report. If the Sponsor has not provided comments to the report within 6 months of draft issue, the report will be finalized by the Testing Facility unless other arrangements are made by the Sponsor.

23. ANIMAL WELFARE

This study will comply with all applicable sections of the Final Rules of the Animal Welfare Act regulations (Code of Federal Regulations, Title 9), the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* from the Office of Laboratory Animal Welfare,[4] and the *Guide for the Care and Use of Laboratory Animals* from the National Research Council.[1] The protocol and any amendments or procedures involving the care or use of animals in this study will be reviewed and approved by the Testing Facility Institutional Animal Care and Use Committee before the initiation of such procedures.

If an animal is determined to be in overt pain/distress, or appears moribund and is beyond the point where recovery appears reasonable, the animal will be euthanized for humane reasons in accordance with the *American Veterinary Medical Association (AVMA) Guidelines on Euthanasia* and with the procedures outlined in the protocol.[5]

By approving this protocol, the Sponsor affirms that there are no acceptable non-animal alternatives for this study, that this study is required by a relevant government regulatory agency and that it does not unnecessarily duplicate any previous experiments.

24. REFERENCES

1 National Research Council. Guide for the Care and Use of Laboratory Animals, Committee for the Update of the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, Division on Earth and Life Sciences; The National Academies Press: Washington, DC, 2011.

2 Olive PL., Banath JP., Durand RE. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cell using the “comet” assay. Radiat. Res., 122(1), 86-94, 1990.

3 Collins AR., The Comet Assay for DNA Damage and Repair; Principles, Applications, and Limitations. Molecular Biotechnology, 26, 249-261, 2004.

4 Office of Laboratory Animal Welfare. *Public Health Services Policy on Humane Care and Use of Laboratory Animals*. Bethesda, MD: National Institutes of Health. March 2015.

5 American Veterinary Medical Association. *AVMA Guidelines on Euthanasia*. March 2013.

TESTING FACILITY APPROVAL

The signature below acknowledges Testing Facility Management's responsibility to the study as defined by the relevant GLP regulations.

Date: 19 Dec 2018

James M. Randazzo, PhD, DABT
Associate Director, Inhalation Toxicology
Testing Facility Management

The signature below indicates that the Study Director approves the study protocol.

Date: 19 DEC 2018

Michael S. Cockburn
Associate Research Scientist, Inhalation Toxicology
Study Director

SPONSOR APPROVAL

The protocol was approved by the Sponsor by email on 11 Dec 2018.

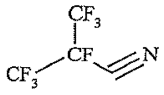
APPENDIX 2

Test Material Information

[redacted] Analytical Laboratory

An ISO9001/2015 Certified Laboratory

Certificate of Analysis

Nominal Product: 

Product Code: [redacted] Mfg. date 03/2017

Product Name: 2,3,3,3-tetrafluoro-2-(trifluoromethyl)propanenitrile

Physical State: Clear and colorless liquid at approximately -17 °C

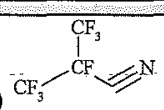
Issue Date: May 1, 2017

The sample of [redacted] was subjected to low temperature $^{1}H/^{19}F$-NMR spectral analyses to determine the purity of the nominal product and to characterize as many impurity components as possible. The qualitative and quantitative compositional results that were derived from the combined $^{1}H/^{19}F$-NMR spectral analyses are summarized below.

TABLE-1

N[redacted] Sample: [redacted], mfg. date 03/2017

Compositional Results by Low Temperature $^{1}H/^{19}F$-NMR Cross Integration Spectral Analysis

Components [1]	$^{1}H/^{19}F$-NMR **Relative Wt.%** Concentrations
1) 	98.95%
2) CF_3-CFH-CF_3	0.78%
3) $CF_3CF_2CF_2$-CN	0.25%
4) Acetone	0.0079%
5) CH_3-CF_2-CN	0.0025%
6) Water	0.0021%
7) C_nH_{2n+2} saturated aliphatic hydrocarbons	0.0005%

1) Trace amounts of a couple other unassigned impurity components are also detected in the NMR spectra.

Analytical Chemist:
[redacted] (NMR)

[redacted] Analytical Laboratory [redacted]

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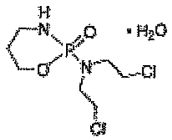
3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Cyclophosphamide monohydrate - bulk package

Product Number:	**C0768**
Batch Number:	**MKCG5464**
Brand:	SIGMA
CAS Number:	6055-19-2
MDL Number:	MFCD00149395
Formula:	C7H15Cl2N2O2P · H2O
Formula Weight:	279.10 g/mol
Storage Temperature:	Store at 2 - 8 °C
Quality Release Date:	07 AUG 2018
Recommended Retest Date:	MAY 2021



Test	Specification	Result
Appearance (Color)	White to Off-White	White
Appearance (Form)	Powder	Powder
Solubility (Color)	Colorless	Colorless
Solubility (Turbidity) 100 mg/ml in H2O	Clear	Clear
Water (by Karl Fischer)	4.7 - 7.8 %	5.6 %
Proton NMR Spectrum	Conforms to Structure	Conforms
Purity (HPLC) Anhydrous Basis	97.0 - 103.0 %	99.1 %
Recommended Retest Period 3 Years	----------	----------

Michael Grady, Manager
Quality Control
Milwaukee, WI US

SIGMA-ALDRICH®

3050 Spruce Street, Saint Louis, MO 63103 USA
Email USA: techserv@sial.com Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:	ETHYL METHANESULFONATE liquid
Product Number:	M0880
Batch Number:	BCBS6100V
Brand:	Sigma
CAS Number:	62-50-0
Formula:	$CH_3SO_3CH_2CH_3$
Formula Weight:	124.16
Quality Release Date:	03 AUG 2016

TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	COLORLESS TO FAINT YELLOW	COLORLESS
APPEARANCE (FORM)	LIQUID	LIQUID
REFRACTIVE INDEX N20/D	1.417 - 1.419	1.418
PROTON NMR SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS

Dr. Claudia Geitner
Manager Quality Control
Buchs, Switzerland

APPENDIX 3

Exposure Atmosphere Generation Report

charles river

FINAL REPORT

Study Phase: Inhalation

Laboratory Project ID [redacted]

Sponsor Reference No. [redacted]

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REPORT APPROVAL

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[redacted] Inhalation Report.pdf MD5 Checksum: F443900E472D633270D6784A2183C9B9

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1. INTRODUCTION

1.1. Objective

The objective of this study was to assess the potential of [redacted] to introduce micronuclei and/or to cause DNA damage in rat liver, lung, kidney, and nasal tissue when administered via nose-only inhalation to Sprague Dawley (Crl:CD[SD]) rats for 6 hours per day for up to 3 consecutive days.

1.2. Study Design

This report presents the inhalation study design, exposure atmosphere generation, and analysis methods used during the conduct of this study.

1.3. Computerized Systems

Critical computerized systems used in the study are listed below. All computerized systems used in the conduct of this study have been validated; when a particular system has not satisfied all requirements, appropriate administrative and procedural controls were implemented to assure the quality and integrity of data.

As Charles River Ashland transitions between various computer systems, the study number may appear as [redacted], [redacted], or [redacted] in the data records and report.

Text Table 1
Critical Computerized Systems

Program/System	Version No.	Description
Deviation Information Library	2.1	Deviations.
DocuSign	19	Collection of Part 11 compliant signature.
Inhalation Exposure Data Collection System (WINH)	1.26	Monitors and records inhalation chamber temperature, relative humidity, ventilation rate, and negative pressure.
Metasys DDC Electronic Environmental Control System	12.04	Controls and monitors animal room environmental conditions.
Microsoft Office 2010 or higher	N/A	Used in conjunction with the publishing software to generate study reports.
WIL Toxicology Data Management System™ (WTDMS™)	Various	In-house developed system used for collection and reporting of in-life and postmortem data.

N/A = not applicable.
Note: Version numbers of WTDMS™ programs used for the study are presented on the report data tables (reporting programs), Study Records (input programs), and Facility Records (release dates).

2. MATERIALS AND METHODS

2.1. Exposure Methods

2.1.1. Exposure Regimen

Filtered air or [redacted] gas atmosphere was administered as daily, 6-hour, nose-only inhalation exposures for up to 3 consecutive days. A detailed description of the study design and exposure schedule is presented in the main report.

Text Table 2 and Text Table 3 present the schedule for the inhalation exposures Phases 1 and 2, respectively.

Text Table 2
Exposure Schedule – Phase 1

	Day	Date	Gender
First Exposure	1	19 Jan 2019	M
First Exposure	2	20 Jan 2019	F
Last Exposure	3	21 Jan 2019	M
Last Exposure	4	22 Jan 2019	F

M = Male; F = Female

Text Table 3
Exposure Schedule – Phase 2

	Day	Date	Gender
First Exposure	1	20 May 2019	M
Last Exposure	2	21 May 2019	M

M = Male; F = Female

2.1.2. Inhalation Exposure System Description

Phase 1 exposures were conducted using 7.9-L stainless steel, conventional nose-only exposure systems (CNOS) with grommets in the exposure ports to engage animal holding tubes and 0.74-L 12-port module CH technologies flow-past (directed-flow) nose-only exposure systems (NOS) were used for Phase 2. One exposure system was dedicated to each group for the duration of the study. Group designations are presented in Text Table 4.

Text Table 4
Study Design

Group:	1	2	3	4
Exposure System:	1	2	3	4
Target Exposure Concentration (ppm):	0	375	750	1500

Air supplied to the nose-only systems was provided from the Inhalation Department breathing quality, in-house HEPA- and charcoal-filtered, temperature- and humidity-controlled supply air source. All nose-only system exhaust passed through the facility exhaust system, which consists of redundant exhaust blowers preceded by activated-charcoal and HEPA-filtration units.

2.1.3. Animal Transport and Placement

All animals were housed in an animal colony room during non-exposure hours. Prior to each exposure, the animals selected for exposure were placed into nose-only restraint tubes in the colony room. For Phase 1, they were transported to either the control group exposure room (Exposure Room 6) or the test substance exposure room (Exposure Room 3). For Phase 2, all exposures animals were transported to Exposure Room 1. Animals were then held in the nose-only restraint tubes for 15 to 44 minutes prior to initiation of exposure. Animals were placed on the nose-only systems, exposed for the requisite duration, and returned to their home cages in the animal colony room. Animals were rotated on a daily basis among the ports of the nose-only system. Food and water were withheld during nose-only restraint tube acclimation and the exposure period.

2.1.4. Exposure Environmental Conditions

The nose-only exposure systems were operated under dynamic conditions. Airflow rates through the exposure systems were based on a portion of the output of the test substance primary container and supply air requirements, and provided a sufficient volume of air for the number of animals exposed. System airflow (ventilation rate) was monitored using a venturi tube and Dwyer Magnehelic® Indicating Transmitter pressure gauge (Dwyer Instruments, Inc.; Michigan City, IN). Each gauge was calibrated for conversion from pressure to airflow in liters per minute through the use of a Dry Test Meter (Model No. DTM 200A, Elster American Meter Co.; Nebraska City, NE).

A temperature and relative humidity transmitter probe (Model No. HMP 110 with Model No. HMT120, Vaisala; Finland) was used to monitor temperature and relative humidity. Temperature, relative humidity, and airflow rate of the exposure atmosphere was continually monitored and recorded at 60-minute intervals during each exposure through the use of the Inhalation Exposure Data Collection System (WINH) and a personal computer.

Daily means for exposure system temperature, relative humidity, and airflow rate over the study are presented in Table 1, Table 3, and Table 5 for Phase 1 of this appendix, respectively, and Table 2, Table 4, and Table 6 for Phase 2 of this appendix, respectively. Text Table 5 and Text Table 6 summarize the grand mean environmental conditions for all exposure systems:

Text Table 5
Mean Exposure System Conditions – Phase 1

Exposure System:	1	2	3	4
Temperature (°C):	21.5	21.7	22.6	22.6
Standard Deviation:	0.29	0.39	0.36	0.45
Relative Humidity (%):	48.5	45.6	45.7	44.0
Standard Deviation:	2.06	2.20	2.61	2.70
System Airflow Rate (LPM):	15	16	16	15
Standard Deviation:	0.0	0.0	0.0	0.0
Total Number of Exposures:	4	4	4	4

Text Table 6
Mean Exposure System Conditions – Phase 2

Exposure System:	1	2	3	4
Temperature (°C):	21.2	20.4	20.6	20.6
Standard Deviation:	0.22	0.10	0.10	0.10
Relative Humidity (%):	50.2	46.9	48.8	50.8
Standard Deviation:	0.28	2.19	1.20	0.85
System Airflow Rate (LPM):	6.8	6.3	6.7	6.6
Standard Deviation:	0.00	0.14	0.14	0.14
Total Number of Exposures:	2	2	2	2

Oxygen content of the exposure atmospheres was measured during the method development phase of the study using a Dräger PAC III equipped with a calibrated oxygen sensor (Serial No. ERRH-0148, Draeger Safety Inc.; Pittsburgh, PA) and a personal air sampler (Casella CEL; Bedford, UK). Oxygen content was 20.9% for all groups for both phases of the study.

2.2. Exposure Atmosphere Generation Methods

2.2.1. Filtered Air Control (Group 1)

The control exposure system (0 ppm) was operated as follows. For Phase 1, supply air was delivered to the nose-only exposure system using a Dwyer rotameter-type flowmeter (Model No. DR4141M). For Phase 2, supply air was delivered to the nose-only exposure system using a Gilmont rotameter-type flowmeter (Model No. 13). Atmosphere exhaust was controlled using a Dwyer rotameter-type flowmeter (Model No. VFB-66-SSV).

2.2.2. Test Substance-Treated (Groups 2, 3, and 4)

The test substance exposure systems were operated as follows. Test substance atmosphere was generated by releasing the test substance gas (1,000,000 ppm) from the original cylinder.

A 2-stage regulator (no model number, Matheson; Montgomeryville, PA) equipped with a Matheson Gauge was used to monitor the outlet pressure of the cylinder. The test substance was delivered from the cylinder to a stainless steel manifold, where it was distributed to each exposure system. The manifold pressure was monitored using a Dwyer Digital Pressure Gauge (Model No. DPGA-07) for Phase 1 and a Dwyer Digital Pressure Gauge (no model number) for Phase 2. The amount of test substance directed to each test substance exposure system from the manifold was controlled using a needle valve and a Gilmont rotameter-type flowmeter (Model No. 10, Barnant Co./Gilmont Instruments; Barrington, IL). Test substance was directed to the inlet of each exposure system, where it mixed with supply air to achieve the desired atmosphere concentrations. Test substance delivery lines were 1/4-inch polyethylene tubing. Test substance passed through a 3-way valve prior to mixing with supply air at the nose-only system inlet. Initiation of the bypass directed test substance to the facility exhaust. This allowed for continual flow of the test substance from the original cylinder in the instances where generation was needed when one (or more) of the exposure systems needed to be bypassed due to staggering of the exposures. For Phase 1, the bypass, a "T" fitting was placed inline where supply air was delivered to the nose-only exposure system using a Dwyer rotameter-type flowmeter (Model No. DR4141M). For Phase 2, a Gilmont rotameter-type flowmeter (Model No. 13) was placed inline where supply air was delivered to the nose-only exposure system. For Phase 2, atmosphere exhaust from the top of the NOS was controlled using a Dwyer rotameter-type flowmeter (Model No. VFB-66-SSV).

Text Table 7 and Text Table 8 summarize the generation parameters used during the animal exposures.

Text Table 7
Exposure System Settings – Phase 1

Exposure System (No.)	Generation Air (Indicated)	Supply Air (LPM)	Total Airflow Rate (LPM)
2	4-5	16.0	16
3	8-9	15.6-17.8	16
4	15-20	15.8	15

Text Table 8
Exposure System Settings – Phase 2

Exposure System (No.)	Generation Air (Indicated)	Supply Air (LPM)	Total Airflow Rate (LPM)
2	1.5-2.5	5.9-6.7	6.8
3	3.0-4.0	6.3-6.8	5.9-6.7
4	5.0-7.0	6.1-6.7	6.1-6.7

3. PARAMETERS EVALUATED

3.1. Nominal Exposure Concentrations

Nominal exposure concentrations were not calculated due to the configuration of the exposure atmosphere generation system, where a manifold system was used to deliver test substance to

multiple systems. Documentation of daily test substance usage was performed by calculating the total amount of test substance consumed during each exposure. The amount of test substance consumed during each exposure was defined as the difference between the weight of the original test substance cylinder prior to and following each exposure.

3.1.1. Analytical/Sampling Methods

Test substance concentration within the exposure systems were sampled and analyzed at 60-minute intervals using a gas chromatograph (GC). Samples were collected from the approximate animal-breathing zone of the exposure system via 1/8-inch Teflon™ tubing. Under the control of the WINH system, sampling and analyses were performed as follows:
The program controlled an external multi-position valve (Model No. E16, Valco Instruments Co., Inc.; Houston, TX) that permitted sequential sampling from the exposure room and each test substance exposure system. Manual bag samples were collected from Exposure System 1 (Control Group) using a Metal Bellows sampling pump (Model No. MB-41, Serial No. 35777, Senior Flexonics Inc., Metal Bellows Div.; Sharon, MA) and 10-L Tedlar® gas bags (Supelco Inc.; Bellefonte, PA). Manual bag sampling was performed due to the location of the control exposure system being located in Exposure Room 6 for Phase 1, separate from Room 3 where the GC was located. The manual bag samples were analyzed on the GC during each sample round for Exposure System 1. For Phase 2, all systems were located in Exposure Room 1 where system sampling was performed.

Gas sampling injection onto the chromatography column occurred via an internal gas-sampling valve with a sample loop, the chromatograph was displayed, and the area under the sample peak was calculated and stored. The WINH system acquired the stored peak area data and used an ln-quadratic equation based on the GC calibration curve to calculate the measured concentration in ppm. The following information summarizes the operational parameters for the GC methods during the individual phases:

Phase 1

Equipment

GC:	Hewlett Packard 5890 Series II
GC Detector:	FID
GC Column:	Agilent Technologies Inc.; DB-5, 30 m × 0.530 mm ID, 1.50-micron film-thickness
Integrator:	Hewlett Packard Model 3396A

GC Gases	Pressure (psig)	Approximate Flow Rate (mL/minute)
Carrier - Helium:	15.0	15 ± 1
Fuel - Hydrogen:	17.5	30 ± 3
Air:	32.5	280 ± 10

GC Temperatures

Initial Column:	125
Detector:	250

Integrator Run Parameters

Chart Zero Offset:	0
Chart Attenuation:	6
Chart Speed:	1.0 cm/min
Peak Area Rejection Value:	0
Peak Threshold:	4
Peak Width:	0.04
Retention Time:	Approximately 0.8 minutes

Phase 2

Equipment

GC:	Hewlett Packard 5890A Series II
GC Detector:	FID
GC Column:	Agilent Technologies Inc.; DB-5, 30 m × 0.530 mm ID, 1.50-micron film-thickness
Integrator:	Hewlett Packard Model 3396A

GC Gases	Pressure (psig)	Approximate Flow Rate (mL/minute)
Carrier - Helium:	15.0	28 ± 1
Fuel - Hydrogen:	21.0	30 ± 3
Air:	36.0	302 ± 10

GC Temperatures

Initial Column:	125
Detector:	250

Integrator Run Parameters

Chart Zero Offset:	0
Chart Attenuation:	6
Chart Speed:	1.0 cm/min
Peak Area Rejection Value:	0
Peak Threshold:	4
Peak Width:	0.04
Retention Time:	Approximately 0.5 minutes

3.1.2. Gas Chromatograph Calibration

The GC was calibrated using gas-phase standards prepared to contain known gas concentrations of the test substance in 10-L Tedlar® Supelco gas bags. A 1,000,000 ppm neat bag was prepared by releasing neat test substance gas from the cylinder to a 10-L Tedlar® gas bag. A known volume of the test substance gas from the 1,000,000 ppm neat bag was injected into a standard bag filled with 8-L of compressed air using a gas tight syringe. Compressed air was measured using a dry test meter Model No. DTM-200A. Concentrations of the gas-phase standards were calculated as follows:

$$\text{Concentration (ppm)} = \frac{\text{IVB} \times \text{NBC}}{\text{IVB} + \text{V}}$$

Where:

IVB = Injection volume from neat bag, mL
NBC = Neat bag concentration, ppm
V = Volume of air added to standard bag, mL

The GC was calibrated with standards prepared and analyzed in triplicate. Using the quadratic formula, the calibration curve was defined by standards prepared at 4 concentrations spanning an appropriate range relative to the target exposure concentrations. The peak area (y) and the theoretical concentrations (x) of the calibration standards were fit with a least-squares regression analysis to the ln-quadratic function:

$$\ln (y) = a \times [\ln (x)]^2 + b \times \ln (x) + c$$

Prior to the initiation of exposure, 1 of the appropriate standards used in the prime calibration curve of the phase was prepared and analyzed to perform a calibration check of the GC. The GC was considered to be in calibration if the analyzed concentration of the prepared standard was within ± 10% of the known concentration. Text Table 9 summarizes the standard preparation parameters, respectively:

Text Table 9
GC Standard Parameters

Standard Concentration (ppm)	Volume of Test Substance Gas (mL)
250	2.0
749	6.0
1248	10.0
2120	17.0

4. RESULTS

4.1. Analyzed Exposure Concentrations

Daily mean analyzed concentrations for each exposure system are presented in Table 4 of this appendix. Text Table 10 and Text Table 11 summarize the overall mean analyzed concentrations for each exposure system.

Text Table 10
Overall Mean Exposure Concentrations – Phase 1

Exposure System:	1	2	3	4
Target Concentration (ppm):	0	375	750	1500
Mean Concentration (ppm):	0	381	753	1501
Standard Deviation:	0.0	6.2	9.9	27.3
Total Number of Exposures:	4	4	4	4

Text Table 11
Overall Mean Exposure Concentrations – Phase 2

Exposure System:	**1**	**2**	**3**	**4**
Target Concentration (ppm):	0	375	750	1500
Mean Concentration (ppm):	0	375	741	1494
Standard Deviation:	0.0	27.6	59.4	84.1
Total Number of Exposures:	2	2	2	2

FIGURES

Figure 1 (Phase 1)
Atmosphere Generation and Exposure System

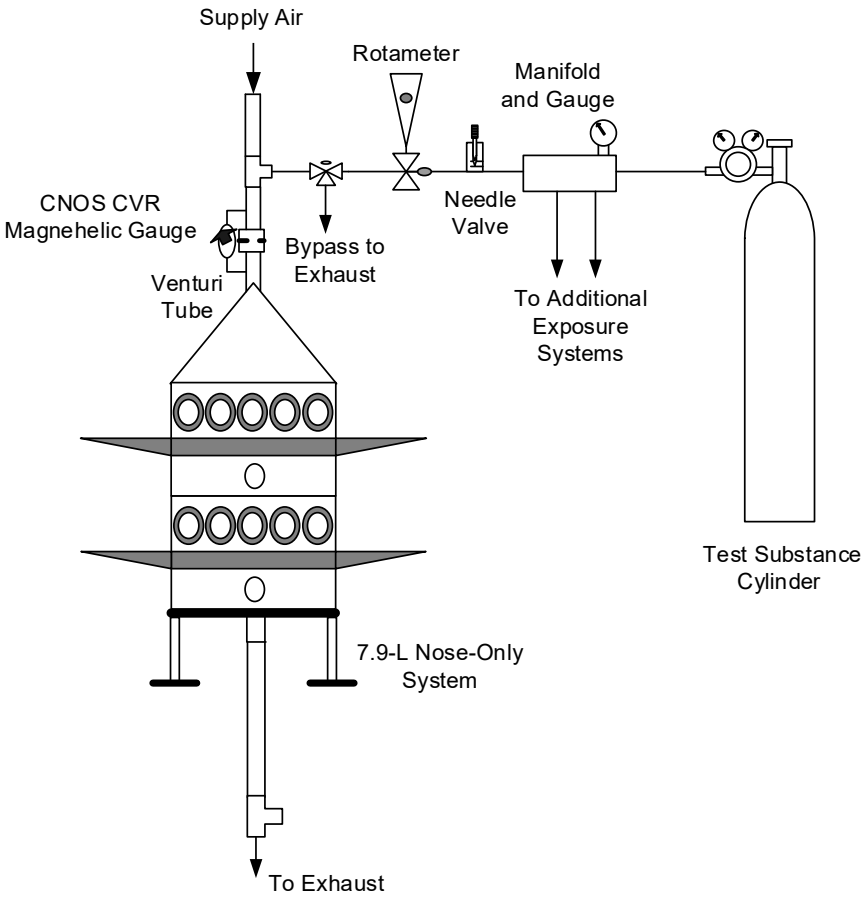
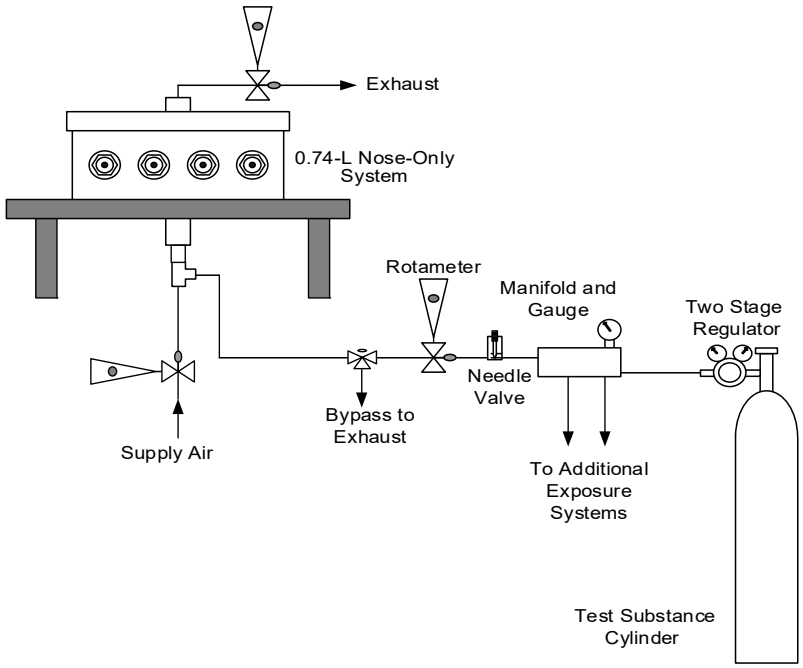


Figure 2 (Phase 2)
Atmosphere Generation and Exposure System



TABLES

INHALATION TABLE 1 (PHASE 1)

PROJECT NO.:[redacted] A COMBINED IN VIVO MICRONUCLEUS AND COMET ASSAY OF [redacted] IN RATS PAGE 1

SPONSOR: [redacted]

DAILY MEAN TEMPERATURE VALUES (°C)

RUN DATE	0 PPM	375 PPM	750 PPM	1500 PPM
19-JAN-2019	21.5	21.2	22.1	22.0
20-JAN-2019	21.9	21.9	22.8	22.9
21-JAN-2019	21.2	22.1	22.9	23.0
22-JAN-2019	21.4	21.6	22.5	22.5
GRAND MEAN:	21.5	21.7	22.6	22.6
SD:	0.29	0.39	0.36	0.45
N:	4	4	4	4

WIL Research Laboratories, LLC
Report File: TemperatureSummary v1.10.rpt (10-12-13), WINH version 1.28 Printed On: 02-Jul-2019 8:33

INHALATION TABLE 2 (PHASE 2)

PROJECT NO.:[redacted] A COMBINED IN VIVO MICRONUCLEUS AND COMET ASSAY OF [redacted] IN RATS PAGE 1

SPONSOR: [redacted]

DAILY MEAN TEMPERATURE VALUES (°C)

RUN DATE	0 PPM	375 PPM	750 PPM	1500 PPM
20-MAY-2019	21.0	20.3	20.5	20.6
21-MAY-2019	21.3	20.4	20.6	20.5
GRAND MEAN:	21.2	20.4	20.6	20.6
SD:	0.22	0.10	0.10	0.10
N:	2	2	2	2

WIL Research Laboratories, LLC
Report File: TemperatureSummary v1.10.rpt (10-12-13), WINH version 1.28 Printed On: 02-Jul-2019 8:34

INHALATION TABLE 3 (PHASE 1)
PROJECT NO.:[redacted] A COMBINED IN VIVO MICRONUCLEUS AND COMET ASSAY OF [redacted] IN RATS PAGE 1
SPONSOR: [redacted]

DAILY MEAN HUMIDITY VALUES (%)

RUN DATE	0 PPM	375 PPM	750 PPM	1500 PPM
19-JAN-2019	50.7	48.8	48.9	47.5
20-JAN-2019	46.2	43.9	43.3	41.1
21-JAN-2019	49.7	44.6	43.8	43.0
22-JAN-2019	47.4	45.0	46.6	44.4
GRAND MEAN:	48.5	45.6	45.7	44.0
SD:	2.06	2.20	2.61	2.70
CV (%):	4.247	4.825	5.711	6.136
N:	4	4	4	4

WIL Research Laboratories, LLC
Report File: HumiditySummary v1.10.rpt (10-15-13), WINH version 1.28 Printed On: 02-Jul-2019 8:27

INHALATION TABLE 4 (PHASE 2)
PROJECT NO.:[redacted] A COMBINED IN VIVO MICRONUCLEUS AND COMET ASSAY OF [redacted] IN RATS PAGE 1
SPONSOR: [redacted]

DAILY MEAN HUMIDITY VALUES (%)

RUN DATE	0 PPM	375 PPM	750 PPM	1500 PPM
20-MAY-2019	50.4	48.4	49.6	51.4
21-MAY-2019	50.0	45.3	47.9	50.2
GRAND MEAN:	50.2	46.9	48.8	50.8
SD:	0.28	2.19	1.20	0.85
CV (%):	0.558	4.670	2.459	1.673
N:	2	2	2	2

WIL Research Laboratories, LLC
Report File: HumiditySummary v1.10.rpt (10-15-13), WINH version 1.28 Printed On: 02-Jul-2019 8:30

INHALATION TABLE 5 (PHASE 1)

PROJECT NO.:[redacted] A COMBINED IN VIVO MICRONUCLEUS AND COMET ASSAY OF [redacted] IN RATS PAGE 1

SPONSOR: [redacted]

DAILY MEAN CVR VALUES (L/MIN)

RUN DATE	0 PPM	375 PPM	750 PPM	1500 PPM
19-JAN-2019	15	16	16	15
20-JAN-2019	15	16	16	15
21-JAN-2019	15	16	16	15
22-JAN-2019	15	16	16	15
GRAND MEAN:	15	16	16	15
SD:	0.0	0.0	0.0	0.0
CV (%):	0.00	0.00	0.00	0.00
N:	4	4	4	4

WIL Research Laboratories, LLC
Report File: CVRSummary v1.08.rpt (10-13-13), WINH version 1.28 Printed On: 02-Jul-2019 8:25

INHALATION TABLE 6 (PHASE 2)

PROJECT NO.: [redacted] A COMBINED IN VIVO MICRONUCLEUS AND COMET ASSAY OF [redacted] IN RATS PAGE 1

SPONSOR: [redacted] DAILY MEAN CVR VALUES (L/MIN)

DATE	GROUP: 0 PPM	375 PPM	750 PPM	1500 PPM
20-MAY-2019	6.8	6.4	6.8	6.7
21-MAY-2019	6.8	6.2	6.6	6.5
GRAND MEAN	6.8	6.3	6.7	6.6
SD	0.00	0.14	0.14	0.14
CV(%)	0.000	2.222	2.090	2.121
N	2	2	2	2

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INHALATION TABLE 7 (PHASE 1)

PROJECT NO.:[redacted] A COMBINED IN VIVO MICRONUCLEUS AND COMET ASSAY OF [redacted] IN RATS PAGE 1

SPONSOR: [redacted]

DAILY MEAN CONCENTRATION VALUES FOR [redacted] (PPM)

RUN DATE		0 PPM	375 PPM	750 PPM	1500 PPM
19-JAN-2019		0	378	753	1,492
	SD	0.0	103.2	22.3	34.7
	CV	NA	27.30	2.96	2.33
	N	6	6	6	6
20-JAN-2019		0	390	740	1,470
	SD	0.0	43.9	18.2	27.9
	CV	NA	11.26	2.46	1.90
	N	6	6	6	6
21-JAN-2019		0	378	755	1,507
	SD	0.0	9.3	3.5	28.4
	CV	NA	2.46	0.46	1.88
	N	6	6	6	6
22-JAN-2019		0	377	764	1,535
	SD	0.0	11.5	11.9	11.2
	CV	NA	3.05	1.56	0.73
	N	6	6	6	6
GRAND MEAN:		0	381	753	1,501
SD:		0.0	6.2	9.9	27.3
CV (%):		NA	1.63	1.31	1.82
N:		4	4	4	4

WIL Research Laboratories, LLC
Report File: CncSummary v1.13.rpt (12-07-15), WINH version 1.28 Printed On: 02-Jul-2019 8:21

INHALATION TABLE 8 (PHASE 2)

PROJECT NO.:[redacted] A COMBINED IN VIVO MICRONUCLEUS AND COMET ASSAY OF [redacted] IN RATS PAGE 1

SPONSOR: [redacted]

DAILY MEAN CONCENTRATION VALUES FOR [redacted] (PPM)

RUN DATE		0 PPM	375 PPM	750 PPM	1500 PPM
20-MAY-2019		0	355	699	1,434
	SD	0.0	180.6	129.1	191.7
	CV	NA	50.87	18.47	13.37
	N	6	10	6	6
21-MAY-2019		0	394	783	1,553
	SD	0.0	65.9	79.9	212.1
	CV	NA	16.73	10.20	13.66
	N	6	6	6	6
GRAND MEAN:		0	375	741	1,494
SD:		0.0	27.6	59.4	84.1
CV (%):		NA	7.36	8.02	5.63
N:		2	2	2	2

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Report File: CncSummary v1.13.rpt (12-07-15), WINH version 1.28 Printed On: 02-Jul-2019 8:23

APPENDIX 4

Individual Animal Data

[redacted]

TABLES EXPLANATION PAGE

All Day(s) referenced throughout the outputs generated are Study Days beginning with Study Day 1, the first day of dosing.

Abbreviations consistent throughout the Summary and Individual Tables.

Note: All of the abbreviations listed on these pages may not be applicable to this report.

Abbreviation	Description
MKD	milligrams/kilograms/day
mg/kg	milligrams/kilograms
ppm	parts per million
% Diff	% Difference from Group 1
tCtrl	Times control (fold change)
1F, 2F, 3F, 4F, 5F	Group 1 Female, Group 2 Female, Group 3 Female, Group 4 Female, Group 5 Female
1M, 2M, 3M, 4M, 5M	Group 1 Male, Group 2 Male, Group 3 Male, Group 4 Male, Group 5 Male
g	grams
kg	kilograms
mg	milligrams
N	Number of values included in analysis
M, F	Male, Female
<, >	Out of range
--	Not scheduled to be performed/dead
-	Not applicable

Phases 1 and 2
Group 1 - 0 ppm
Group 2 - 375 ppm
Group 3 - 750 ppm
Group 4 - 1500 ppm
Group 5 - 20/200 MKD

Abbreviation	Description	Abbreviation	Description
ANIC	Animal not in cage or incorrect cage during measurement	OA	Omitted activity
AVS	Suspected aberrant value	REHO	Animal rehoused during measurement interval
COME	See comment value excluded	REPL	Animal replaced during measurement interval
COMI	See comment value included	Sup	Suppress
COMM	Comment added	TARE	Balance tared
Exc	Exclude	Temp	Temperature
Int.	Interval	TERR	Technical error
NA	Not applicable	UPTD	Unable to perform due to technical difficulty
NC	Not calculable	UTD	Unable to determine
No.	Animal number	Wt	Weight
NSCH	Not scheduled to be performed		

CLINICAL OBSERVATIONS

Abbreviation	Description	Abbreviation	Description
0	White	A	Slight group housed
1	Slight	B	Moderate group housed
2	Moderate	C	Severe group housed
3	Severe	D	Scab ended
4	Black	DE	Detailed examination
5	Blue	G	Lesion ended
6	Brown	L	Lesion present
7	Clear	M	Mass present
8	Green	N	Severity not applicable
9	Red	S	Scab present
-	Severity not recorded	X	Present
!	Comment present	Y	Yellow

BODY WEIGHTS

Abbreviation	Description	Abbreviation	Description
AVS	Suspected aberrant value	TERR	Technical Error
E	Excluded	UPTD	Unable to perform due to technical difficulty
OA	Omitted Activity	X	Excluded from mean
%UD	%		

FOOD CONSUMPTION

Abbreviation	Description	Abbreviation	Description
AFE	Animal found with no food during measurement interval – exclude	Nr	Not reported due to animal replacement
AFNF	Animal found with no food during measurement interval – include	ONEG	Original value negative, animal did not eat
ANH	Animal found with no hopper during measurement interval	Pf	Powdered food
ANW	Animal found with no water during measurement interval	Sf	Supplemental food
Ar	Animals rehoused	SPIL	Spilled food by animal
AVS	Aberrant value suppressed/excluded	UTD	Unable to determine
Bf	Bowl on floor	W	Wet or contaminated food
Fd	Food deprived	Wa	Water added
FSNC	Food supplementation given during interval, value not calculable	WETF	Wet or contaminated food
NC	Not calculable	X	Excluded from mean
Np	Not scheduled to be performed		

MORTALITY

Abbreviation	Description	Abbreviation	Description
0–2 Ho	Removal symptom entered 0–2 hours postdosing	PostRx	Observation postdosing
ACCD	Accidental death	PreRx	Observation predosing
AD	Accidental death	REC	Recovery euthanasia
AM SIRT	Mortality/moribundity check in the morning	REL	Released
CSO	Cage side observation	TE	Terminal euthanasia
DE	Detailed examination	TERM	Terminal euthanasia
FD	Found dead	UE	Unscheduled euthanasia
INTM	Interim euthanasia	UNSC	Unscheduled euthanasia
PM SIRT	Mortality/moribundity check in the afternoon		

Table 4.1 Individual Mortality: Phase 1

[redacted]

Group	Dose Level	Sex	Animal	Cage	Removal Day	Removal Week	Removal Date	Removal Time	Time Slot	Removal Symptom	Pathology Reason
1	0 ppm	Male	1001	501	3	1	21JAN2019	14:01	.	TE	TERM
			1002	501	3	1	21JAN2019	14:02	.	TE	TERM
			1003	502	3	1	21JAN2019	14:03	.	TE	TERM
			1004	502	3	1	21JAN2019	14:05	.	TE	TERM
			1005	503	3	1	21JAN2019	14:06	.	TE	TERM
			1006	503	3	1	21JAN2019	14:07	.	TE	TERM
1	0 ppm	Female	1501	504	3	1	22JAN2019	14:37	.	TE	TERM
			1502	504	3	1	22JAN2019	14:37	.	TE	TERM
			1503	505	3	1	22JAN2019	14:39	.	TE	TERM
			1504	505	3	1	22JAN2019	14:38	.	TE	TERM
			1505	506	3	1	22JAN2019	14:40	.	TE	TERM
			1506	506	3	1	22JAN2019	14:41	.	TE	TERM
2	375 ppm	Male	2001	507	3	1	21JAN2019	14:56	.	TE	TERM
			2002	507	3	1	21JAN2019	14:56	.	TE	TERM
			2003	508	3	1	21JAN2019	14:57	.	TE	TERM
			2004	508	3	1	21JAN2019	14:57	.	TE	TERM
			2005	509	3	1	21JAN2019	14:57	.	TE	TERM
			2006	509	3	1	21JAN2019	14:57	.	TE	TERM
2	375 ppm	Female	2501	510	3	1	22JAN2019	14:55	.	TE	TERM
			2502	510	3	1	22JAN2019	14:54	.	TE	TERM
			2503	511	3	1	22JAN2019	14:55	.	TE	TERM
			2504	511	3	1	22JAN2019	14:55	.	TE	TERM
			2505	512	3	1	22JAN2019	14:56	.	TE	TERM
			2506	512	3	1	22JAN2019	14:55	.	TE	TERM
3	750 ppm	Male	3001	513	3	1	21JAN2019	15:27	.	TE	TERM
			3002	513	3	1	21JAN2019	15:27	.	TE	TERM
			3003	514	3	1	21JAN2019	15:29	.	TE	TERM
			3004	514	3	1	21JAN2019	15:30	.	TE	TERM
			3005	515	3	1	21JAN2019	15:32	.	TE	TERM
			3006	515	3	1	21JAN2019	15:32	.	TE	TERM
3	750 ppm	Female	3501	516	3	1	22JAN2019	16:10	.	TE	TERM
			3502	516	3	1	22JAN2019	16:10	.	TE	TERM

Table 4.1 Individual Mortality: Phase 1

[redacted]

Group	Dose Level	Sex	Animal	Cage	Removal Day	Removal Week	Removal Date	Removal Time	Time Slot	Removal Symptom	Pathology Reason
3	750 ppm	Female	3503	517	3	1	22JAN2019	16:10	.	TE	TERM
			3504	517	3	1	22JAN2019	16:11	.	TE	TERM
			3505	518	3	1	22JAN2019	16:11	.	TE	TERM
			3506	518	3	1	22JAN2019	16:11	.	TE	TERM
4	1500 ppm	Male	4001	519	3	1	21JAN2019	16:13	.	TE	TERM
			4002	519	3	1	21JAN2019	16:13	.	TE	TERM
			4003	520	3	1	21JAN2019	16:13	.	TE	TERM
			4004	520	3	1	21JAN2019	16:13	.	TE	TERM
			4005	521	3	1	21JAN2019	16:14	.	TE	TERM
			4006	521	3	1	21JAN2019	16:14	.	TE	TERM
4	1500 ppm	Female	4501	522	3	1	22JAN2019	16:12	.	TE	TERM
			4502	522	3	1	22JAN2019	16:11	.	TE	TERM
			4503	523	3	1	22JAN2019	16:12	.	TE	TERM
			4504	523	3	1	22JAN2019	16:12	.	TE	TERM
			4505	524	3	1	22JAN2019	16:13	.	TE	.
			4506	524	3	1	22JAN2019	16:12	.	TE	TERM
5	20/200 MKD	Male	5001	525	3	1	21JAN2019	12:45	.	TE	TERM
			5002	525	3	1	21JAN2019	12:45	.	TE	TERM
			5003	526	3	1	21JAN2019	12:45	.	TE	TERM
			5004	526	3	1	21JAN2019	12:45	.	TE	TERM
			5005	527	3	1	21JAN2019	12:45	.	TE	TERM
			5006	527	3	1	21JAN2019	12:45	.	TE	TERM
5	20/200 MKD	Female	5501	528	3	1	22JAN2019	12:53	0-2 Ho	TE	TERM
			5502	528	3	1	22JAN2019	12:53	0-2 Ho	TE	TERM
			5503	529	3	1	22JAN2019	12:53	0-2 Ho	TE	TERM
			5504	529	3	1	22JAN2019	12:53	0-2 Ho	TE	TERM
			5505	530	3	1	22JAN2019	12:54	0-2 Ho	TE	TERM
			5506	530	3	1	22JAN2019	12:54	0-2 Ho	TE	TERM

Table 4.2 Individual Clinical Observations: Phase 1

[redacted]

750 ppm Sex: Male	Observation Type: Toxicology Observations	Day(s) Relative to Start Date						
		2 DE	3 DE					
3006	Skin, Scab, Periorbital, Left	.	X					

X=Present

Table 4.2 Individual Clinical Observations: Phase 1

[redacted]

0 ppm Sex: Female	Observation Type: Toxicology Observations	Day(s) Relative to Start Date						
		2 DE	3 DE					
1504	Teeth, Broken	X	X					

X=Present

Table 4.2 Individual Clinical Observations: Phase 1

[redacted]

1500 ppm Sex: Female	Observation Type: Toxicology Observations	Day(s) Relative to Start Date						
		2 DE	3 DE					
4502	Fur, Staining, Urogenital, Yellow	.	X					

X=Present

Table 4.3 Individual Body Weights: Phase 1

[redacted]
Sex: Male Bodyweight (g)

0 ppm	Day(s) Relative to Start Date	
	1	3
1001	234	243
1002	221	231
1003	246	254
1004	225	231
1005	234	240
1006	229	243
Mean	231.5	240.3
SD	8.7	8.7
N	6	6

Table 4.3 Individual Body Weights: Phase 1

[redacted]
Sex: Male Bodyweight (g)

375 ppm	Day(s) Relative to Start Date	
	1	3
2001	226	233
2002	241	250
2003	227	234
2004	241	245
2005	225	225
2006	219	224
Mean	229.8	235.2
SD	9.1	10.5
N	6	6
%Diff	-0.7	-2.1

Table 4.3 Individual Body Weights: Phase 1

[redacted]
Sex: Male Bodyweight (g)

750 ppm	Day(s) Relative to Start Date	
	1	3
3001	231	226
3002	237	239
3003	217	218
3004	227	223
3005	225	227
3006	241	238
Mean	229.7	228.5
SD	8.6	8.4
N	6	6
%Diff	-0.8	-4.9

Table 4.3 Individual Body Weights: Phase 1

[redacted]
Sex: Male Bodyweight (g)

1500 ppm	Day(s) Relative to Start Date	
	1	3
4001	237	238
4002	246	242
4003	222	215
4004	229	229
4005	217	205
4006	237	238
Mean	231.3	227.8
SD	10.7	14.8
N	6	6
%Diff	-0.1	-5.2

Table 4.3 Individual Body Weights: Phase 1

[redacted]

Sex: Male Bodyweight (g)

20/200 MKD	Day(s) Relative to Start Date	
	1	3
5001	211	197
5002	222	212
5003	243	221
5004	229	217
5005	232	216
5006	235	217
Mean	228.7	213.3
SD	11.1	8.5
N	6	6
%Diff	-1.2	-11.2

Table 4.3 Individual Body Weights: Phase 1

[redacted]

Sex: Female Bodyweight (g)

0 ppm	Day(s) Relative to Start Date	
	1	3
1501	191	203
1502	184	189
1503	178	185
1504	197	192
1505	193	206
1506	185	187
Mean	188.0	193.7
SD	6.9	8.8
N	6	6

Table 4.3 Individual Body Weights: Phase 1

[redacted]

Sex: Female Bodyweight (g)

375 ppm	Day(s) Relative to Start Date	
	1	3
2501	200	206
2502	206	201
2503	180	170
2504	190	186
2505	192	194
2506	183	179
Mean	191.8	189.3
SD	9.9	13.6
N	6	6
%Diff	2.0	-2.2

Table 4.3 Individual Body Weights: Phase 1

[redacted]

Sex: Female Bodyweight (g)

750 ppm	Day(s) Relative to Start Date	
	1	3
3501	208	206
3502	182	182
3503	174	168
3504	189	186
3505	188	187
3506	193	195
Mean	189.0	187.3
SD	11.4	12.7
N	6	6
%Diff	0.5	-3.3

Table 4.3 Individual Body Weights: Phase 1

[redacted]

Sex: Female Bodyweight (g)

1500 ppm	Day(s) Relative to Start Date	
	1	3
4501	177	176
4502	167	171
4503	195	195
4504	192	187
4505	181	174
4506	188	188
Mean	183.3	181.8
SD	10.4	9.5
N	6	6
%Diff	-2.5	-6.1

Table 4.3 Individual Body Weights: Phase 1

[redacted]

Sex: Female Bodyweight (g)

20/200 MKD	Day(s) Relative to Start Date	
	1	3
5501	184	167
5502	197	174
5503	200	189
5504	186	174
5505	185	177
5506	195	193
Mean	191.2	179.0
SD	7.0	9.9
N	6	6
%Diff	1.7	-7.6

Table 4.4 Individual Body Weight Gains (g): Phase 1

[redacted]
Sex: Male Bodyweight Gain (Interval)

0 ppm	Day(s) Relative to Start Date
	1 → 3
1001	9
1002	10
1003	8
1004	6
1005	6
1006	14
Mean	8.8
SD	3.0
N	6

Table 4.4 Individual Body Weight Gains (g): Phase 1

[redacted]
Sex: Male Bodyweight Gain (Interval)

375 ppm	Day(s) Relative to Start Date
	1 → 3
2001	7
2002	9
2003	7
2004	4
2005	0
2006	5
Mean	5.3
SD	3.1
N	6

Table 4.4 Individual Body Weight Gains (g): Phase 1

[redacted]
Sex: Male Bodyweight Gain (Interval)

750 ppm	Day(s) Relative to Start Date
	1 → 3
3001	-5
3002	2
3003	1
3004	-4
3005	2
3006	-3
Mean	-1.2
SD	3.2
N	6

Table 4.4 Individual Body Weight Gains (g): Phase 1

[redacted]
Sex: Male Bodyweight Gain (Interval)

1500 ppm	Day(s) Relative to Start Date
	1 → 3
4001	1
4002	-4
4003	-7
4004	0
4005	-12
4006	1
Mean	-3.5
SD	5.2
N	6

Table 4.4 Individual Body Weight Gains (g): Phase 1

[redacted]
Sex: Male Bodyweight Gain (Interval)

20/200 MKD	Day(s) Relative to Start Date
	1 → 3
5001	-14
5002	-10
5003	-22
5004	-12
5005	-16
5006	-18
Mean	-15.3
SD	4.3
N	6

Table 4.4 Individual Body Weight Gains (g): Phase 1

[redacted]

Sex: Female Bodyweight Gain (Interval)

0 ppm	Day(s) Relative to Start Date
	1 → 3
1501	12
1502	5
1503	7
1504	-5
1505	13
1506	2
Mean	5.7
SD	6.7
N	6

Table 4.4 Individual Body Weight Gains (g): Phase 1

[redacted]

Sex: Female Bodyweight Gain (Interval)

375 ppm	Day(s) Relative to Start Date
	1 → 3
2501	6
2502	-5
2503	-10
2504	-4
2505	2
2506	-4
Mean	-2.5
SD	5.6
N	6

Table 4.4 Individual Body Weight Gains (g): Phase 1

[redacted]

Sex: Female Bodyweight Gain (Interval)

750 ppm	Day(s) Relative to Start Date
	1 → 3
3501	-2
3502	0
3503	-6
3504	-3
3505	-1
3506	2
Mean	-1.7
SD	2.7
N	6

Table 4.4 Individual Body Weight Gains (g): Phase 1

[redacted]

Sex: Female Bodyweight Gain (Interval)

1500 ppm	Day(s) Relative to Start Date
	1 → 3
4501	-1
4502	4
4503	0
4504	-5
4505	-7
4506	0
Mean	-1.5
SD	3.9
N	6

Table 4.4 Individual Body Weight Gains (g): Phase 1

[redacted]

Sex: Female Bodyweight Gain (Interval)

20/200 MKD	Day(s) Relative to Start Date
	1 → 3
5501	-17
5502	-23
5503	-11
5504	-12
5505	-8
5506	-2
Mean	-12.2
SD	7.3
N	6

Table 4.5 Individual Food Consumption: Phase 1

[redacted]
Sex: Male Food Mean Daily Consumption (g/animal/day)

0 ppm	Day(s) Relative to Start Date
	1 → 3
1001	20
1002	20
1003	19
1004	19
1005	21
1006	21
Mean	19.58
SD	0.79
N	6

Table 4.5 Individual Food Consumption: Phase 1

[redacted]
Sex: Male Food Mean Daily Consumption (g/animal/day)

375 ppm	Day(s) Relative to Start Date
	1 → 3
2001	18
2002	18
2003	16
2004	16
2005	16
2006	16
Mean	16.83
SD	1.10
N	6
%Diff	-14.04

Table 4.5 Individual Food Consumption: Phase 1

[redacted]
Sex: Male Food Mean Daily Consumption (g/animal/day)

750 ppm	Day(s) Relative to Start Date
	1 → 3
3001	16
3002	16
3003	14
3004	14
3005	16
3006	16
Mean	15.00
SD	1.18
N	6
%Diff	-23.40

Table 4.5 Individual Food Consumption: Phase 1

[redacted]

Sex: Male Food Mean Daily Consumption (g/animal/day)

1500 ppm	Day(s) Relative to Start Date
	1 → 3
4001	13
4002	13
4003	10
4004	10
4005	11
4006	11
Mean	11.33
SD	1.15
N	6
%Diff	-42.13

Table 4.5 Individual Food Consumption: Phase 1

[redacted]
Sex: Male Food Mean Daily Consumption (g/animal/day)

20/200 MKD	Day(s) Relative to Start Date
	1 → 3
5001	10
5002	10
5003	9
5004	9
5005	10
5006	10
Mean	9.75
SD	0.59
N	6
%Diff	-50.21

Table 4.5 Individual Food Consumption: Phase 1

[redacted]

Sex: Female Food Mean Daily Consumption (g/animal/day)

0 ppm	Day(s) Relative to Start Date
	1 → 3
1501	15
1502	15
1503	16
1504	16
1505	15
1506	15
Mean	15.08
SD	0.56
N	6

Table 4.5 Individual Food Consumption: Phase 1

[redacted]

Sex: Female Food Mean Daily Consumption (g/animal/day)

375 ppm	Day(s) Relative to Start Date
	1 → 3
2501	14
2502	14
2503	13
2504	13
2505	13
2506	13
Mean	13.33
SD	0.34
N	6
%Diff	-11.60

Table 4.5 Individual Food Consumption: Phase 1

[redacted]

Sex: Female Food Mean Daily Consumption (g/animal/day)

750 ppm	Day(s) Relative to Start Date
	1 → 3
3501	11
3502	11
3503	10
3504	10
3505	11
3506	11
Mean	10.50
SD	0.45
N	6
%Diff	-30.39

Table 4.5 Individual Food Consumption: Phase 1

[redacted]

Sex: Female Food Mean Daily Consumption (g/animal/day)

1500 ppm	Day(s) Relative to Start Date
	1 → 3
4501	8
4502	8
4503	10
4504	10
4505	8
4506	8
Mean	8.42
SD	0.85
N	6
%Diff	-44.20

Table 4.5 Individual Food Consumption: Phase 1

[redacted]

Sex: Female Food Mean Daily Consumption (g/animal/day)

20/200 MKD	Day(s) Relative to Start Date
	1 → 3
5501	7
5502	7
5503	9
5504	9
5505	9
5506	9
Mean	8.00
SD	0.77
N	6
%Diff	-46.96

Table 4.6 Individual Mortality: Phase 2

[redacted]

Group	Dose Level	Sex	Animal	Cage	Removal Day	Removal Week	Removal Date	Removal Time	Time Slot	Removal Symptom	Pathology Reason
1	0 ppm	Male	1007	535	2	1	21MAY2019	13:06	.	TE	TERM
			1008	535	2	1	21MAY2019	13:06	.	TE	TERM
			1009	536	2	1	21MAY2019	13:06	.	TE	TERM
			1010	536	2	1	21MAY2019	13:07	.	TE	TERM
			1011	537	2	1	21MAY2019	13:07	.	TE	TERM
			1012	537	2	1	21MAY2019	13:07	.	TE	TERM
2	375 ppm	Male	2007	538	2	1	21MAY2019	15:06	.	TE	TERM
			2008	538	2	1	21MAY2019	15:06	.	TE	TERM
			2009	539	2	1	21MAY2019	15:06	.	TE	TERM
			2010	539	2	1	21MAY2019	15:06	.	TE	TERM
			2011	540	2	1	21MAY2019	15:06	.	TE	TERM
			2012	540	2	1	21MAY2019	15:06	.	TE	TERM
3	750 ppm	Male	3007	541	2	1	21MAY2019	15:06	.	TE	TERM
			3008	541	2	1	21MAY2019	15:06	.	TE	TERM
			3009	542	2	1	21MAY2019	15:07	.	TE	TERM
			3010	542	2	1	21MAY2019	15:07	.	TE	TERM
			3011	543	2	1	21MAY2019	15:07	.	TE	TERM
			3012	543	2	1	21MAY2019	15:07	.	TE	TERM
4	1500 ppm	Male	4007	544	2	1	21MAY2019	15:07	.	TE	TERM
			4008	544	2	1	21MAY2019	15:07	.	TE	TERM
			4009	545	2	1	21MAY2019	15:07	.	TE	TERM
			4010	545	2	1	21MAY2019	15:07	.	TE	TERM
			4011	546	2	1	21MAY2019	15:08	.	TE	TERM
			4012	546	2	1	21MAY2019	15:08	.	TE	TERM
5	200 MKD	Male	5007	547	2	1	21MAY2019	12:31	.	TE	TERM
			5008	547	2	1	21MAY2019	12:31	.	TE	TERM
			5009	548	2	1	21MAY2019	12:32	.	TE	TERM
			5010	548	2	1	21MAY2019	12:32	.	TE	TERM
			5011	549	2	1	21MAY2019	12:33	.	TE	TERM
			5012	549	2	1	21MAY2019	12:33	.	TE	TERM

Table 4.7 Individual Clinical Observations: Phase 2

[redacted]

	Observation Type: All Types	Day(s) Relative to Start: Day 1 to 2
0 ppm	No Abnormalities Detected for All Animals	
375 ppm	No Abnormalities Detected for All Animals	
750 ppm	No Abnormalities Detected for All Animals	
1500 ppm	No Abnormalities Detected for All Animals	
200 MKD	No Abnormalities Detected for All Animals	

Table 4.8 Individual Body Weights: Phase 2

[redacted]
Sex: Male Bodyweight (g)

0 ppm	Day(s) Relative to Start Date	
	1	2
1007	248	256
1008	249	251
1009	232	233
1010	282	281
1011	222	226
1012	263	265
Mean	249.3	252.0
SD	21.5	20.3
N	6	6

Table 4.8 Individual Body Weights: Phase 2

[redacted]
Sex: Male Bodyweight (g)

375 ppm	Day(s) Relative to Start Date	
	1	2
2007	260	258
2008	234	238
2009	243	243
2010	254	254
2011	267	260
2012	261	256
Mean	253.2	251.5
SD	12.4	8.9
N	6	6
%Diff	1.5	-0.2

Table 4.8 Individual Body Weights: Phase 2

[redacted]
Sex: Male Bodyweight (g)

750 ppm	Day(s) Relative to Start Date	
	1	2
3007	262	259
3008	273	275
3009	261	267
3010	245	247
3011	236	241
3012	260	255
Mean	256.2	257.3
SD	13.3	12.5
N	6	6
%Diff	2.7	2.1

Table 4.8 Individual Body Weights: Phase 2

[redacted]

Sex: Male Bodyweight (g)

1500 ppm	Day(s) Relative to Start Date	
	1	2
4007	248	241
4008	242	247
4009	259	248
4010	267	249
4011	269	258
4012	249	230
Mean	255.7	245.5
SD	11.0	9.4
N	6	6
%Diff	2.5	-2.6

Table 4.8 Individual Body Weights: Phase 2

[redacted]
Sex: Male Bodyweight (g)

200 MKD	Day(s) Relative to Start Date	
	1	2
5007	237	229
5008	257	235
5009	273	258
5010	252	242
5011	252	241
5012	261	234
Mean	255.3	239.8
SD	11.9	10.1
N	6	6
%Diff	2.4	-4.8

Table 4.9 Individual Body Weight Gains (g): Phase 2

[redacted]
Sex: Male Bodyweight Gain (Interval)

0 ppm	Day(s) Relative to Start Date
	1 → 2
1007	8
1008	2
1009	1
1010	-1
1011	4
1012	2
Mean	2.7
SD	3.1
N	6

Table 4.9 Individual Body Weight Gains (g): Phase 2

[redacted]

Sex: Male Bodyweight Gain (Interval)

375 ppm	Day(s) Relative to Start Date
	1 → 2
2007	-2
2008	4
2009	0
2010	0
2011	-7
2012	-5
Mean	-1.7
SD	3.9
N	6

Table 4.9 Individual Body Weight Gains (g): Phase 2

[redacted]

Sex: Male Bodyweight Gain (Interval)

750 ppm	Day(s) Relative to Start Date
	1 → 2
3007	-3
3008	2
3009	6
3010	2
3011	5
3012	-5
Mean	1.2
SD	4.4
N	6

Table 4.9 Individual Body Weight Gains (g): Phase 2

[redacted]
Sex: Male Bodyweight Gain (Interval)

1500 ppm	Day(s) Relative to Start Date
	1 → 2
4007	-7
4008	5
4009	-11
4010	-18
4011	-11
4012	-19
Mean	-10.2
SD	8.7
N	6

Table 4.9 Individual Body Weight Gains (g): Phase 2

[redacted]
Sex: Male Bodyweight Gain (Interval)

200 MKD	Day(s) Relative to Start Date
	1 → 2
5007	-8
5008	-22
5009	-15
5010	-10
5011	-11
5012	-27
Mean	-15.5
SD	7.5
N	6

Table 4.10 Individual Food Consumption: Phase 2

[redacted]
Sex: Male Food Mean Daily Consumption (g/animal/day)

0 ppm	Day(s) Relative to Start Date
	1 → 2
1007	19
1008	19
1009	21
1010	21
1011	18
1012	18
Mean	18.83
SD	1.37
N	6

Table 4.10 Individual Food Consumption: Phase 2

[redacted]
Sex: Male Food Mean Daily Consumption (g/animal/day)

375 ppm	Day(s) Relative to Start Date
	1 → 2
2007	17
2008	17
2009	6
2010	6
2011	3
2012	3
Mean	8.33
SD	6.85
N	6
%Diff	-55.75

Table 4.10 Individual Food Consumption: Phase 2

[redacted]
Sex: Male Food Mean Daily Consumption (g/animal/day)

750 ppm	Day(s) Relative to Start Date
	1 → 2
3007	21
3008	21
3009	4
3010	4
3011	16
3012	16
Mean	13.17
SD	7.81
N	6
%Diff	-30.09

Table 4.10 Individual Food Consumption: Phase 2

[redacted]
Sex: Male Food Mean Daily Consumption (g/animal/day)

1500 ppm	Day(s) Relative to Start Date
	1 → 2
4007	18
4008	18
4009	7
4010	7
4011	7
4012	7
Mean	10.17
SD	5.68
N	6
%Diff	-46.02

Table 4.10 Individual Food Consumption: Phase 2

[redacted]
Sex: Male Food Mean Daily Consumption (g/animal/day)

200 MKD	Day(s) Relative to Start Date
	1 → 2
5007	5
5008	5
5009	9
5010	9
5011	7
5012	7
Mean	6.67
SD	1.57
N	6
%Diff	-64.60

APPENDIX 5

Micronucleus Report

FINAL PRINCIPAL INVESTIGATOR'S CONTRIBUTING REPORT

Study Title
A Combined *In Vivo* Micronucleus and Comet Assay of [redacted] in Sprague Dawley Rats

Study Subtitle
Peripheral Blood Micronucleus Evaluation

Test Site
BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Reference Number
AF56EM.129FLOWPBGLP.BTL

Author
Megan Young, PhD

Study Completion Report Date
15 May 2019

Sponsor
[redacted]

Testing Facility
Charles River Laboratories Ashland, LLC
1407 George Road
Ashland, OH 44805

Test Facility Study Number
[redacted]

Sponsor Reference Number
[redacted]

1. STATEMENT OF COMPLIANCE

Reference No. AF56EM.129FLOWPBGLP.BTL was conducted in compliance with the following regulations: United States Code of Federal Regulations, Title 40, Parts 160 and 792: Good Laboratory Practice Standards and as accepted by Regulatory Authorities throughout the European Union (OECD Principles of Good Laboratory Practice), Japan (MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

Megan Young, PhD
Principal Investigator

15 May 2019
Date

2. QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AF56EM.129FLOWPBGLP.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this portion of the multi site study was performed in accordance with the regulation(s) listed below and conducted according to the client study protocol/statement of work and relevant BioReliance Standard Operating Procedures.

US EPA Good Laboratory Standards 40CFR 160
US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this portion of the multi site study.

Insp. Dates (From/To)		Phase Inspected	To Principal Investigator	To Test Site Management	To Study Director & Facility Management
01-Feb-2019	01-Feb-2019	Slide Preparation	06-Mar-2019	06-Mar-2019	06-Mar-2019
04-Mar-2019	07-Mar-2019	Data/Draft Report	07-Mar-2019	07-Mar-2019	26-Apr-2019
05-Mar-2019	06-Mar-2019	Protocol Review	06-Mar-2019	06-Mar-2019	06-Mar-2019
06-Mar-2019	06-Mar-2019	Protocol Amendment Review	06-Mar-2019	06-Mar-2019	06-Mar-2019
09-Apr-2019	10-Apr-2019	Final Report	10-Apr-2019	10-Apr-2019	15-May-2019
15-May-2019	15-May-2019	Protocol Amendment Review	15-May-2019	15-May-2019	15-May-2019

The Final Report for this portion of the multi site study identified above describes the methods and procedures and attests that the reported results accurately reflect the raw data.

E-signature

Quality Assurance: Alan Tarwater 15-May-2019 6:23 pm GMT
Reason for signature: QA Approval

Printed by:Alan Tarwater
Printed on:15-May-19

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4. STUDY INFORMATION

Study Conduct

Sponsor:	[redacted]
Sponsor's Authorized Representative:	[redacted]
Testing Facility:	Charles River Laboratories Ashland, LLC 1407 George Road Ashland, OH 44805
Study Director:	Michael S. Cockburn
Test Site:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Reference No.:	AF56EM.129FLOWPBGLP.BTL
Test Facility Study No.:	[redacted]

Samples

Test Substance Identification:	[redacted]
Description of Material Received at BioReliance:	Frozen Opaque blackish-red liquid (blood samples)
Receipt Condition:	Dry Ice
Receipt Dates:	30 & 31 January 2019

Study Dates

Study Initiation Date:	19 December 2018
First Day of Data Collection for Micronucleus Evaluation:	01 February 2019
Experimental Completion Date (End of Micronucleus Evaluation):	01 February 2019

Key Personnel

Principal Investigator:	Megan Young, PhD
Test Site Management:	Rohan Kulkarni, MSc, PhD Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Ryan Hamilton, MS
Report Writer:	Joan Huynh, BS

5. SUMMARY

Peripheral blood samples were evaluated for the test substance's ability to increase the incidence of micronuclei formation in peripheral blood reticulocytes (RETs) as compared to the control.

Rat peripheral blood samples from six rats per group/sex were shipped to BioReliance. Upon receipt at BioReliance, the peripheral blood samples were washed with buffer solution, stained with a solution containing RNase, FITC-conjugated anti-CD 71 antibodies, and PE-conjugated anti-CD 61 antibodies, and then stained with propidium iodide for evaluation using flow cytometry.

There was no significant increase in the number of micronuclei in the test substance dosed animals compared to the control in both males and females. The control values were compatible with the expected range of %MnRETs. There was a statistically significant increase in MnRETs in the positive control compared to the concurrent control. All criteria for a valid assay were met.

Under the conditions of this study, the administration of [redacted] at doses up to and including dose of 1500ppm was concluded to be negative in the Micronucleus assay.

6. PURPOSE

The purpose of this work was to evaluate the ability of the test substance to increase the incidence of micronuclei formation in peripheral blood as compared to the control.

Historical control data are found in Appendix I.

7. PLAN OF WORK

The main study was conducted at the test facility. The study design is presented in the following table:

Group Number	Treatment	Target Exposure Concentration (ppm)	**Positive Control Concentration (mg/mL)**	**Positive Control Dose Volume (mL/kg)**	Animal Number	
					Males	Females
1	Filtered Air	0	NA	NA	1001-1006	1501-1506
2	[redacted]	375	NA	NA	2001-2006	2501-2506
3	[redacted]	750	NA	NA	3001-3006	3501-3506
4	[redacted]	1500	NA	NA	4001-4006	4501-4506
5	Positive Control	CP: 20 mg/kg/day	2	10	5001-5006	5501-5506
		EMS: 200 mg/kg/day	20	10		

The preparation of all articles, dosing of animals, observation of animals, and collection of peripheral blood samples is addressed in the study protocol (Study Number [redacted]) and protocol amendment.

Peripheral blood was collected from 6 rats/group/sex, and two peripheral blood samples were prepared for each animal.

Shipment of Blood Samples

Two fixed blood samples per animal per time point (6 animals per group/sex) stored in Long Term Storage Solution (LTSS) were shipped to the Test Site. Appropriate documentation was included in the shipment and is included in the raw data file.

Upon receipt, the fixed blood samples in LTSS were delivered to the laboratory for storage at -65 to -90ºC until flow cytometric processing.

Detection of Micronucleated Reticulocytes with Flow Cytometry

Five samples/group/sex in LTSS were thawed at room temperature, washed with ice cold 1% FBS solution to remove the fixative and maintained on wet ice. The cells were then pelleted by centrifugation, and the supernatant was poured off leaving a small amount of supernatant with the pellet. The cells were re-suspended and 20 µL of suspension was added to 80 µL of staining solution containing RNase, FITC-conjugated anti-CD 71 antibodies and PE-

conjugated anti-CD 61 antibodies. The samples were incubated at 2-8ºC for 30 minutes, re-suspended, then incubated at room temperature for an additional 30 minutes.

The frequency of micronucleated reticulocytes in peripheral blood was analyzed after flow cytometry calibration using Malaria infected biostandard and negative control standards provided in the Litron kit. 20,000 RETs per animal were analyzed when possible.

Remaining samples were discarded prior to report finalization

Calculation of Flow Cytometric Analysis

The proportion of reticulocytes to the total number of cells scored (%RETs) was determined for each animal and treatment group. This calculation was carried out as indicated below:

$$\%RET = \frac{(UL + UR) \times 100}{(UL + UR + LL + LR)}$$

UL: The number of events in the upper-left quadrant
UR: The number of events in the upper-right quadrant
LL: The number of events in the lower-left quadrant
LR: The number of events in the lower-right quadrant

The %RETs served as a parameter of the test substance cytotoxicity in peripheral blood. A decrease in this ratio in the test substance groups, as compared to the control (Sham), indicated a toxic effect of the test substance, while an increase represented a sign of recovery from earlier toxic insult.

The quantization of the MnRETs in peripheral blood was expressed as the percentage of MnRETs per total number of cells evaluated. The %MnRETs was presented for each animal, and the mean ± standard deviation was calculated and presented for each treatment group as follows:

$$\%MnRET = \frac{(UR) \times 100}{(UL + UR)}$$

Statistical Analysis

Statistical analysis was performed on the micronucleus frequency (%MnRET) and %RET using animal as unit. The mean and standard deviation of %MnRET and %RET were presented for each treatment group.

The use of parametric or non-parametric statistical methods in the evaluation of data was based on the variation between groups. The group variances for micronucleus frequency for the control and test substance groups at the respective sampling time were compared using Levene's test (significance level of $p \leq 0.05$). Since the variation between groups was found

not to be significant, a parametric one-way ANOVA was performed followed by a Dunnett's post-hoc analysis to compare each dose group to the concurrent control.

A linear regression analysis was conducted to assess dose responsiveness in the test substance treated groups ($p \leq 0.01$).

A pair-wise comparison (Student's T-test) was used to compare the positive control group to the concurrent control group.

Criteria for Determination of a Valid Test

A target of 20,000 RETs/animal was analyzed for the presence of micronuclei (MnRETs). The proportion of reticulocytes to total number of cells scored (%RETs) was determined for each animal and treatment group. The %RETs served as a parameter of the test substance cytotoxicity in peripheral blood. A reduction in the RET proportions to less than 5% of the control value was considered excessively toxic and the animal data was excluded from evaluation.

Negative Controls

The group mean frequency of MnRETs should ideally be within the 95% control limits of the distribution of the historical negative control database. If the concurrent Filtered Air control fall outside the 95% control limits, they may be acceptable as long as these data are not extreme outliers (indicative of experimental or human error).

Positive Controls

The frequency of MnRETs for the scoring controls must be significantly greater than the concurrent control ($p \leq 0.05$).

Evaluation of Test Results

A test substance was considered to have induced a positive response if:

a) at least one of the substance doses exhibited a statistically significant increase when compared with the concurrent Filtered Air control ($p \leq 0.05$), and
b) when multiple doses were examined at a particular sampling time, the increase was dose-related ($p \leq 0.01$ and $R^2 \geq 70\%$), and
c) results of the group mean or of the individual animals in at least one group were outside the 95% control limit of the historical negative control data.

A test substance was considered to have induced a clear negative response if none of the criteria for a positive response were met and there was evidence that the bone marrow was exposed to the test substance (unless intravenous administration was used).

Electronic Data Collection Systems

Electronic systems used for the collection or analysis of data included, but was not limited to, the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel (Microsoft Corporation)	Calculations
Excel	Statistics
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting
Flow Cytometry and BD FACSDiva™ Software	Sample Analysis

Records and Archives

All materials generated BioReliance from this study will be transferred to a Charles River archive

Deviations

No deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

8. RESULTS AND DISCUSSION

The incidence of MnRETs per total RETs scored (20,000 RETs/animal, when possible) and the proportion of reticulocytes to total cells (%RETs), summarized and presented in Table 1. Individual animal data is presented in Table 2.

Based on peripheral blood analysis, the following were observed:

- No appreciable reductions in the RET proportions in the test substance treated groups for both males and females compared to the control group were observed indicating the test substance did not induce cytotoxicity.

- Group variances for the mean of the micronucleus frequency in the control and test substance groups of both males and females were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p > 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.

- No statistically significant increase in the incidence of MnRETs in the test substance treated groups of both males and females was observed relative to the control group ($p > 0.05$, ANOVA followed by Dunnett's post-hoc analysis).

- The positive control of both males and females induced a statistically significant increase in the incidence of MnRETs (Student's t-test, $p < 0.05$).

- The number of MnRETs in the control groups of both males and females did not exceed the historical control range (Appendix I).

Based upon this, all criteria for a valid test were met based on testing facility protocol.

9. CONCLUSION

Under the conditions of this study, the administration of [redacted] at doses up to and including dose of 1500ppm was concluded to be negative in the Micronucleus assay.

10. DATA TABLES

Table 1: Summary of Peripheral Blood Micronucleus Analysis

Treatment	Gender	Time (Hrs)	Animals	%RET (Mean +/- SD)	Cytotoxicity (%)	%MnRET (Mean +/- SD)	Total Cells Scored MnRET	Total Cells Scored RET
Filtered Air								
0 ppm	M	1-3	5	3.90 ± 0.46	---	0.13 ± 0.04	125	99875
0 ppm	F	1-3	5	1.43 ± 0.17	---	0.12 ± 0.01	119	99881
[redacted]								
375 ppm	M	1-3	5	3.65 ± 0.67	-6	0.12 ± 0.04	122	99879
375 ppm	F	1-3	5	1.78 ± 0.65	24	0.09 ± 0.02	91	99919
750 ppm	M	1-3	5	3.56 ± 0.35	-9	0.12 ± 0.02	116	99947
750 ppm	F	1-3	5	1.80 ± 0.12	26	0.08 ± 0.02	84	99917
1500 ppm	M	1-3	5	3.33 ± 0.83	-15	0.13 ± 0.04	128	99872
1500 ppm	F	1-3	5	1.60 ± 0.36	12	0.12 ± 0.04	124	99876
CP/EMS								
20/200 mg/kg/day	M	1-3	5	1.71 ± 1.22*	-56	1.44 ± 1.25*	1312	92919
20/200 mg/kg/day	F	1-3	5	0.24 ± 0.12**	-83	1.73 ± 0.56**	630	40364

*p < 0.05 or **p < 0.01, One-Way ANOVA with Post-Hoc Dunnett's Test or T-Test
1-3 Hrs MnRET Male GLM P-value = 0.951, R-sqr = 2.07%
1-3 Hrs MnRET Female GLM P-value = 0.056, R-sqr = 36.83%

Table 2: Induction of Micronucleated Reticulocytes in Peripheral Blood
1-3 Hour

Treatment	Sex	Animal No.	RET%	Micronucleated RET		
				MnRET	RET	%
Filtered Air	M	1001	4.32	13	19987	0.07
0 ppm		1002	4.06	28	19972	0.14
		1003	3.19	28	19972	0.14
		1004	3.73	32	19968	0.16
		1005	4.21	24	19976	0.12
Filtered Air	F	1501	1.38	22	19978	0.11
0 ppm		1502	1.62	23	19977	0.12
		1503	1.32	26	19974	0.13
		1504	1.24	21	19979	0.11
		1505	1.60	27	19973	0.14
[redacted]	M	2001	3.84	17	19983	0.09
375 ppm		2002	4.56	22	19978	0.11
		2003	2.68	31	19970	0.16
		2004	3.56	33	19967	0.17
		2005	3.62	19	19981	0.10
[redacted]	F	2501	2.72	15	19985	0.08
375 ppm		2502	1.88	19	19981	0.10
		2503	1.28	18	19982	0.09
		2504	1.05	15	19995	0.08
		2505	1.95	24	19976	0.12
[redacted]	M	3001	3.91	23	19977	0.12
750 ppm		3002	3.63	22	19978	0.11
		3003	2.98	26	19975	0.13
		3004	3.54	27	19973	0.14
		3005	3.72	18	20044	0.09
[redacted]	F	3501	1.83	12	19989	0.06
750 ppm		3502	1.98	14	19986	0.07
		3503	1.65	23	19977	0.12
		3504	1.72	21	19979	0.11
		3505	1.80	14	19986	0.07

RET – reticulocytes; MnRET – micronucleated reticulocytes

Table 2: Induction of Micronucleated Reticulocytes in Peripheral Blood
1-3 Hour

Treatment	Sex	Animal No.	RET%	Micronucleated RET		
				MnRET	RET	%
[redacted]	M	4001	4.36	38	19962	0.19
1500 ppm		4002	3.95	20	19980	0.10
		4003	2.66	22	19978	0.11
		4004	3.29	18	19982	0.09
		4005	2.39	30	19970	0.15
[redacted]	F	4501	1.94	23	19977	0.12
1500 ppm		4502	1.42	32	19968	0.16
		4503	1.13	33	19967	0.17
		4504	1.99	21	19979	0.11
		4505	1.50	15	19985	0.08
CP/EMS	M	5001	0.41	324	13907	2.28
20/200 mg/kg/day		5002	1.89	44	19956	0.22
		5003	0.94	625	19375	3.13
		5004	1.70	252	19748	1.26
		5006	3.61	67	19933	0.34
CP/EMS	F	5501	0.18	84	5690	1.46
20/200 mg/kg/day		5502	0.29	166	9921	1.65
		5503	0.17	129	5847	2.16
		5504	0.43	148	14705	1.00
		5505	0.12	103	4201	2.39

RET – reticulocytes; MnRET – micronucleated reticulocytes

11. APPENDIX I: Historical Control

Micronucleus Test Using Flow Methodology in Rat Peripheral Blood Lymphocytes
Historical Control Values
2015-2017

Historical Vehicle Control[1] in Male Rats (Peripheral Blood)

	Individual Animals		Studies	
	RET%	**MN%**	**RET%**	**MN%**
N	155	155	30	30
Mean[3]	3.99	0.07	4.05	0.07
SD	1.43	0.04	1.25	0.04
95% UCL	6.84	0.16	6.55	0.14
95% LCL	1.14	0.00	1.55	0.00
Max[4]	9.76	0.22	6.55	0.14
Min[4]	0.87	0.01	1.17	0.03

Historical Positive Control[2] in Male Rats (Peripheral Blood)

	Individual Animals		Studies	
	RET%	**MN%**	**RET%**	**MN%**
N	135	135	29	29
Mean[3]	0.54	1.14	0.55	1.16
SD	0.61	0.77	0.60	0.67
95% UCL	1.77	2.69	1.75	2.50
95% LCL	0.00	0.00	0.00	0.00
Max[4]	3.30	6.59	2.58	3.43
Min[4]	0.01	0.14	0.02	0.19

Note: Since no appreciable differences in the induction of MnRETs by different vehicles and solvents (test substance carriers) and different routes of administration were observed, this table contains data from carriers and routes of administration widely used during the conduct of contract studies in the period of 2015 thru 2017 at BioReliance.

Routes of administration: intraperitoneal (IP), intravenous (IV), oral gavage (PO), subcutaneous (SC).
Peripheral blood collection time: 48 hours post-final dose.
[1]Vehicles: water, water soluble vehicles (methylcellulose, carboxymethylcellulose, dextrose), saline, corn oil and other vehicles.
[2]Positive control: Cyclophosphamide monohydrate (CP) dosed orally.
[3]Average of the RET ratio observed out of up to 20,000 RETs scored per animal for the total number of animals used from 2015 to 2017; average of the number of MnRETs observed out of up to 20,000 RETs for the total number of animals used; average of number of MnRETs observed out of up to 20,000 RETs for total number of studies.
[4]Minimum and maximum range of the RET ratio observed out of up to 20,000 RETs scored per animal; the minimum and maximum range of MnRETs observed out of up to 20,000 RETs for the total number of animals used and the minimum and maximum range of MnRETs observed out of up to 20,000 RETs for the total number of studies.
Formula: 95% control limit ranges = mean ± 2 x standard deviation

Historical Vehicle Control[1] in Female Rats (Peripheral Blood)

	Individual Animals		Studies	
	RET%	**MN%**	**RET%**	**MN%**
N	58	58	11	11
Mean[3]	1.78	0.08	1.78	0.08
SD	0.70	0.05	0.60	0.04
95% UCL	3.19	0.19	2.99	0.16
95% LCL	0.37	0.00	0.58	0.00
Max[4]	2.98	0.28	2.89	0.15
Min[4]	0.56	0.00	0.85	0.02

Historical Positive Control[2] in Female Rats (Peripheral Blood)

	Individual Animals		Studies	
	RET%	**MN%**	**RET%**	**MN%**
N	48	47	10	10
Mean[3]	0.16	0.95	0.16	0.97
SD	0.26	0.84	0.24	0.73
95% UCL	0.68	2.64	0.64	2.43
95% LCL	0.00	0.00	0.00	0.00
Max[4]	1.29	3.64	0.82	2.74
Min[4]	0.00	0.00	0.02	0.23

Note: Since no appreciable differences in the induction of MnRETs by different vehicles and solvents (test substance carriers) and different routes of administration were observed, this table contains data from carriers and routes of administration widely used during the conduct of contract studies in the period of 2015 thru 2017 at BioReliance.

Routes of administration: intraperitoneal (IP), intravenous (IV), oral gavage (PO), subcutaneous (SC).
Peripheral blood collection time: 48 hours post-final dose.
[1]Vehicles: water, water soluble vehicles (methylcellulose, carboxymethylcellulose, dextrose), saline, corn oil and other vehicles.
[2]Positive control: Cyclophosphamide monohydrate (CP) dosed orally.
[3]Average of the RET ratio observed out of up to 20,000 RETs scored per animal for the total number of animals used from 2015 to 2017; average of the number of MnRETs observed out of up to 20,000 RETs for the total number of animals used; average of number of MnRETs observed out of up to 20,000 RETs for total number of studies.
[4]Minimum and maximum range of the RET ratio observed out of up to 20,000 RETs scored per animal; the minimum and maximum range of MnRETs observed out of up to 20,000 RETs for the total number of animals used and the minimum and maximum range of MnRETs observed out of up to 20,000 RETs for the total number of studies.
Formula: 95% control limit ranges = mean ± 2 x standard deviation

APPENDIX 6

In Vivo Comet Report

FINAL PRINCIPAL INVESTIGATOR'S CONTRIBUTING REPORT

Study Title
A Combined *In Vivo* Micronucleus and Comet Assay of [redacted] in Sprague Dawley Rats

Study Subtitle
***In Vivo* Comet Assay Slide Evaluation**

Author
Shannon Bruce, MFS

Study Completion Date
22 October 2019

Test Site
BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850, USA

BioReliance Reference Number
AF56EM.151.BTL

Sponsor
[redacted]

Testing Facility
Charles River Laboratories Ashland, LLC
1407 George Road
Ashland, OH 44805

Testing Facility Study Number
[redacted]

Sponsor Reference Number
[redacted]

1. STATEMENT OF COMPLIANCE

This *in-vivo* Comet assay, identified by BioReliance Reference No. AF56EM.151.BTL, was conducted in compliance with the following regulations: United States Code of Federal Regulations, Title 40, Parts 160 and 792: Good Laboratory Practice Standards, and as accepted by Regulatory Authorities throughout the European Union (OECD Principles of Good Laboratory Practice), Japan (MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

Shannon Wilson Bruce
Shannon Bruce, MFS
Principal Investigator

22 Oct 2019
Date

2. QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AF56EM.151.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this portion of the multi site study was performed in accordance with the regulation(s) listed below and conducted according to the client study protocol/statement of work and relevant BioReliance Standard Operating Procedures.

US EPA Good Laboratory Standards 40CFR 160
US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this portion of the multi site study.

Insp. Dates (From/To)		Phase Inspected	To Principal Investigator	To Test Site Management	To Study Director & Facility Management
05-Mar-2019	06-Mar-2019	Protocol Review	06-Mar-2019	06-Mar-2019	06-Mar-2019
06-Mar-2019	06-Mar-2019	Protocol Amendment Review	06-Mar-2019	06-Mar-2019	06-Mar-2019
06-Mar-2019	06-Mar-2019	Cell Handling	06-Mar-2019	06-Mar-2019	06-Mar-2019
08-Apr-2019	14-Apr-2019	Data/Draft Report	14-Apr-2019	14-Apr-2019	15-Apr-2019
15-May-2019	15-May-2019	Protocol Amendment Review	15-May-2019	15-May-2019	15-May-2019
07-Jun-2019	07-Jun-2019	Scoring	07-Jun-2019	07-Jun-2019	07-Jun-2019
15-Jul-2019	19-Jul-2019	Data/Draft Report	19-Jul-2019	19-Jul-2019	19-Jul-2019
11-Oct-2019	11-Oct-2019	Final Report	11-Oct-2019	11-Oct-2019	11-Oct-2019

The Final Report for this portion of the multi site study identified above describes the methods and procedures and attests that the reported results accurately reflect the raw data.

E-signature

Quality Assurance: Jeannie Eberle 21-Oct-2019 4:39 pm GMT
Reason for signature: QA Approval

Printed by:Jeannie Eberle
Printed on:21-Oct-19

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3. STUDY INFORMATION

Study Conduct

Sponsor:	[redacted]
Sponsor Representative:	[redacted]
Testing Facility:	Charles River Laboratories Ashland, LLC 1407 George Road Ashland, OH 44805
Study Director:	Michael S. Cockburn
Alternate Contact:	Jeffrey T. Weinberg, BS
Management Contact:	James M. Randazzo, PhD, DABT
Testing Facility Study No.:	[redacted]
Test Site for Comet Assay:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850, USA
BioReliance Reference No.:	AF56EM.151.BTL
Sponsor Reference No.:	[redacted]

Samples

Test Substance Identification:	[redacted]
Description of Material Received at BioReliance:	Microscope slides
Storage Conditions:	Room temperature, protected from light, with desiccant
Receipt Date:	29 January 2019 (Phase 1) 29 May 2019 (Phase 2)

Study Dates

Study Initiation Date:	19 December 2018

First Day of Data Collection - Phase 1 (at BioReliance):	21 January 2019
Last Day of Data Collection – Phase 1 (at BioReliance):	27 March 2019
First Day of Data Collection - Phase 2 (at BioReliance):	31 May 2019
Last Day of Data Collection – Phase 2 (at BioReliance):	06 June 2019

Key Personnel

Principal Investigator:	Shannon Bruce, MFS BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
Test Site Management:	Rohan Kulkarni, MSc, PhD Director, Genetic Toxicology Study Management
Test Site Laboratory Supervisor (Comet Assay, Preparation and Scoring of Slides):	Tiffany Rae Blaylock, BS
Report Writer:	Cristian Ciubotaru, MS

4. SUMMARY

The Comet Assay was conducted on kidney, liver, lung and nasal cells obtained from five Sprague Dawley rats/sex/group. During the initial assay (Phase 1), animals in Group 1 were exposed via nose-only inhalation to filtered air (negative control, 0 ppm); animals in Groups 2-4 were exposed via nose-only inhalation to the vaporized test substance ([redacted], at 375 ppm, 750 ppm or 1500 ppm, respectively). The exposure period for Groups 1-4 was 6 hours/day, for three consecutive days. Positive control animals (Group 5) were dosed by oral gavage with cyclophosphamide (CP, 20 mg/kg/day) formulated in deionized water, on Days 1 and 2; the same animals were dosed with ethyl methanesulfonate by oral gavage (EMS, 200 mg/kg/day) formulated in 0.9% saline, on Days 2 and 3. All treatments for Group 5 animals were administered via oral gavage, at a dose volume of 10 mL/kg.

The rats were euthanized on Day 3, between 2-4 hours after the final exposure. The kidney, liver, lung and nasal cells were harvested at the Testing Facility for processing to single cell suspensions. Once Comet slide preparation and electrophoresis were completed and the cells were fixed, the multi-well slides were shipped at ambient temperature to BioReliance for staining and scoring.

Kidney - Phase 1

For males, statistically significant increases in %Tail DNA were observed when compared to the vehicle control values for all test substance dose levels; however, the increases were within the historical control range and were evaluated as not biologically relevant (a dose response was also observed).

For females, no statistically significant increases in %Tail DNA were observed when compared to the vehicle control values. One vehicle control animal and one mid dose animal had individual %Tail DNA values above the historical control range, but the group mean ranges were all within the historical control range; no concentration-response was observed.

For both sexes, the group mean vehicle control value was within the historical control range; however, the group mean positive control (CP/EMS) was not statistically significant when compared to the concurrent group mean vehicle control and was not compatible with the historical control range for CP/EMS dosed animals.

Liver - Phase 1

For males, no statistically significant increases in %Tail DNA were observed when compared to the vehicle control values, and no concentration-response was observed. The group mean vehicle control value was within the historical control range.

For females, no statistically significant increases in %Tail DNA were observed when compared to the vehicle control values, and no concentration-response was observed. However, all

animals in the mid concentration group had %Tail DNA values above the historical control range; in addition, the group mean vehicle control value was above the historical control range.

For males, the group mean positive control was statistically significant when compared to the concurrent group mean vehicle control and was compatible with the historical control range.

For females, the group mean positive control was not statistically significant when compared to the concurrent group mean vehicle control and was not compatible with the historical positive control range.

Lung - Phase 1

For males, statistically significant increases in %Tail DNA were observed when compared to the vehicle control values for all test substance exposure levels; however, these increases were all within historical control range and have been evaluated as not biologically relevant (a concentration-response was also observed). The group mean vehicle control value was slightly below the current historical control range.

For females, a statistically significant increase in %Tail DNA was observed in the mid concentration- group, when compared to the vehicle control values. This increase was above the historical control range; however, no concentration-response was observed. One vehicle control animal and all test substance-treated animals had %Tail DNA values above the historical control range. The group mean vehicle control value was slightly above the current historical control range.

For both sexes, the group mean positive control was not statistically significant when compared to the concurrent group mean vehicle control and was not compatible with the historical control range.

Nasal Cavity - Phase 1

For males, a statistically significant increase in %Tail DNA was observed in the mid concentration group, when compared to the vehicle control values. This increase was within historical control range and has been evaluated as not biologically relevant; in addition, no concentration-response was observed.

For females, no statistically significant increases in %Tail DNA were observed when compared to the vehicle control values; also, no concentration-response was observed.

For both sexes, the group mean vehicle control value was within the historical control range; however, the group mean positive control was not statistically significant when compared to the concurrent group mean vehicle control and was not compatible with the historical control range.

Due to a lack of positive response in the Comet assay from the positive controls, a second phase was added to this study. Only males were used in Phase 2, since no difference in systemic

toxicity was noted between males and females during Phase 1. The treatments administered in Phase 2 were similar with Phase 1, with the following exceptions: males in Groups 1-4 were exposed for only 2 consecutive days (6 hours/day); Group 5 males were treated with EMS only, on Days 1 and 2. The terminal procedures were similar but were performed on Day 2.

Male Kidney - Phase 2

No statistically significant increases in %Tail DNA were observed when compared to the concurrent vehicle control for all test article concentration levels. No concentration-response was observed. The group mean vehicle control %Tail DNA was within historical control range. The group mean positive control %Tail DNA was statistically significant when compared to the concurrent group mean vehicle control and was compatible with the historical control range.

Male Liver - Phase 2

A statistically significant increase in %Tail DNA was observed in the mid concentration group (750 ppm) when compared to the concurrent vehicle control; however, this increase was within historical control range and has been evaluated as not biologically relevant. No concentration-response was observed. The group mean vehicle control %Tail DNA was within the historical control range. The group mean positive control %Tail DNA was statistically significant when compared to the concurrent group mean vehicle control and was compatible with the historical control range.

Male Lung - Phase 2

No statistically significant increases in %Tail DNA were observed when compared to the concurrent vehicle control for all test article concentration levels. No concentration-response was observed. The group mean vehicle control %Tail DNA was within the historical control range. The group mean positive control %Tail DNA was statistically significant when compared to the concurrent group mean vehicle control and was compatible with the historical control range.

Male Nasal Cavity - Phase 2

No statistically significant increases in %Tail DNA were observed in the nasal cavity when compared to the concurrent vehicle control. No concentration-response was observed. The group mean vehicle control %Tail DNA was within the historical control range. The group mean positive control %Tail DNA was statistically significant when compared to the concurrent group mean vehicle control and was compatible with the historical control range.

In conclusion, during Phase 1 of the assay (both sexes), the test substance, [redacted], could be evaluated as negative (non-DNA damaging) in male liver cells only. For the remaining tissues tested, the assay did not meet all the acceptance criteria (specifically as related to the positive control treatment), therefore was considered invalid. However, during Phase 2 of the assay (males only), the test substance has been evaluated as negative (non-DNA

damaging) during the *in vivo* alkaline Comet assay; all valid assay criteria were met, for all tissues tested. Therefore, under the conditions of these studies, [redacted] is considered to be non-DNA damaging as evaluated by the comet assay.

5. PURPOSE

The purpose of this portion of the study was to evaluate the ability of the test substance ([redacted]) to induce DNA damage in liver, lung, kidney and nasal tissue, when administered via nose-only inhalation to Sprague Dawley rats, for 6 hours per day, for up to 3 consecutive days.

The Comet assay was conducted according to OECD Guideline 489.

6. PLAN OF WORK

The main study was conducted at the Testing Facility. The study design relevant to the Comet assay (Phase 1) is presented in the following table:

Group	Treatment	Target Exposure Concentration* (ppm)	Dose Volume (mL/kg)	Number of Rats/Sex
1	Filtered Air	0	NA	6
2	[redacted]	375	NA	6
3	[redacted]	750	NA	6
4	[redacted]	1500	NA	6
5	Positive Control	EMS[a] 200 mg/kg/day	10	6
		CP[b] 20 mg/kg/day	10	

NA = Not applicable.
* Filtered air (negative control group) and vaporized test substance were administered via nose-only inhalation for 3 consecutive days (6 hrs/day), Days 1-3.
[a] = Ethyl methanesulfonate, positive control for Comet assay, administered on Days 2 and 3.
[b] = Cyclophosphamide monohydrate, positive control for Micronucleus assay, administered on Days 1 and 2.

The study design relevant to the Phase 2 of the assay is presented in the following table:

Group	Treatment	Target Exposure Concentration* (ppm)	Dose Volume (mL/kg)	Number of Male Rats
1	Filtered Air	0	NA	6
2	[redacted]	375	NA	6
3	[redacted]	750	NA	6
4	[redacted]	1500	NA	6
5	Positive Control	EMS[a] 200 mg/kg/day	10	6

NA = Not applicable.
* Filtered air (negative control group) and vaporized test substance were administered via nose-only inhalation for 2 consecutive days (6 hrs/day), Days 1 and 2.
[a] = Ethyl methanesulfonate, positive control for Comet assay, administered on Days 1 and 2.

The preparation of all articles, dosing of animals and observation of animals is addressed in the main study report.

Tissue Collection for Comet Assay

The following work was performed at the Testing Facility.

Surviving animals were anesthetized on Day 3 (Phase 1) or on Day 2 (Phase 2), between 2 to 4 hours after the final exposure, by isoflurane inhalation followed by exsanguination (to complete euthanasia). Immediately following euthanasia, kidney, liver, lung and nasal cells samples were collected for the Comet assay from 5 animals/sex/group.

Sections of kidney, liver, lung and nasal tissue were placed in 3 mL chilled mincing solution (Hanks' balanced salt solution with EDTA and DMSO), then minced with fine scissors to release the cells. The cell suspensions were strained into pre-labeled conical polypropylene tubes through a cell strainer and were kept on wet ice during preparation of the multi-well slides.

Preparation of Comet Multi-well Slides

Preparation of Multi-well Slides

From each cell suspension, a 2.5 µL aliquot was mixed with 75 µL of low melting agarose. The cell/agarose suspension was applied to microscope multi-well slides. Commercially purchased (Trevigen®) pre-treated, multi-well slides were used, and these slides have 20 individual circular areas, referred to as wells. The multi-well slides were kept at 2-8°C for at least 15 minutes to allow the gel to solidify. Multi-well slides were identified with a random code that reflects the study number, group, animal number, and organ/tissue. At least two 20-well slides were prepared per animal per tissue. Three wells were used in scoring and the other wells were designated as a backup. Following solidification of agarose, the multi-well slides were placed in jars containing lysis solution.

Lysis

Following solidification of agarose, the multi-well slides were submerged in a cold solution composed of a commercially available lysis solution supplemented with 10% DMSO, on the day of use. The multi-well slides were kept in this solution at least overnight at 2-8°C.

Unwinding

After cell lysis, slides/wells were washed with neutralization buffer (0.4 M tris hydroxymethyl aminomethane in purified water, pH ~7.5) and placed in the electrophoresis chamber. The chamber reservoirs were slowly filled with alkaline buffer, composed of 300 mM sodium hydroxide and 1 mM EDTA (disodium) in purified water; the pH was >13. All multi-well slides remained in the buffer for 20 minutes at 2-10°C, protected from light, allowing DNA to unwind.

Electrophoresis

Using the same buffer, electrophoresis was conducted for 30 minutes at 0.7 V/cm, at 2-12°C and protected from light (except as noted in the Deviations section). The electrophoresis time was constant for all multi-well slides.

Neutralization

After completion of electrophoresis, the multi-well slides were removed from the electrophoresis chamber and washed with neutralization buffer for at least 10 minutes. The multi-well slides (gels) were then dehydrated with 200-proof ethanol for at least 5 minutes, then air dried for at least 2 hours and stored at room temperature with desiccant. These multi-well slides were shipped at ambient temperature to BioReliance by overnight shipment; upon receipt, the slides were logged in by the Test Site's repository.

Staining

The following work was performed at the Test Site (BioReliance).

Multi-well slides were stained with a DNA stain (i.e., Sybr-gold™) prior to scoring. The stain solution was prepared by diluting 1 µL of Sybr-gold™ stain in 15 mL of 1xTBE (tris-boric acid EDTA buffer solution).

Scoring of Comet Multi-well Slides

Three wells per organ/animal were used. Fifty randomly selected, non-overlapping cells per slide/well were scored, resulting in a total of 150 cells (when possible) evaluated per animal for DNA damage, using the fully validated automated scoring system Comet Assay IV from Perceptive Instruments Ltd. (UK).

The following endpoints of DNA damage were assessed and measured:

- Comet Tail Migration; defined as the distance from the perimeter of the Comet head to the last visible point in the tail.
- % Tail DNA; (also known as % tail intensity or % DNA in tail); defined as the percentage of DNA fragments present in the tail.
- Tail Moment (also known as Olive Tail moment); defined as the product of the amount of DNA in the tail and the tail length [(% Tail DNA x Tail Length)/100; Olive et al. 1990)].

Each slide/well was also examined for indications of cytotoxicity. The rough estimate of the percentage of "clouds" was determined by scanning 150 cells per animal, when possible (percentage of "clouds" was calculated by adding the total number of clouds for all multi-well slides scored, dividing by the total number of cells scored and multiplying by 100). The "clouds", also known as "hedgehogs", are a morphological indication of highly damaged cells

often associated with severe genotoxicity, necrosis or apoptosis. A "cloud" is produced when almost the entire cell DNA is in the tail of the comet and the head is reduced in size, almost nonexistent (Collins, 2004). "Clouds" with visible gaps between the nuclei and the comet tail were excluded from comet image analysis.

Multi-well slides were discarded prior to Comet report finalization.

Statistical Analysis

The median %Tail DNA for the Comets scored on each slide was determined and the mean of the median values was calculated for each animal. The mean of the individual animal was then used to calculate a group mean.

To quantify the test substance-related effects on DNA damage, the following statistical analysis was performed:

- The use of parametric or non-parametric statistical methods in evaluation of data was based on the variances between groups. The group variances for % Tail DNA generated for the negative control and test substance-treated groups were compared using Levene's test (significant level of $p < 0.05$). Since the variances between groups were found to be not significant, a parametric one-way ANOVA followed by a Dunnett's post-hoc test was performed (significant level of $p < 0.05$).

- A linear regression analysis was conducted to assess concentration- responsiveness in the test substance-treated groups ($p < 0.01$).

- A pair-wise comparison (Student's T-test, $p < 0.05$) was used to compare the positive control group against the concurrent negative control group.

Criteria for a Valid Test

Negative controls

For each tissue analyzed, the DNA damage (% Tail DNA) in the negative control group was expected to be within the historical vehicle/negative control range for that tissue.

Positive Controls

The group mean for the % Tail DNA must be significantly greater than the concurrent negative control ($p < 0.05$), and the response should be comparable with those observed in the historical positive control data base.

Evaluation of Test Results

Once the criteria for a valid assay were met, the results were evaluated as detailed below.

Means of 150 counts of % tail DNA, Tail moment, and Tail migration were presented for each animal and each organ. The mean and standard deviation of the mean values for % tail DNA were presented for each treatment group.

Statistical analysis was performed only for % tail DNA. All conclusions were based on sound scientific judgment.

A test substance was considered to have induced a positive response in a particular tissue if:

a) at least one of the groups mean for the % tail DNA of the test substance doses exhibited a statistically significant increase when compared with the concurrent negative control ($p < 0.05$), and

b) when multiple doses were examined at a particular sampling time, the increase was dose-related ($p < 0.01$), and

c) results of the group mean or of individual animals from at least one group were outside the distribution of the historical vehicle/negative control data for that tissue.

A test substance was considered to have induced a clear negative response if none of the criteria for a positive response were met, and there was direct or indirect evidence supportive of exposure or toxicity at the target tissue.

A test substance was considered to have induced an equivocal response if the response was neither clearly positive nor clearly negative; to establishing the biological relevance of an equivocal result, data may be evaluated by expert judgment and/or further investigations. Any additional work would only be carried out in consultation with (and at the request of) the Sponsor.

Electronic Data Collection Systems

Electronic systems used for the collection or analyses of data included, but were not limited to, the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test substance/Slide Tracking
Excel (Microsoft Corporation)	Calculations
Minitab	Statistics
Kaye Lab Watch Monitoring System (Kaye GE)	Environmental Monitoring
BRIQS	Deviations and audit reporting
Comet Assay IV (Perceptive Instruments)	Scoring of Slides

Records and Archives

All study-specific raw data, electronic data, documentation, protocol, retained samples and specimens, and final reports will be archived by no later than the date of final report issue. All

materials generated by BioReliance from this study will be transferred to a Charles River archive.

Deviations

The following deviations from the protocol and the assay-method SOPs were noted (BRIQS = BioReliance Integrated Quality System).

BRIQS Event #348858: Electrophoresis in the Trevigen chamber was supposed to be done at 0.7 V/cm, corresponding to 14 V and 170 to 200 mA, based on chamber size. In deviation of the protocol and SOP OPGT0802, Electrophoresis Run No. 9 (Phase 1) was done at 0.65 V/cm (corresponding to 13 V and 199 mA, both at the start and the end of the run). Although the technician attempted to correct the problem (adding electrophoresis buffer to the maximum volume allowed by the chamber, and even replacing most of the buffer in the chamber with a new batch), the voltage did not increase. Electrophoresis Run No. 9 contained female slides for vehicle control (all organs) and for liver (mid dose only).

This deviation had no impact on study or the interpretation of the study results. The excursion was small; none of the affected slides had abnormal responses to the conditions, and all values were within the expected ranges.

BRIQS Event #363489: The voltage and allowable amperage range for an electrophoresis run using the 10-slide Trevigen electrophoresis chamber is 14V and 170 to 200 mA. In deviation from protocol and SOP OPGT0802, the amperage at the start and end of Electrophoresis Run No. 2 (Phase 2) containing the B (backup) slides was 163 mA. Although the technician attempted to correct the problem (additional alkaline electrophoresis buffer was added to the chamber in increments and amperage rechecked, until the maximum chamber volume of 850 mL was reached), the amperage did not increase.

This deviation had no impact on study or the interpretation of the study results, since the backup slides were not scored for evaluation of comet.

7. RESULTS AND DISCUSSION

Kidney – Phase 1

The %Tail DNA in kidney cells is summarized for each treatment group and presented in Table 1A (males) and Table 2 (females). Median values for the %Tail DNA**,** Tail moment and Tail migration (µm) for kidney cells are calculated per 150 cells for each animal and are presented in Table 9A (males) and Table 10 (females).

For males, the scoring results and statistical analysis of data indicated the following:

- The means of 'clouds' in the test substance groups were ≤ 0.8%, which was somewhat comparable with the mean of clouds in the negative control group (0.0%).
- Group variances for mean of medians of the %Tail DNA in the negative control and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p \geq 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.
- A statistically significant increase in %Tail DNA (DNA damage) was observed in all test substance groups, relative to the concurrent negative control group (ANOVA followed by Dunnett's post-hoc analysis, $p < 0.05$). However, the test substance group means were within the historical control range for kidneys, therefore these results were considered biologically irrelevant.
- A concentration-dependent increase in the %Tail DNA was observed across the test substance groups (regression analysis, $p < 0.01$).
- The positive control did not induce a statistically significant increase in the %Tail DNA in kidney cells as compared to the negative control group (Student's t-test, $p \geq 0.05$); additionally, the positive control group mean was not compatible with the historical control range.
- In the negative control group, the group mean %Tail DNA was below the historical vehicle/negative control range for kidney (Appendix I).

For females, the scoring results and statistical analysis of data indicated the following:

- The means of 'clouds' in the test substance groups were ≤ 0.6%, which was comparable with the mean of clouds in the negative control group (0.2%).
- Group variances for mean of medians of the % Tail DNA in the negative control and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p \geq 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.
- A statistically significant decrease in %Tail DNA (DNA damage) was observed in the high and low exposure groups, relative to the concurrent negative control group

(ANOVA followed by Dunnett's post-hoc analysis, $p < 0.05$); these results were considered biologically irrelevant. One vehicle control animal and one mid exposure animal had individual %Tail DNA values above the historical control range, but the group mean ranges were all within the historical control range.

- No concentration-dependent increase in the %Tail DNA was observed across the test substance groups (regression analysis, $p \geq 0.01$).
- The positive control did not induce a statistically significant increase in the %Tail DNA in kidney cells as compared to the negative control group (Student's t-test, $p \geq 0.05$); additionally, the positive control group mean was not compatible with the historical control range.
- In the negative control group, the group mean %Tail DNA was within the historical vehicle/negative control range for kidney (Appendix I).

These results indicate that the criteria for a valid test were not met, for either sex.

Kidney – Phase 2

The %Tail DNA in male kidney cells is summarized for each treatment group and presented in Table 1B; median values for the %Tail DNA**,** Tail moment and Tail migration (µm) for male kidney cells are calculated per 150 cells for each animal and are presented in Table 9B.

For males, the scoring results and statistical analysis of data indicated the following:

- The means of 'clouds' in the test substance groups were ≤ 1.2%, which was comparable with the mean of clouds in the negative control group (1.8%).
- Group variances for mean of medians of the %Tail DNA in the negative control and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p \geq 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.
- No statistically significant response in the %Tail DNA (DNA damage) was observed in the test substance groups relative to the concurrent negative control group (ANOVA followed by Dunnett's post-hoc analysis, $p \geq 0.05$).
- No concentration-dependent increase in the %Tail DNA was observed across the test substance groups (regression analysis, $p \geq 0.01$).
- The positive control induced a statistically significant increase in the %Tail DNA in liver cells as compared to the negative control group (Student's t-test, $p < 0.05$).
- In the negative control group, the group mean %Tail DNA was within the historical vehicle/negative control range for kidney (Appendix I).

These results indicate that all criteria for a valid test, as specified in the protocol, were met.

Liver – Phase 1

The %Tail DNA in liver cells is summarized for each treatment group and presented in Table 3A (males) and Table 4 (females). Median values for the %Tail DNA**,** Tail moment and Tail migration (µm) for liver cells are calculated per 150 cells for each animal and are presented in Table 11A (males) and Table 12 (females).

For males, the scoring results and statistical analysis of data indicated the following:

- The means of 'clouds' in the test substance groups were ≤ 1.0%, which was somewhat comparable with the mean of clouds in the negative control group (0.0%).
- Group variances for mean of medians of the %Tail DNA in the negative control and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p \geq 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.
- No statistically significant response in the %Tail DNA (DNA damage) was observed in the test substance groups relative to the concurrent negative control group (ANOVA followed by Dunnett's post-hoc analysis, $p \geq 0.05$).
- No concentration-dependent increase in the %Tail DNA was observed across the test substance groups (regression analysis, $p \geq 0.01$).
- The positive control induced a statistically significant increase in the %Tail DNA in liver cells as compared to the negative control group (Student's t-test, $p < 0.05$).
- In the negative control group, the group mean %Tail DNA was within the historical vehicle/negative control range for liver (Appendix I).

For females, the scoring results and statistical analysis of data indicated the following:

- The means of 'clouds' in the test substance groups were ≤ 0.6%, which was comparable with the mean of clouds in the negative control group (0.0%).
- Group variances for mean of medians of the %Tail DNA in the negative control and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p \geq 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.
- A statistically significant decrease in %Tail DNA (DNA damage) was observed in the high and low exposure groups, relative to the concurrent negative control group (ANOVA followed by Dunnett's post-hoc analysis, $p < 0.05$); these results were considered biologically irrelevant. All animals in the mid exposure group had %Tail DNA values above the historical control range; in addition, the group mean vehicle control value was above the historical control range.

- No concentration-dependent increase in the %Tail DNA was observed across the test substance groups (regression analysis, $p \geq 0.01$).
- The positive control did not induce a statistically significant increase in the %Tail DNA in liver cells as compared to the negative control group (Student's t-test, $p \geq 0.05$); additionally, the positive control group mean was below the historical control range.
- In the negative control group, the group mean %Tail DNA was higher than the historical vehicle/negative control range for liver (Appendix I).

These results indicate that all criteria for a valid test, as specified in the protocol, were met for males; however, the tissue response was considered invalid for females.

Liver – Phase 2

The %Tail DNA in male liver cells is summarized for each treatment group and presented in Table 3B; median values for the %Tail DNA**,** Tail moment and Tail migration (µm) for male liver cells are calculated per 150 cells for each animal and are presented in Table 11B.

For males, the scoring results and statistical analysis of data indicated the following:

- The means of 'clouds' in the test substance groups were ≤ 0.6%, which was comparable with the mean of clouds in the negative control group (0.0%).
- Group variances for mean of medians of the %Tail DNA in the negative control and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p \geq 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.
- A statistically significant increase in %Tail DNA was observed in the mid concentration group, 750 ppm, when compared to the vehicle control (ANOVA followed by Dunnett's post-hoc analysis, $p < 0.05$); however, this increase was within the historical control range, therefore has been evaluated as not biologically relevant.
- No concentration-dependent increase in the %Tail DNA was observed across the test substance groups (regression analysis, $p \geq 0.01$).
- The positive control induced a statistically significant increase in the %Tail DNA in liver cells as compared to the negative control group (Student's t-test, $p < 0.05$).
- In the negative control group, the group mean %Tail DNA was within the historical vehicle/negative control range for liver (Appendix I).

These results indicate that all criteria for a valid test, as specified in the protocol, were met.

Lung – Phase 1

The %Tail DNA in lung cells is summarized for each treatment group and presented in Table 5A (males) and Table 6 (females). Median values for the %Tail DNA**,** Tail moment and Tail migration (µm) for lung cells are calculated per 150 cells for each animal and are presented in Table 13A (males) and Table 14 (females).

For males, the scoring results and statistical analysis of data indicated the following:

- The means of 'clouds' in the test substance groups were ≤ 5.4%, generally higher than the mean of clouds in the negative control group (0.6%).
- Group variances for mean of medians of the %Tail DNA in the negative control and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p \geq 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.
- A statistically significant increase in %Tail DNA (DNA damage) was observed in all test substance groups, relative to the concurrent negative control group (ANOVA followed by Dunnett's post-hoc analysis, $p < 0.05$). However, the test substance group means were within the historical control range for lung, therefore these results were considered biologically irrelevant.
- A concentration-dependent increase in the %Tail DNA was observed across the test substance groups (regression analysis, $p < 0.01$).
- The positive control did not induce a statistically significant increase in the %Tail DNA in lung cells as compared to the negative control group (Student's t-test, $p \geq 0.05$); additionally, the positive control group mean was not compatible with the historical control range.
- In the negative control group, the group mean %Tail DNA was below the historical vehicle/negative control range for lung (Appendix I).

For females, the scoring results and statistical analysis of data indicated the following:

- The means of 'clouds' in the test substance groups were ≤ 0.8%, which was comparable with the mean of clouds in the negative control group (0.2%).
- Group variances for mean of medians of the %Tail DNA in the negative control and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p \geq 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.
- Although ANOVA was not significant ($p \geq 0.05$), Dunnett's test flagged a statistically significant increase in %Tail DNA for the mid dose group, relative to the concurrent negative control group ($p < 0.05$). One vehicle control animal and all test substance-treated animals had %Tail DNA values above the historical

control range. For all test substance groups, the means were higher than the historical vehicle/negative control range for lung.

- No concentration-dependent increase in the %Tail DNA was observed across the test substance groups (regression analysis, $p \geq 0.01$).
- The positive control did not induce a statistically significant increase in the %Tail DNA in lung cells as compared to the negative control group (Student's t-test, $p \geq 0.05$); additionally, the positive control group mean was not compatible with the historical control range.
- In the negative control group, the group mean %Tail DNA was higher than the historical vehicle/negative control range for lung (Appendix I).

These results indicate that the criteria for a valid test were not met, for either sex.

Lung – Phase 2

The %Tail DNA in male lung cells is summarized for each treatment group and presented in Table 5B; median values for the %Tail DNA**,** Tail moment and Tail migration (µm) for male lung cells are calculated per 150 cells for each animal and are presented in Table 13B.

For males, the scoring results and statistical analysis of data indicated the following:

- The means of 'clouds' in the test substance groups were ≤ 6.4%, which was comparable with the mean of clouds in the negative control group (7.0%).
- Group variances for mean of medians of the %Tail DNA in the negative control and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p \geq 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.
- No statistically significant response in the %Tail DNA (DNA damage) was observed in the test substance groups relative to the concurrent negative control group (ANOVA followed by Dunnett's post-hoc analysis, $p \geq 0.05$).
- No concentration-dependent increase in the %Tail DNA was observed across the test substance groups (regression analysis, $p \geq 0.01$).
- The positive control induced a statistically significant increase in the %Tail DNA in liver cells as compared to the negative control group (Student's t-test, $p < 0.05$).
- In the negative control group, the group mean %Tail DNA was within the historical vehicle/negative control range for lung (Appendix I).

These results indicate that all criteria for a valid test, as specified in the protocol, were met.

Nasal Cells – Phase 1

The %Tail DNA in nasal cells is summarized for each treatment group and presented in Table 7A (males) and Table 8 (females). Median values for the %Tail DNA**,** Tail moment and Tail migration (µm) for nasal cells are calculated per 150 cells for each animal and are presented in Table 15A (males) and Table 16 (females).

For males, the scoring results and statistical analysis of data indicated the following:

- The means of 'clouds' in the test substance groups were ≤ 20.2%, generally higher than the mean of clouds in the negative control group (3.6%).
- Group variances for mean of medians of the %Tail DNA in the negative control and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p \geq 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.
- A statistically significant increase in %Tail DNA (DNA damage) was observed in the mid exposure group, relative to the concurrent negative control group (ANOVA followed by Dunnett's post-hoc analysis, $p < 0.05$). However, this group mean was within the historical control range for nasal cells, therefore the result was considered biologically irrelevant.
- No concentration-dependent increase in the %Tail DNA was observed across the test substance groups (regression analysis, $p \geq 0.01$).
- The positive control did not induce a statistically significant increase in the %Tail DNA in nasal cells as compared to the negative control group (Student's t-test, $p \geq 0.05$); additionally, the positive control group mean was not compatible with the historical control range.
- In the negative control group, the group mean %Tail DNA was within the historical vehicle/negative control range for nasal cells (Appendix I).

For females, the scoring results and statistical analysis of data indicated the following:

- The means of 'clouds' in the test substance groups were ≤ 32.4%, comparable with the mean of clouds in the negative control group (28.0%).
- Group variances for mean of medians of the %Tail DNA in the negative control and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p \geq 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.
- A statistically significant decrease in %Tail DNA (DNA damage) was observed in the high and low exposure groups, relative to the concurrent negative control group (ANOVA followed by Dunnett's post-hoc analysis, $p < 0.05$); these results were considered biologically irrelevant.

- No concentration-dependent increase in the %Tail DNA was observed across the test substance groups (regression analysis, $p \geq 0.01$).
- The positive control did not induce a statistically significant increase in the %Tail DNA in nasal cells as compared to the negative control group (Student's t-test, $p \geq 0.05$); additionally, the positive control group mean was not compatible with the historical control range.
- In the negative control group, the group mean %Tail DNA was within the historical vehicle/negative control range for nasal cells (Appendix I).

These results indicate that the criteria for a valid test were not met, for either sex.

Nasal Cells – Phase 2

The %Tail DNA in male nasal cells is summarized for each treatment group and presented in Table 7B; median values for the %Tail DNA**,** Tail moment and Tail migration (µm) for male nasal cells are calculated per 150 cells for each animal and are presented in Table 15B.

For males, the scoring results and statistical analysis of data indicated the following:

- The means of 'clouds' in the test substance groups were ≤ 28.2%, which was somewhat comparable with the mean of clouds in the negative control group (11.0%).
- Group variances for mean of medians of the %Tail DNA in the negative control and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p \geq 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.
- No statistically significant response in the %Tail DNA (DNA damage) was observed in the test substance groups relative to the concurrent negative control group (ANOVA followed by Dunnett's post-hoc analysis, $p \geq 0.05$).
- No concentration-dependent increase in the %Tail DNA was observed across the test substance groups (regression analysis, $p \geq 0.01$).
- The positive control induced a statistically significant increase in the %Tail DNA in liver cells as compared to the negative control group (Student's t-test, $p < 0.05$).
- In the negative control group, the group mean %Tail DNA was within the historical vehicle/negative control range for nasal cells (Appendix I).

These results indicate that all criteria for a valid test, as specified in the protocol, were met.

8. CONCLUSION

During Phase 1 of the assay (both sexes), the test substance, [redacted], could be evaluated as negative (non-DNA damaging) in male liver cells only. For the remaining tissues tested, the assay did not meet all the acceptance criteria (especially as related to the positive control treatment), therefore was considered invalid. During Phase 2 (males only), the test substance has been evaluated as negative (non-DNA damaging) during the *in vivo* alkaline Comet assay; all valid assay criteria were met, for all tissues tested.

9. REFERENCES

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Olive PL., Banath JP., Durand RE. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cell using the "comet" assay. *Radiat. Res.,* 122(1), 86-94, 1990

10. DATA TABLES

Table 1A: %Tail DNA in Male Kidney Cells - Phase 1

Samples Collected 2 to 4 Hours Post Final Exposure Completion

Treatment (6 Hours of Exposure)	Number of Animals	Group Mean (% of Clouds)	Tail DNA (%)[A] Mean ± S.D.
Negative Control:			
(Filtered Air)	5	0.0	0.07 ± 0.02
Test Substance:			
[redacted] (375 ppm)	5	0.0	0.39[@#] ± 0.09
[redacted] (750 ppm)	5	0.4	0.36[@#] ± 0.18
[redacted] (1500 ppm)	5	0.8	0.40[@#] ± 0.22
Positive Control:			
EMS, 200 mg/kg[B] CP, 20 mg/kg[C]	5	0.0	0.09 ± 0.02

A = Mean of 5 animals means of medians
B = Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.
C = Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on Days 1 and 2.
S.D. = Standard Deviation
@ $p < 0.01$ (regression analysis): Statistically significant relative to the negative control.
# $p \leq 0.05$ (ANOVA, Dunnett's post hoc); Statistically significant increase relative to the negative control.

Table 1B: %Tail DNA in Male Kidney Cells - Phase 2

Samples Collected 2 to 4 Hours Post Final Exposure Completion

Treatment (6 Hours of Exposure)	Number of Animals	Group Mean (% of Clouds)	Tail DNA (%)[A] Mean ± S.D.
Negative Control:			
(Filtered Air)	5	1.8	1.55 ± 0.68
Test Substance:			
[redacted] (375 ppm)	5	1.0	2.51 ± 0.89
[redacted] (750 ppm)	5	1.0	2.53 ± 0.97
[redacted] (1500 ppm)	5	1.2	0.81 ± 0.25
Positive Control:			
EMS, 200 mg/kg[B]	5	11.6	30.86* ± 3.76

A = Mean of 5 animals means of medians
B = Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.
S.D. = Standard Deviation
*$p \leq 0.05$ (Student's t-test); Statistically significant increase relative to the negative control.

Table 2: %Tail DNA in Female Kidney Cells - Phase 1

Samples Collected 2 to 4 Hours Post Final Exposure Completion

Treatment (6 Hours of Exposure)	Number of Animals	Group Mean (% of Clouds)	Tail DNA (%)[A] Mean ± S.D.
Negative Control:			
(Filtered Air)	5	0.2	2.36 ± 0.89
Test Substance:			
[redacted] (375 ppm)	5	0.6	0.45[#] ± 0.30
[redacted] (750 ppm)	5	0.4	2.13 ± 0.52
[redacted] (1500 ppm)	5	0.2	0.71[#] ± 0.27
Positive Control:			
EMS, 200 mg/kg[B] CP, 20 mg/kg[C]	5	0.0	0.23 ± 0.07

A = Mean of 5 animals means of medians
B = Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.
C = Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on Days 1 and 2.
S.D. = Standard Deviation
# $p \leq 0.05$ (ANOVA, Dunnett's post hoc); Statistically significant decrease relative to the negative control.

Table 3A: %Tail DNA in Male Liver Cells - Phase 1

Samples Collected 2 to 4 Hours Post Final Exposure Completion

Treatment (6 Hours of Exposure)	Number of Animals	Group Mean (% of Clouds)	Tail DNA (%)[A] Mean ± S.D.
Negative Control:			
(Filtered Air)	5	0.0	0.06 ± 0.04
Test Substance:			
[redacted] (375 ppm)	5	0.2	0.24 ± 0.21
[redacted] (750 ppm)	5	1.0	0.09 ± 0.08
[redacted] (1500 ppm)	5	0.6	0.12 ± 0.04
Positive Control:			
EMS, 200 mg/kg[B] CP, 20 mg/kg[C]	5	0.0	0.10* ± 0.03

A = Mean of 5 animals means of medians
B = Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.
C = Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on Days 1 and 2.
S.D. = Standard Deviation
*$p \leq 0.05$ (Student's t-test); Statistically significant increase relative to the negative control.

Table 3B: %Tail DNA in Male Liver Cells - Phase 2

Samples Collected 2 to 4 Hours Post Final Exposure Completion

Treatment (6 Hours of Exposure)	Number of Animals	Group Mean (% of Clouds)	Tail DNA (%)[A] Mean ± S.D.
Negative Control:			
(Filtered Air)	5	0.0	0.16 ± 0.14
Test Substance:			
[redacted] (375 ppm)	5	0.6	0.14 ± 0.11
[redacted] (750 ppm)	5	0.4	0.58# ± 0.36
[redacted] (1500 ppm)	5	0.4	0.11 ± 0.03
Positive Control:			
EMS, 200 mg/kg[B]	5	12.6	30.57* ± 4.94

A = Mean of 5 animals means of medians
B = Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.
S.D. = Standard Deviation
# $p \leq 0.05$ (ANOVA, Dunnett's post hoc); Statistically significant increase relative to the negative control.
*$p \leq 0.05$ (Student's t-test); Statistically significant increase relative to the negative control.

Table 4: %Tail DNA in Female Liver Cells - Phase 1

Samples Collected 2 to 4 Hours Post Final Exposure Completion

Treatment (6 Hours of Exposure)	Number of Animals	Group Mean (% of Clouds)	Tail DNA (%)[A] Mean ± S.D.
Negative Control:			
(Filtered Air)	5	0.0	2.30 ± 0.83
Test Substance:			
[redacted] (375 ppm)	5	0.6	0.38[#] ± 0.15
[redacted] (750 ppm)	5	0.0	2.76 ± 1.08
[redacted] (1500 ppm)	5	0.0	0.60[#] ± 0.20
Positive Control:			
EMS, 200 mg/kg[B] CP, 20 mg/kg[C]	5	0.2	0.32 ± 0.17

A = Mean of 5 animals means of medians
B = Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.
C = Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on Days 1 and 2.
S.D. = Standard Deviation
# $p \leq 0.05$ (ANOVA, Dunnett's post hoc); Statistically significant decrease relative to the negative control.

Table 5A: %Tail DNA in Male Lung Cells - Phase 1

Samples Collected 2 to 4 Hours Post Final Exposure Completion

Treatment (6 Hours of Exposure)	Number of Animals	Group Mean (% of Clouds)	Tail DNA (%)[A] Mean ± S.D.
Negative Control:			
(Filtered Air)	5	0.6	0.01 ± 0.00
Test Substance:			
[redacted] (375 ppm)	5	4.4	0.18[@#] ± 0.11
[redacted] (750 ppm)	5	5.0	0.27[@#] ± 0.06
[redacted] (1500 ppm)	5	5.4	0.40[@#] ± 0.12
Positive Control:			
EMS, 200 mg/kg[B] CP, 20 mg/kg[C]	5	0.0	0.01 ± 0.00

A = Mean of 5 animals means of medians
B = Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.
C = Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on Days 1 and 2.
S.D. = Standard Deviation
@ $p < 0.01$ (regression analysis): Statistically significant relative to the negative control.
# $p \leq 0.05$ (ANOVA, Dunnett's post hoc); Statistically significant increase relative to the negative control.

Table 5B: %Tail DNA in Male Lung Cells – Phase 2

Samples Collected 2 to 4 Hours Post Final Exposure Completion

Treatment (6 Hours of Exposure)	Number of Animals	Group Mean (% of Clouds)	Tail DNA (%)[A] Mean ± S.D.
Negative Control:			
(Filtered Air)	5	7.0	0.56 ± 0.69
Test Substance:			
[redacted] (375 ppm)	5	6.4	0.22 ± 0.12
[redacted] (750 ppm)	5	5.4	0.78 ± 0.26
[redacted] (1500 ppm)	5	4.8	0.26 ± 0.12
Positive Control:			
EMS, 200 mg/kg[B]	5	11.2	26.48* ± 3.76

A = Mean of 5 animals means of medians
B = Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.
S.D. = Standard Deviation
*$p \leq 0.05$ (Student's t-test); Statistically significant increase relative to the negative control.

Table 6: %Tail DNA in Female Lung Cells - Phase 1

Samples Collected 2 to 4 Hours Post Final Exposure Completion

Treatment (6 Hours of Exposure)	Number of Animals	Group Mean (% of Clouds)	Tail DNA (%)[A] Mean ± S.D.
Negative Control:			
(Filtered Air)	5	0.2	1.54 ± 0.64
Test Substance:			
[redacted] (375 ppm)	5	0.8	2.36 ± 0.71
[redacted] (750 ppm)	5	0.2	3.10[#] ± 1.33
[redacted] (1500 ppm)	5	0.2	2.47 ± 0.71
Positive Control:			
EMS, 200 mg/kg[B] CP, 20 mg/kg[C]	5	0.0	0.50 ± 0.20

A = Mean of 5 animals means of medians
B = Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.
C = Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on Days 1 and 2.
S.D. = Standard Deviation
# Statistically significant increase (Dunnett's post hoc, $p \leq 0.05$) relative to the negative control.

Table 7A: %Tail DNA in Male Nasal Cells - Phase 1

Samples Collected 2 to 4 Hours Post Final Exposure Completion

Treatment (6 Hours of Exposure)	Number of Animals	Group Mean (% of Clouds)	Tail DNA (%)[A] Mean ± S.D.
Negative Control:			
(Filtered Air)	5	3.6	0.02 ± 0.01
Test Substance:			
[redacted] (375 ppm)	5	19.2	0.18 ± 0.20
[redacted] (750 ppm)	5	16.8	0.38[#] ± 0.22
[redacted] (1500 ppm)	5	20.2	0.24 ± 0.09
Positive Control:			
EMS, 200 mg/kg[B] CP, 20 mg/kg[C]	5	0.0	0.01 ± 0.01

A = Mean of 5 animals means of medians
B = Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.
C = Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on Days 1 and 2.
S.D. = Standard Deviation
# $p \leq 0.05$ (ANOVA, Dunnett's post hoc); Statistically significant increase relative to the negative control.

Table 7B: %Tail DNA in Male Nasal Cells – Phase 2

Samples Collected 2 to 4 Hours Post Final Exposure Completion

Treatment (6 Hours of Exposure)	Number of Animals	Group Mean (% of Clouds)	Tail DNA (%)[A] Mean ± S.D.
Negative Control:			
(Filtered Air)	5	11.0	0.40 ± 0.18
Test Substance:			
[redacted] (375 ppm)	5	17.8	0.70 ± 0.50
[redacted] (750 ppm)	5	16.8	0.65 ± 0.84
[redacted] (1500 ppm)	5	28.2	0.34 ± 0.25
Positive Control:			
EMS, 200 mg/kg[B]	5	37.6	24.32* ± 3.72

A = Mean of 5 animals means of medians
B = Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.
S.D. = Standard Deviation
*$p \leq 0.05$ (Student's t-test); Statistically significant increase relative to the negative control.

Table 8: %Tail DNA in Female Nasal Cells - Phase 1

Samples Collected 2 to 4 Hours Post Final Exposure Completion

Treatment (6 Hours of Exposure)	Number of Animals	Group Mean (% of Clouds)	Tail DNA (%)[A] Mean ± S.D.
Negative Control:			
(Filtered Air)	5	28.0	0.95 ± 0.55
Test Substance:			
[redacted] (375 ppm)	5	30.0	0.36[#] ± 0.11
[redacted] (750 ppm)	5	32.4	0.61 ± 0.31
[redacted] (1500 ppm)	5	28.2	0.18[#] ± 0.04
Positive Control:			
EMS, 200 mg/kg[B] CP, 20 mg/kg[C]	5	4.4	0.82 ± 1.37

A = Mean of 5 animals means of medians
B = Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.
C = Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on Days 1 and 2.
S.D. = Standard Deviation
# $p \leq 0.05$ (ANOVA, Dunnett's post hoc); Statistically significant decrease relative to the negative control.

Table 9A: DNA Damage Data in Male Kidney Cells - Phase 1

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Negative Control: Filtered Air	1001	0	0.01	0.00	0.04	0.01	0.00	0.09
			0.01	0.00	0.12			
			0.01	0.00	0.10			
	1002	0	0.00	0.00	0.05	0.00	0.00	0.08
			0.01	0.00	0.06			
			0.01	0.00	0.12			
	1003	0	0.01	0.00	0.10	0.01	0.00	0.09
			0.01	0.00	0.04			
			0.01	0.00	0.12			
	1004	0	0.00	0.00	0.05	0.00	0.00	0.04
			0.00	0.00	0.05			
			0.00	0.00	0.03			
	1005	0	0.00	0.00	0.03	0.00	0.00	0.04
			0.00	0.00	0.04			
			0.00	0.00	0.04			

[A] Mean of median of 150 cells scored per animal

Table 9A: DNA Damage Data in Male Kidney Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	2001	0	0.01	0.41	0.09	0.04	7.95	0.37
			0.06	11.92	0.45			
			0.06	11.51	0.57			
	2002	0	0.05	6.99	0.30	0.06	9.18	0.47
			0.07	7.40	0.56			
			0.08	13.16	0.55			
[redacted] (375 ppm)	2003	0	0.03	2.47	0.22	0.06	9.46	0.34
			0.07	11.92	0.35			
			0.08	13.98	0.44			
	2004	0	0.02	2.06	0.22	0.06	5.48	0.48
			0.08	7.81	0.63			
			0.09	6.58	0.59			
	2005	0	0.02	1.23	0.17	0.04	7.54	0.27
			0.04	9.46	0.27			
			0.05	11.92	0.36			

[A] Mean of median of 150 cells scored per animal

Table 9A: DNA Damage Data in Male Kidney Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	3001	1	0.04	14.39	0.26	0.08	16.17	0.58
			0.08	16.45	0.53			
			0.13	17.68	0.95			
	3002	0	0.08	14.39	0.55	0.09	16.45	0.55
			0.09	18.50	0.42			
			0.10	16.45	0.67			
[redacted] (750 ppm)	3003	1	0.03	11.10	0.22	0.04	11.92	0.25
			0.04	12.75	0.27			
	3004	0	0.04	13.16	0.19	0.04	13.16	0.20
			0.03	12.75	0.19			
			0.04	13.57	0.22			
	3005	0	0.05	15.63	0.26	0.04	11.24	0.25
			0.02	1.23	0.14			
			0.06	16.86	0.35			

[A] Mean of median of 150 cells scored per animal

Table 9A: DNA Damage Data in Male Kidney Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	4001	0	0.03	6.17	0.27	0.03	7.13	0.23
			0.03	4.52	0.22			
			0.03	10.69	0.21			
	4002	1	0.08	13.16	0.70	0.09	16.58	0.67
			0.09	17.27	0.54			
			0.11	19.33	0.77			
	4003	1	0.08	17.68	0.55	0.11	19.60	0.61
[redacted] (1500 ppm)			0.09	17.27	0.54			
			0.14	23.85	0.74			
	4004	1	0.04	13.57	0.29	0.04	15.49	0.25
			0.04	15.21	0.23			
			0.04	17.68	0.22			
	4005	1	0.04	13.16	0.24	0.04	14.67	0.24
			0.03	14.80	0.21			
			0.05	16.04	0.28			

[A] Mean of median of 150 cells scored per animal

Table 9A: DNA Damage Data in Male Kidney Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post-Last Dose

Treatment (10 mL/kg/treatment)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Positive Control:	5001	0	0.01	0.00	0.06	0.01	0.00	0.10
			0.01	0.00	0.10			
			0.01	0.00	0.13			
	5002	0	0.01	0.00	0.08	0.01	0.00	0.10
			0.02	0.00	0.18			
			0.00	0.00	0.04			
EMS 200 mg/kg[B]	5003	0	0.00	0.00	0.04	0.00	0.00	0.06
			0.01	0.00	0.08			
CP 20mg/kg[C]			0.01	0.00	0.05			
	5004	0	0.01	0.00	0.08	0.01	0.00	0.09
			0.00	0.00	0.05			
			0.01	0.00	0.13			
	5005	0	0.01	0.00	0.11	0.01	0.00	0.09
			0.01	0.00	0.10			
			0.00	0.00	0.05			

[A] Mean of median of 150 cells scored per animal

[B] Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.

[C] Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on days 1 and 2.

Table 9B: DNA Damage Data in Male Kidney Cells - Phase 2

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Negative Control: Filtered Air	1007	1	0.19	27.96	1.37	0.18	28.51	1.06
			0.20	25.90	0.98			
			0.15	31.66	0.83			
	1008	2	0.12	24.67	0.76	0.20	34.13	1.03
			0.22	34.54	0.99			
			0.25	43.17	1.34			
	1009	1	0.11	25.90	0.65	0.21	33.03	1.05
			0.28	35.36	1.32			
			0.23	37.83	1.18			
	1010	5	0.32	40.71	1.91	0.46	50.71	2.35
			0.54	54.69	2.68			
			0.52	56.74	2.47			
	1011	0	0.59	49.75	2.75	0.45	46.46	2.23
			0.39	46.05	2.09			
			0.35	43.59	1.86			

[A] Mean of median of 150 cells scored per animal

Table 9B: DNA Damage Data in Male Kidney Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test Article:	2007	1	0.25	25.90	1.72	0.22	23.99	1.33
			0.11	14.39	0.86			
			0.30	31.66	1.40			
	2008	0	0.26	31.25	1.38	0.31	32.89	1.88
			0.36	32.07	2.41			
			0.32	35.36	1.86			
[redacted] (375 ppm)	2009	3	0.45	42.76	2.85	0.51	42.35	2.72
			0.62	39.47	3.00			
			0.47	44.82	2.32			
	2010	0	0.65	49.75	3.87	0.53	46.05	3.16
			0.52	46.05	3.17			
			0.43	42.35	2.44			
	2011	1	0.45	41.94	2.38	0.63	46.19	3.46
			0.63	47.70	3.24			
			0.80	48.93	4.75			

[A] Mean of median of 150 cells scored per animal

Table 9B: DNA Damage Data in Male Kidney Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test Article:	3007	1	0.19	24.26	1.65	0.28	34.68	1.70
			0.25	34.54	1.36			
			0.41	45.23	2.08			
	3008	1	0.35	32.48	1.84	0.31	34.40	1.73
			0.36	39.47	2.08			
			0.23	31.25	1.27			
[redacted] (750 ppm)	3009	1	0.57	47.29	3.84	0.45	45.50	2.96
			0.44	51.81	3.31			
			0.34	37.42	1.75			
	3010	1	0.45	50.99	2.41	0.45	50.30	2.27
			0.47	53.87	2.42			
			0.43	46.05	1.96			
	3011	1	0.78	51.81	4.57	0.82	58.39	4.00
			1.05	71.96	4.14			
			0.63	51.40	3.28			

[A] Mean of median of 150 cells scored per animal

Table 9B: DNA Damage Data in Male Kidney Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test Article:	4007	0	0.13	17.27	0.96	0.12	20.83	0.70
			0.12	23.44	0.57			
			0.10	21.79	0.57			
	4008	1	0.24	30.84	1.39	0.21	28.23	1.20
			0.21	25.90	1.11			
			0.20	27.96	1.10			
[redacted] (1500 ppm)	4009	1	0.12	25.90	0.66	0.17	30.98	0.87
			0.25	32.48	1.19			
			0.14	34.54	0.76			
	4010	1	0.13	31.25	0.53	0.13	33.03	0.54
			0.11	32.48	0.44			
			0.16	35.36	0.64			
	4011	3	0.19	34.95	0.77	0.18	32.62	0.76
			0.27	39.47	1.07			
			0.07	23.44	0.43			

[A] Mean of median of 150 cells scored per animal

Table 9B: DNA Damage Data in Male Kidney Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post-Last Dose

Treatment (10 mL/kg/treatment)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Positive Control:	5008	9	4.12	28.37	26.07	5.29	37.28	29.87
			5.49	40.30	30.75			
			6.27	43.17	32.80			
	5007	13	3.96	29.61	25.27	4.58	34.68	27.25
			4.34	32.89	26.70			
			5.46	41.53	29.80			
EMS 200 mg/kg[B]	5009	8	4.30	32.48	24.98	4.98	41.67	27.92
			4.76	44.00	26.80			
			5.87	48.52	31.98			
	5010	13	6.33	44.41	32.41	6.38	44.13	32.94
			7.02	48.93	35.32			
			5.81	39.06	31.07			
	5012	15	5.47	37.01	30.45	7.70	50.16	36.30
			8.79	52.22	39.80			
			8.84	61.27	38.66			

[A] Mean of median of 150 cells scored per animal

[B] Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on study days 1 and 2.

Table 10: DNA Damage Data in Female Kidney Cells - Phase 1

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Negative Control: Filtered Air	1501	1	0.47	16.04	4.34	0.44	17.27	3.83
			0.37	17.68	3.14			
			0.48	18.09	4.01			
	1502	0	0.15	7.81	1.38	0.23	10.83	1.73
			0.35	13.16	2.48			
			0.18	11.51	1.34			
	1503	0	0.13	5.76	0.96	0.19	8.63	1.64
			0.26	12.75	2.29			
			0.18	7.40	1.66			
	1504	0	0.24	5.35	2.47	0.29	10.28	2.48
			0.33	14.39	2.75			
			0.28	11.10	2.21			
	1505	0	0.18	7.40	1.54	0.28	12.75	2.14
			0.33	16.45	2.29			
			0.31	14.39	2.58			

[A] Mean of median of 150 cells scored per animal

Table 10: DNA Damage Data in Female Kidney Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	2501	0	0.08	2.88	0.63	0.04	2.60	0.31
			0.02	2.88	0.18			
			0.01	2.06	0.10			
	2502	1	0.04	4.93	0.31	0.05	6.85	0.35
			0.08	12.75	0.52			
			0.03	2.88	0.22			
	2503	1	0.02	4.11	0.13	0.05	7.68	0.35
[redacted] (375 ppm)			0.08	12.75	0.55			
			0.06	6.17	0.37			
	2504	0	0.03	6.58	0.19	0.05	10.28	0.27
			0.06	14.39	0.41			
			0.04	9.87	0.22			
	2505	1	0.15	10.69	0.95	0.15	14.80	0.98
			0.12	16.45	0.82			
			0.18	17.27	1.19			

[A] Mean of median of 150 cells scored per animal

Table 10: DNA Damage Data in Female Kidney Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	3501	0	0.34	16.04	2.35	0.38	21.24	2.77
			0.42	23.44	2.87			
			0.40	24.26	3.10			
	3502	0	0.14	19.33	0.82	0.28	23.99	1.59
			0.41	27.55	2.33			
			0.28	25.08	1.62			
[redacted] (750 ppm)	3503	1	0.44	32.07	2.66	0.39	27.28	2.50
			0.35	26.73	2.44			
			0.37	23.03	2.41			
	3504	1	0.18	18.09	1.10	0.25	25.77	1.65
			0.34	30.84	2.36			
			0.23	28.37	1.48			
	3505	0	0.22	25.08	1.75	0.34	27.14	2.11
			0.37	27.14	1.88			
			0.45	29.19	2.71			

[A] Mean of median of 150 cells scored per animal

Table 10: DNA Damage Data in Female Kidney Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	4501	1	0.06	9.05	0.45	0.08	13.84	0.49
			0.07	15.21	0.46			
			0.10	17.27	0.57			
	4502	0	0.10	15.21	0.70	0.11	17.82	0.71
			0.10	18.91	0.73			
			0.15	19.33	0.69			
[redacted] (1500 ppm)	4504	0	0.07	13.98	0.40	0.07	16.17	0.43
			0.05	18.09	0.28			
			0.10	16.45	0.60			
	4506	0	0.09	16.04	0.63	0.13	18.09	0.79
			0.10	17.68	0.64			
			0.19	20.56	1.11			
	4505	0	0.11	22.20	0.64	0.18	25.90	1.11
			0.25	30.84	1.48			
			0.19	24.67	1.20			

[A] Mean of median of 150 cells scored per animal

Table 10: DNA Damage Data in Female Kidney Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post-Last Dose

Treatment (10 mL/kg/treatment)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Positive Control:	5501	0	0.01	0.00	0.14	0.01	0.00	0.19
			0.01	0.00	0.17			
			0.02	0.00	0.26			
	5502	0	0.01	0.00	0.13	0.03	0.00	0.29
			0.01	0.00	0.11			
			0.06	0.00	0.63			
EMS 200 mg/kg[B] CP 20mg/kg[C]	5503	0	0.03	0.00	0.37	0.03	0.00	0.32
			0.03	0.00	0.28			
			0.06	0.00	0.62			
			0.00	0.00	0.02			
	5504	0	0.01	0.00	0.17	0.01	0.00	0.15
			0.01	0.00	0.21			
			0.00	0.00	0.08			
	5505	0	0.02	0.00	0.50	0.01	0.00	0.21
			0.01	0.00	0.06			
			0.00	0.00	0.06			

[A] Mean of median of 150 cells scored per animal

[B] Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.

[C] Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on days 1 and 2.

Table 11A: DNA Damage Data in Male Liver Cells - Phase 1

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Negative Control:	1001	0	0.00	0.00	0.08	0.01	0.00	0.12
			0.02	0.00	0.26			
			0.00	0.00	0.02			
			0.00	0.00	0.02			
	1002	0	0.00	0.00	0.03	0.00	0.00	0.03
			0.00	0.00	0.01			
			0.00	0.00	0.04			
Filtered Air	1003	0	0.01	0.00	0.05	0.00	0.00	0.03
			0.00	0.00	0.02			
			0.00	0.00	0.02			
	1004	0	0.00	0.00	0.01	0.00	0.00	0.05
			0.01	0.00	0.05			
			0.00	0.00	0.02			
			0.00	0.00	0.12			
	1005	0	0.01	0.00	0.06	0.00	0.00	0.05
			0.01	0.00	0.07			
			0.00	0.00	0.03			

[A] Mean of median of 150 cells scored per animal

Table 11A: DNA Damage Data in Male Liver Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	2001	0	0.04	0.41	0.34	0.02	0.69	0.17
			0.01	1.64	0.11			
			0.00	0.00	0.05			
	2002	0	0.00	0.00	0.00	0.01	0.00	0.06
			0.00	0.00	0.01			
			0.01	0.00	0.17			
[redacted] (375 ppm)	2003	0	0.00	0.00	0.05	0.01	1.92	0.10
			0.02	2.06	0.14			
			0.02	3.70	0.12			
	2004	0	0.00	0.00	0.03	0.07	2.47	0.58
			0.07	4.52	0.66			
			0.13	2.88	1.05			
	2005	1	0.06	2.88	0.42	0.03	2.06	0.27
			0.00	0.00	0.10			
			0.04	3.29	0.30			

[A] Mean of median of 150 cells scored per animal

Table 11A: DNA Damage Data in Male Liver Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	3001	3	0.00	0.00	0.03	0.00	0.51	0.03
			0.01	2.06	0.07			
			0.00	0.00	0.01			
			0.00	0.00	0.01			
	3002	1	0.00	0.41	0.04	0.01	1.23	0.06
			0.00	0.82	0.04			
			0.01	2.47	0.09			
[redacted] (750 ppm)	3003	0	0.00	0.00	0.02	0.00	1.92	0.03
			0.00	0.00	0.02			
			0.01	5.76	0.07			
	3004	0	0.02	6.17	0.13	0.01	6.03	0.10
			0.01	6.58	0.08			
			0.01	5.35	0.08			
	3005	1	0.04	12.75	0.24	0.03	8.91	0.21
			0.04	8.63	0.29			
			0.02	5.35	0.11			

[A] Mean of median of 150 cells scored per animal

Table 11A: DNA Damage Data in Male Liver Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	4001	0	0.01	2.88	0.07	0.02	7.26	0.11
			0.03	8.63	0.16			
			0.02	10.28	0.11			
	4002	1	0.03	9.05	0.17	0.02	7.40	0.15
			0.01	5.35	0.11			
			0.02	7.81	0.17			
[redacted] (1500 ppm)	4003	0	0.03	9.87	0.20	0.03	11.38	0.18
			0.03	11.51	0.17			
			0.03	12.75	0.17			
	4004	1	0.02	6.58	0.10	0.02	9.59	0.12
			0.02	11.10	0.13			
			0.02	11.10	0.13			
	4005	1	0.01	9.87	0.07	0.01	9.87	0.06
			0.01	10.28	0.07			
			0.01	9.46	0.05			

[A] Mean of median of 150 cells scored per animal

Table 11A: DNA Damage Data in Male Liver Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post-Last Dose

Treatment (10 mL/kg/treatment)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Positive Control:	5001	0	0.00	0.00	0.05	0.01	0.00	0.05
			0.01	0.00	0.06			
			0.00	0.00	0.05			
	5002	0	0.01	0.00	0.04	0.01	0.14	0.08
			0.02	0.41	0.14			
			0.01	0.00	0.07			
EMS 200 mg/kg[B]	5003	0	0.01	0.00	0.13	0.01	0.00	0.12
			0.02	0.00	0.12			
CP 20mg/kg[C]			0.01	0.00	0.09			
	5004	0	0.02	0.00	0.13	0.01	0.00	0.12
			0.01	0.00	0.11			
			0.01	0.00	0.11			
	5005	0	0.01	0.41	0.10	0.01	0.34	0.12
			0.02	0.21	0.18			
			0.01	0.41	0.08			

[A] Mean of median of 150 cells scored per animal

[B] Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.

[C] Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on days 1 and 2.

Table 11B: DNA Damage Data in Male Liver Cells - Phase 2

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Negative Control: Filtered Air	1007	0	0.01	10.28	0.03	0.01	10.83	0.04
			0.01	11.10	0.06			
			0.01	11.10	0.04			
	1008	0	0.01	11.10	0.05	0.02	12.20	0.07
			0.02	12.34	0.09			
			0.02	13.16	0.08			
	1009	0	0.02	16.45	0.10	0.03	17.54	0.17
			0.05	17.27	0.22			
			0.03	18.91	0.19			
	1010	0	0.02	12.75	0.11	0.02	13.16	0.10
			0.02	12.34	0.09			
			0.02	14.39	0.10			
	1011	0	0.09	21.38	0.40	0.09	22.07	0.39
			0.08	20.15	0.45			
			0.09	24.67	0.34			

[A] Mean of median of 150 cells scored per animal

Table 11B: DNA Damage Data in Male Liver Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test Article:	2007	1	0.01	11.10	0.07	0.01	10.83	0.07
			0.02	11.10	0.10			
			0.01	10.28	0.05			
	2008	1	0.00	10.28	0.03	0.01	10.69	0.03
			0.01	10.69	0.04			
			0.00	11.10	0.03			
[redacted] (375 ppm)	2009	0	0.03	12.75	0.15	0.03	14.53	0.13
			0.02	12.75	0.10			
			0.04	18.09	0.15			
	2010	0	0.02	16.04	0.13	0.03	16.58	0.13
			0.03	18.50	0.16			
			0.02	15.21	0.09			
	2011	1	0.05	18.91	0.22	0.07	21.52	0.33
			0.09	25.49	0.43			
			0.07	20.15	0.33			

[A] Mean of median of 150 cells scored per animal

Table 11B: DNA Damage Data in Male Liver Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test Article:	3007	0	0.05	14.80	0.27	0.04	16.45	0.20
			0.03	17.68	0.16			
			0.03	16.86	0.17			
	3008	0	0.09	26.32	0.49	0.08	23.99	0.42
			0.08	24.26	0.43			
			0.07	21.38	0.35			
[redacted] (750 ppm)	3009	1	0.24	33.72	1.13	0.21	32.62	1.01
			0.22	33.31	1.10			
			0.17	30.84	0.80			
	3010	0	0.07	22.62	0.40	0.08	23.16	0.36
			0.06	22.62	0.25			
			0.11	24.26	0.41			
	3011	1	0.20	31.66	0.95	0.19	33.85	0.91
			0.22	37.83	0.97			
			0.15	32.07	0.80			

[A] Mean of median of 150 cells scored per animal

Table 11B: DNA Damage Data in Male Liver Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test Article:	4007	0	0.01	9.05	0.07	0.01	10.42	0.06
			0.01	11.10	0.05			
			0.01	11.10	0.06			
	4008	1	0.02	14.80	0.13	0.02	14.39	0.13
			0.02	13.57	0.14			
			0.02	14.80	0.12			
	4009	0	0.02	12.75	0.12	0.02	13.57	0.12
[redacted] (1500 ppm)			0.02	14.39	0.13			
			0.02	13.57	0.12			
	4010	1	0.03	16.86	0.16	0.02	13.84	0.11
			0.02	12.75	0.09			
			0.01	11.92	0.07			
	4011	0	0.03	16.86	0.16	0.03	16.17	0.15
			0.03	19.33	0.14			
			0.03	12.34	0.14			

[A] Mean of median of 150 cells scored per animal

Table 11B: DNA Damage Data in Male Liver Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post-Last Dose

Treatment (10 mL/kg/treatment)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Positive Control:	5008	19	4.13	25.49	25.44	4.57	31.52	26.64
			5.35	38.24	29.24			
			4.23	30.84	25.25			
	5007	3	4.48	32.48	25.02	4.45	35.36	24.89
			4.57	37.01	26.00			
			4.30	36.60	23.67			
EMS 200 mg/kg[B]	5009	11	5.10	32.07	29.52	5.72	40.57	30.18
			6.45	46.46	30.74			
			5.61	43.17	30.26			
	5010	15	8.46	46.88	34.84	8.46	50.71	35.70
			8.32	49.75	35.61			
			8.59	55.51	36.64			
	5012	15	7.00	44.82	33.17	7.96	50.71	35.43
			8.51	53.04	36.48			
			8.38	54.28	36.63			

[A] Mean of median of 150 cells scored per animal

[B] Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on study days 1 and 2.

Table 12: DNA Damage Data in Female Liver Cells - Phase 1

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Negative Control: Filtered Air	1501	0	0.10	1.23	1.05	0.18	3.29	1.55
			0.23	5.76	1.96			
			0.19	2.88	1.65			
	1502	0	0.07	1.23	0.86	0.26	6.99	2.23
			0.33	9.87	2.78			
			0.37	9.87	3.06			
	1503	0	0.08	1.23	0.86	0.22	6.72	1.92
			0.25	9.46	2.19			
			0.33	9.46	2.69			
	1504	0	0.48	8.22	4.25	0.46	10.55	3.72
			0.34	9.87	3.07			
			0.57	13.57	3.83			
	1505	0	0.03	0.41	0.21	0.25	5.35	2.07
			0.30	8.22	2.33			
			0.43	7.40	3.67			

[A] Mean of median of 150 cells scored per animal

Table 12: DNA Damage Data in Female Liver Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	2501	0	0.02	0.41	0.19	0.04	2.06	0.32
			0.04	2.88	0.39			
			0.06	2.88	0.39			
	2502	0	0.04	3.70	0.32	0.04	2.88	0.31
			0.04	1.64	0.38			
			0.04	3.29	0.23			
[redacted] (375 ppm)	2503	1	0.04	4.52	0.44	0.03	1.85	0.30
			0.03	0.82	0.22			
			0.01	0.21	0.24			
	2504	0	0.03	4.52	0.25	0.05	5.62	0.34
			0.09	11.51	0.61			
			0.03	0.82	0.17			
	2505	2	0.12	9.46	0.74	0.11	9.87	0.65
			0.13	11.92	0.69			
			0.08	8.22	0.51			

[A] Mean of median of 150 cells scored per animal

Table 12: DNA Damage Data in Female Liver Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	3501	0	0.36	10.28	2.76	0.44	13.98	3.27
			0.46	19.74	3.08			
			0.50	11.92	3.97			
	3502	0	0.22	6.17	1.67	0.18	8.09	1.46
			0.17	10.28	1.35			
			0.14	7.81	1.35			
	3503	0	0.27	11.92	2.26	0.35	15.63	2.70
			0.44	15.63	3.29			
			0.35	19.33	2.55			
[redacted] (750 ppm)	3504	0	0.31	11.92	2.33	0.26	10.01	2.11
			0.23	4.52	2.33			
			0.23	13.57	1.68			
	3505	0	0.65	18.91	5.26	0.54	19.60	4.26
			0.40	17.27	3.19			
			0.58	22.62	4.35			

[A] Mean of median of 150 cells scored per animal

Table 12: DNA Damage Data in Female Liver Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	4501	0	0.05	6.17	0.45	0.06	8.77	0.48
			0.09	8.22	0.58			
			0.05	11.92	0.40			
	4502	0	0.13	14.39	0.93	0.13	14.67	0.83
			0.13	13.98	0.76			
			0.13	15.63	0.79			
	4503	0	0.11	16.04	0.74	0.13	14.39	0.78
			0.11	11.92	0.69			
[redacted] (1500 ppm)			0.17	15.21	0.90			
	4504	0	0.06	11.51	0.63	0.07	12.47	0.56
			0.05	13.16	0.27			
			0.10	12.75	0.78			
	4505	0	0.04	12.75	0.27	0.06	14.12	0.37
			0.06	14.80	0.31			
			0.07	14.80	0.54			

[A] Mean of median of 150 cells scored per animal

Table 12: DNA Damage Data in Female Liver Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post-Last Dose

Treatment (10 mL/kg/treatment)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Positive Control:	5501	0	0.00	0.00	0.02	0.00	0.00	0.06
			0.00	0.00	0.03			
			0.01	0.00	0.13			
	5502	0	0.01	0.00	0.12	0.03	0.00	0.34
			0.04	0.00	0.58			
			0.04	0.00	0.33			
EMS 200 mg/kg[B]	5503	0	0.02	0.00	0.42	0.02	0.00	0.25
			0.02	0.00	0.21			
CP 20mg/kg[C]			0.01	0.00	0.11			
	5504	0	0.06	0.00	0.50	0.05	0.00	0.45
			0.09	0.00	0.74			
			0.01	0.00	0.12			
	5505	1	0.00	0.00	0.12	0.05	0.00	0.49
			0.05	0.00	0.54			
			0.09	0.00	0.81			

[A] Mean of median of 150 cells scored per animal

[B] Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.

[C] Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on days 1 and 2.

Table 13A: DNA Damage Data in Male Lung Cells - Phase 1

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Negative Control:	1001	0	0.00	0.46	0.02	0.00	0.15	0.01
			0.00	0.00	0.01			
			0.00	0.00	0.00			
	1002	0	0.00	0.46	0.01	0.00	0.15	0.01
			0.00	0.00	0.01			
			0.00	0.00	0.01			
Filtered Air	1003	0	0.00	0.23	0.01	0.00	0.38	0.01
			0.00	0.46	0.03			
			0.00	0.46	0.01			
	1004	1	0.00	0.00	0.01	0.00	0.00	0.01
			0.00	0.00	0.01			
			0.00	0.00	0.00			
	1005	2	0.00	0.00	0.01	0.00	0.00	0.01
			0.00	0.00	0.01			
			0.00	0.00	0.01			

[A] Mean of median of 150 cells scored per animal

Table 13A: DNA Damage Data in Male Lung Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	2001	0	0.00	0.00	0.00	0.00	0.08	0.01
			0.00	0.00	0.01			
			0.00	0.23	0.01			
	2002	4	0.02	5.05	0.09	0.06	9.19	0.30
			0.16	17.46	0.72			
			0.01	5.05	0.10			
	2003	8	0.00	2.76	0.03	0.03	11.18	0.16
[redacted] (375 ppm)			0.03	14.70	0.18			
			0.05	16.08	0.26			
	2004	5	0.01	2.76	0.06	0.03	7.50	0.18
			0.05	11.94	0.27			
			0.05	7.81	0.22			
	2005	5	0.01	5.51	0.06	0.04	9.34	0.23
			0.06	12.86	0.37			
			0.04	9.65	0.28			

[A] Mean of median of 150 cells scored per animal

Table 13A: DNA Damage Data in Male Lung Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	3001	2	0.05	13.32	0.30	0.06	15.77	0.30
			0.05	15.16	0.25			
			0.07	18.84	0.34			
	3002	7	0.06	12.40	0.30	0.04	12.25	0.22
			0.03	11.49	0.12			
			0.04	12.86	0.23			
[redacted] (750 ppm)	3003	3	0.06	14.24	0.41	0.04	11.64	0.26
			0.04	11.03	0.19			
			0.03	9.65	0.16			
	3004	5	0.05	18.84	0.21	0.05	15.31	0.20
			0.04	14.70	0.18			
			0.05	12.40	0.22			
	3005	8	0.16	24.35	0.56	0.09	20.06	0.36
			0.06	17.92	0.26			
			0.05	17.92	0.26			

[A] Mean of median of 150 cells scored per animal

Table 13A: DNA Damage Data in Male Lung Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	4001	4	0.12	16.54	0.57	0.08	15.77	0.36
			0.05	12.86	0.23			
			0.06	17.92	0.28			
	4002	5	0.09	16.54	0.39	0.07	18.38	0.32
			0.04	14.24	0.19			
			0.10	24.35	0.38			
[redacted] (1500 ppm)	4003	6	0.03	15.16	0.16	0.12	20.06	0.53
			0.09	21.59	0.34			
			0.25	23.43	1.08			
	4004	7	0.02	12.40	0.09	0.06	19.30	0.28
			0.10	18.38	0.53			
			0.05	27.11	0.23			
	4005	5	0.07	19.30	0.31	0.13	22.66	0.52
			0.11	23.43	0.38			
			0.21	25.27	0.88			

[A] Mean of median of 150 cells scored per animal

Table 13A: DNA Damage Data in Male Lung Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post-Last Dose

Treatment (10 mL/kg/treatment)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Positive Control:	5001	0	0.00	0.00	0.01	0.00	0.15	0.01
			0.00	0.00	0.00			
			0.00	0.46	0.01			
	5002	0	0.00	0.00	0.01	0.00	0.15	0.01
			0.00	0.00	0.01			
			0.00	0.46	0.01			
EMS 200 mg/kg[B]	5003	0	0.00	0.00	0.01	0.00	0.00	0.01
			0.00	0.00	0.01			
CP 20mg/kg[C]			0.00	0.00	0.01			
	5004	0	0.00	0.00	0.01	0.00	0.00	0.01
			0.00	0.00	0.00			
			0.00	0.00	0.01			
	5005	0	0.00	0.46	0.02	0.00	0.15	0.01
			0.00	0.00	0.01			
			0.00	0.00	0.01			

[A] Mean of median of 150 cells scored per animal

[B] Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.

[C] Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on days 1 and 2.

Table 13B: DNA Damage Data in Male Lung Cells - Phase 2

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Negative Control: Filtered Air	1007	5	0.03	11.92	0.16	0.03	11.65	0.17
			0.03	11.10	0.13			
			0.03	11.92	0.23			
	1008	4	0.01	6.99	0.07	0.01	10.14	0.10
			0.02	11.10	0.15			
			0.01	12.34	0.08			
	1009	6	0.02	13.16	0.10	0.03	14.25	0.22
			0.03	14.39	0.21			
			0.05	15.21	0.35			
	1010	4	0.08	20.56	0.43	0.11	21.11	0.56
			0.15	22.20	0.73			
			0.10	20.56	0.50			
	1011	16	0.31	32.48	2.10	0.27	33.99	1.76
			0.25	28.37	1.45			
			0.25	41.12	1.74			

[A] Mean of median of 150 cells scored per animal

Table 13B: DNA Damage Data in Male Lung Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test Article:	2007	4	0.00	3.70	0.02	0.01	6.85	0.08
			0.01	5.76	0.09			
			0.02	11.10	0.13			
	2008	5	0.02	13.57	0.18	0.02	12.61	0.13
			0.01	11.92	0.07			
			0.03	12.34	0.15			
[redacted] (375 ppm)	2009	9	0.02	16.04	0.15	0.03	15.49	0.21
			0.04	13.57	0.22			
			0.03	16.86	0.27			
	2010	8	0.03	15.63	0.38	0.04	15.90	0.27
			0.04	19.33	0.25			
			0.03	12.75	0.19			
	2011	6	0.11	20.97	0.54	0.07	18.91	0.39
			0.07	19.74	0.41			
			0.04	16.04	0.23			

[A] Mean of median of 150 cells scored per animal

Table 13B: DNA Damage Data in Male Lung Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test Article:	3007	4	0.15	18.91	0.67	0.09	17.82	0.47
			0.07	20.97	0.39			
			0.04	13.57	0.33			
	3008	3	0.08	13.57	0.41	0.12	22.89	0.67
			0.19	30.84	1.05			
			0.11	24.26	0.54			
[redacted] (750 ppm)	3009	7	0.10	20.97	0.60	0.13	21.52	0.78
			0.17	22.62	1.05			
			0.12	20.97	0.69			
	3010	7	0.15	38.24	1.24	0.12	28.78	0.80
			0.10	23.44	0.59			
			0.11	24.67	0.57			
	3011	6	0.25	30.43	1.08	0.26	36.32	1.17
			0.21	32.07	1.03			
			0.33	46.46	1.41			

[A] Mean of median of 150 cells scored per animal

Table 13B: DNA Damage Data in Male Lung Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test Article:	4007	5	0.07	21.38	0.50	0.05	16.45	0.35
			0.02	13.57	0.17			
			0.05	14.39	0.38			
	4008	3	0.01	9.05	0.08	0.01	10.01	0.13
			0.01	10.28	0.10			
			0.02	10.69	0.20			
[redacted] (1500 ppm)	4009	5	0.03	12.75	0.23	0.03	12.88	0.17
			0.03	12.34	0.13			
			0.03	13.57	0.15			
	4010	6	0.06	16.04	0.29	0.05	17.41	0.24
			0.03	11.92	0.19			
			0.06	24.26	0.24			
	4011	5	0.05	11.92	0.21	0.08	15.49	0.41
			0.07	14.80	0.49			
			0.11	19.74	0.53			

[A] Mean of median of 150 cells scored per animal

Table 13B: DNA Damage Data in Male Lung Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post-Last Dose

Treatment (10 mL/kg/treatment)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Positive Control:	5008	12	4.09	31.66	25.53	5.24	37.55	28.32
			6.06	44.41	30.24			
			5.57	36.60	29.17			
	5007	9	3.82	31.25	22.66	3.78	33.44	21.92
			3.57	38.24	20.64			
			3.93	30.84	22.45			
EMS 200 mg/kg[B]	5009	11	3.57	32.07	22.51	3.82	34.13	23.29
			3.99	34.54	23.96			
			3.89	35.77	23.39			
	5010	9	4.17	37.83	24.53	5.12	42.90	27.89
			5.15	39.47	28.09			
			6.03	51.40	31.05			
	5012	15	4.89	38.24	28.01	5.85	44.27	30.97
			6.45	48.52	33.13			
			6.20	46.05	31.77			

[A] Mean of median of 150 cells scored per animal

[B] Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on study days 1 and 2.

Table 14: DNA Damage Data in Female Lung Cells - Phase 1

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Negative Control: Filtered Air	1501	1	0.05	1.23	0.54	0.22	2.47	1.93
			0.42	4.11	3.62			
			0.20	2.06	1.63			
	1502	0	0.14	0.82	1.53	0.18	1.51	1.73
			0.29	2.06	2.77			
			0.11	1.64	0.90			
	1503	0	0.06	0.41	0.53	0.13	1.23	1.27
			0.21	1.23	2.00			
			0.13	2.06	1.27			
	1504	0	0.04	1.23	0.43	0.06	1.23	0.56
			0.08	1.23	0.52			
			0.07	1.23	0.72			
	1505	0	0.33	2.88	2.57	0.27	2.33	2.19
			0.22	1.23	1.59			
			0.26	2.88	2.41			

[A] Mean of median of 150 cells scored per animal

Table 14: DNA Damage Data in Female Lung Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	2501	0	0.43	3.70	4.28	0.19	2.33	1.82
			0.04	1.23	0.48			
			0.09	2.06	0.69			
	2502	1	0.31	5.35	2.68	0.31	6.44	2.71
			0.35	6.58	3.53			
			0.28	7.40	1.91			
	2503	1	0.27	11.92	2.12	0.31	13.71	2.16
[redacted] (375 ppm)			0.41	17.27	3.00			
			0.25	11.92	1.38			
	2504	1	0.48	12.75	3.78	0.48	13.16	3.42
			0.38	11.92	2.74			
			0.60	14.80	3.74			
	2505	1	0.25	16.86	1.54	0.26	17.00	1.70
			0.36	21.38	2.51			
			0.17	12.75	1.05			

[A] Mean of median of 150 cells scored per animal

Table 14: DNA Damage Data in Female Lung Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	3501	0	0.18	4.52	1.49	0.29	8.77	2.39
			0.48	11.10	3.96			
			0.21	10.69	1.73			
	3502	0	0.34	16.04	2.29	0.32	18.37	1.88
			0.41	22.20	2.34			
			0.20	16.86	1.01			
[redacted] (750 ppm)	3503	1	0.61	15.21	4.75	0.76	23.85	5.26
			0.93	30.02	6.44			
			0.74	26.32	4.58			
	3504	0	0.38	11.92	2.70	0.39	16.31	2.53
			0.22	11.51	1.66			
			0.57	25.49	3.24			
	3505	0	0.70	35.77	2.99	0.62	27.14	3.44
			0.68	28.37	3.78			
			0.49	17.27	3.55			

[A] Mean of median of 150 cells scored per animal

Table 14: DNA Damage Data in Female Lung Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	4501	0	0.36	20.15	1.95	0.28	22.34	1.87
			0.35	27.96	2.69			
			0.14	18.91	0.98			
	4502	0	0.32	20.97	2.26	0.29	22.20	1.90
			0.24	21.79	1.33			
			0.31	23.85	2.10			
[redacted] (1500 ppm)	4503	0	0.29	20.15	1.90	0.33	23.44	2.13
			0.37	22.62	2.02			
			0.34	27.55	2.46			
	4504	1	0.47	22.62	2.99	0.45	24.67	3.07
			0.48	26.73	3.62			
			0.40	24.67	2.62			
	4505	0	0.45	27.55	2.27	0.53	31.80	3.39
			0.62	37.83	4.04			
			0.52	30.02	3.87			

[A] Mean of median of 150 cells scored per animal

Table 14: DNA Damage Data in Female Lung Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post-Last Dose

Treatment (10 mL/kg/treatment)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Positive Control:	5501	0	0.05	1.64	0.47	0.03	1.10	0.38
			0.03	0.41	0.33			
			0.02	1.23	0.35			
	5502	0	0.03	1.23	0.32	0.04	1.23	0.37
			0.04	1.23	0.38			
			0.05	1.23	0.40			
EMS 200 mg/kg[B]	5503	0	0.04	1.23	0.46	0.04	1.23	0.41
			0.03	0.41	0.42			
CP 20mg/kg[C]			0.05	2.06	0.34			
	5504	0	0.01	1.23	0.33	0.07	1.37	0.84
			0.04	0.82	0.27			
			0.17	2.06	1.91			
	5505	0	0.05	1.23	0.47	0.05	1.51	0.49
			0.08	1.64	0.69			
			0.03	1.64	0.31			

[A] Mean of median of 150 cells scored per animal

[B] Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.

[C] Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on days 1 and 2.

Table 15A: DNA Damage Data in Male Nasal Cells - Phase 1

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Negative Control:	1001	0	0.00	0.00	0.02	0.00	0.00	0.01
			0.00	0.00	0.01			
			0.00	0.00	0.00			
	1002	9	0.00	0.00	0.01	0.00	0.23	0.02
			0.00	0.69	0.02			
			0.00	0.00	0.03			
Filtered Air	1003	4	0.00	0.00	0.00	0.01	1.23	0.04
			0.02	3.68	0.11			
			0.00	0.00	0.01			
	1004	1	0.00	0.00	0.00	0.00	0.00	0.01
			0.00	0.00	0.02			
			0.00	0.00	0.02			
	1005	4	0.00	0.00	0.01	0.00	0.00	0.01
			0.00	0.00	0.01			
			0.00	0.00	0.02			

[A] Mean of median of 150 cells scored per animal

Table 15A: DNA Damage Data in Male Nasal Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	2001	31	0.01	3.22	0.06	0.01	5.21	0.06
			0.01	5.51	0.03			
			0.02	6.89	0.08			
	2002	15	0.00	1.38	0.03	0.01	5.05	0.08
			0.01	1.38	0.08			
			0.03	12.40	0.13			
[redacted] (375 ppm)	2003	7	0.02	5.05	0.10	0.01	3.37	0.07
			0.00	0.00	0.01			
			0.02	5.05	0.10			
	2004	11	0.00	1.84	0.02	0.03	10.11	0.13
			0.02	12.86	0.10			
			0.07	15.62	0.28			
	2005	32	0.18	12.40	1.19	0.09	12.40	0.54
			0.04	12.40	0.20			
			0.04	12.40	0.21			

[A] Mean of median of 150 cells scored per animal

Table 15A: DNA Damage Data in Male Nasal Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	3001	22	0.03	11.94	0.17	0.07	14.40	0.37
			0.04	12.40	0.23			
			0.14	18.84	0.72			
	3002	17	0.04	17.00	0.21	0.04	15.31	0.24
			0.02	12.40	0.10			
			0.06	16.54	0.40			
[redacted] (750 ppm)	3003	14	0.06	15.16	0.26	0.05	16.08	0.31
			0.05	17.46	0.43			
			0.05	15.62	0.23			
	3004	12	0.06	17.00	0.21	0.06	17.00	0.24
			0.09	21.13	0.37			
			0.03	12.86	0.14			
	3005	19	0.32	30.32	1.40	0.17	24.35	0.77
			0.13	25.73	0.61			
			0.05	17.00	0.29			

[A] Mean of median of 150 cells scored per animal

Table 15A: DNA Damage Data in Male Nasal Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	4001	17	0.04	16.54	0.17	0.07	20.52	0.26
			0.09	27.11	0.35			
			0.08	17.92	0.27			
	4002	23	0.07	23.43	0.29	0.05	19.30	0.21
			0.05	15.16	0.19			
			0.03	19.30	0.15			
[redacted] (1500 ppm)	4003	24	0.08	22.97	0.33	0.05	18.84	0.20
			0.04	17.92	0.15			
			0.03	15.62	0.12			
	4004	22	0.07	22.51	0.25	0.10	23.89	0.39
			0.18	31.70	0.74			
			0.05	17.46	0.17			
	4005	15	0.03	14.24	0.09	0.04	15.93	0.16
			0.04	13.78	0.16			
			0.05	19.75	0.22			

[A] Mean of median of 150 cells scored per animal

Table 15A: DNA Damage Data in Male Nasal Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post-Last Dose

Treatment (10 mL/kg/treatment)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Positive Control:	5001	0	0.00	0.00	0.00	0.00	0.08	0.01
			0.00	0.00	0.01			
			0.00	0.23	0.01			
	5002	0	0.00	0.00	0.03	0.00	0.15	0.01
			0.00	0.46	0.01			
			0.00	0.00	0.00			
EMS 200 mg/kg[B]	5003	0	0.00	0.00	0.00	0.00	0.00	0.00
			0.00	0.00	0.00			
CP 20mg/kg[C]			0.00	0.00	0.00			
	5004	0	0.00	0.92	0.03	0.00	2.76	0.03
			0.01	7.35	0.06			
			0.00	0.00	0.02			
	5005	0	0.00	0.00	0.01	0.00	0.15	0.01
			0.00	0.46	0.02			
			0.00	0.00	0.01			

[A] Mean of median of 150 cells scored per animal

[B] Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.

[C] Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on days 1 and 2.

Table 15B: DNA Damage Data in Male Nasal Cells - Phase 2

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Negative Control:	1007	10	0.01	12.75	0.06	0.04	13.16	0.21
			0.03	13.16	0.24			
			0.06	13.57	0.32			
	1008	6	0.19	28.37	0.89	0.10	22.62	0.59
			0.08	27.14	0.54			
			0.05	12.34	0.33			
Filtered Air	1009	12	0.03	16.04	0.14	0.07	22.20	0.35
			0.13	33.31	0.71			
			0.04	17.27	0.19			
	1010	11	0.02	13.98	0.10	0.05	15.63	0.27
			0.06	19.33	0.35			
			0.06	13.57	0.35			
	1011	16	0.04	20.15	0.18	0.09	20.56	0.60
			0.17	23.85	1.31			
			0.08	17.68	0.33			

[A] Mean of median of 150 cells scored per animal

Table 15B: DNA Damage Data in Male Nasal Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test Article:	2007	12	0.14	23.85	0.68	0.24	27.14	1.47
			0.49	33.72	3.06			
			0.10	23.85	0.67			
	2008	25	0.12	19.74	0.96	0.08	20.01	0.56
			0.06	19.33	0.30			
			0.06	20.97	0.41			
[redacted] (375 ppm)	2009	15	0.08	20.97	0.67	0.06	18.91	0.38
			0.06	21.38	0.35			
			0.02	14.39	0.12			
	2010	28	0.05	18.50	0.26	0.04	15.21	0.20
			0.05	12.75	0.21			
			0.03	14.39	0.14			
	2011	9	0.25	31.66	1.80	0.14	22.62	0.89
			0.10	20.56	0.57			
			0.06	15.63	0.30			

[A] Mean of median of 150 cells scored per animal

Table 15B: DNA Damage Data in Male Nasal Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test Article:	3007	15	0.01	10.69	0.06	0.01	9.05	0.04
			0.00	9.87	0.04			
			0.00	6.58	0.02			
	3008	23	0.02	11.10	0.12	0.01	10.83	0.09
			0.01	10.28	0.08			
			0.01	11.10	0.08			
[redacted] (750 ppm)	3009	17	0.15	22.20	1.13	0.37	33.44	1.96
			0.32	31.66	2.14			
			0.63	46.46	2.62			
	3010	21	0.07	29.61	0.39	0.17	25.49	1.03
			0.09	16.04	0.92			
			0.33	30.84	1.77			
	3011	8	0.01	9.46	0.14	0.01	10.01	0.11
			0.01	10.28	0.02			
			0.02	10.28	0.17			

[A] Mean of median of 150 cells scored per animal

Table 15B: DNA Damage Data in Male Nasal Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test Article:	4007	23	0.02	11.10	0.12	0.02	11.92	0.12
			0.02	9.05	0.10			
			0.02	15.63	0.13			
	4008	23	0.10	13.57	0.75	0.12	14.25	0.71
			0.05	11.51	0.29			
			0.20	17.68	1.08			
[redacted] (1500 ppm)	4009	35	0.09	15.63	0.55	0.04	16.45	0.27
			0.02	16.04	0.12			
			0.03	17.68	0.13			
	4010	35	0.08	27.14	0.65	0.05	18.37	0.47
			0.03	15.21	0.16			
			0.05	12.75	0.59			
	4011	25	0.06	16.86	0.23	0.03	13.29	0.12
			0.01	12.75	0.09			
			0.01	10.28	0.04			

[A] Mean of median of 150 cells scored per animal

Table 15B: DNA Damage Data in Male Nasal Cells - Phase 2 (Continued)

Samples Collected 2 to 4 hours Post-Last Dose

Treatment (10 mL/kg/treatment)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Positive Control:	5008	67	3.85	35.36	21.77	3.89	36.87	22.83
			4.43	42.35	24.77			
			3.39	32.89	21.95			
	5007	61	2.92	30.84	19.11	3.25	32.35	20.87
			3.29	31.25	21.62			
			3.53	34.95	21.87			
EMS 200 mg/kg[B]	5009	25	3.84	37.83	22.59	3.80	35.36	22.42
			4.07	32.89	24.08			
			3.49	35.36	20.59			
	5010	12	3.84	32.48	22.45	4.44	41.80	25.06
			4.59	42.35	26.36			
			4.89	50.58	26.38			
	5012	23	5.08	52.22	27.28	6.04	55.78	30.41
			6.40	58.80	32.34			
			6.65	56.33	31.62			

[A] Mean of median of 150 cells scored per animal

[B] Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on study days 1 and 2.

Table 16: DNA Damage Data in Female Nasal Cells - Phase 1

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Negative Control: Filtered Air	1501	7	0.04	3.22	0.57	0.06	5.51	0.67
			0.08	6.43	0.82			
			0.06	6.89	0.62			
	1502	16	0.01	0.00	0.17	0.22	8.27	1.88
			0.33	10.11	2.86			
			0.34	14.70	2.62			
	1503	32	0.03	2.30	0.24	0.07	3.83	0.75
			0.12	5.97	1.59			
			0.06	3.22	0.42			
	1504	69	0.13	7.35	1.19	0.10	4.44	0.96
			0.11	2.76	0.93			
			0.07	3.22	0.76			
	1505	16	0.06	0.92	0.67	0.04	0.61	0.48
			0.04	0.46	0.43			
			0.02	0.46	0.35			

[A] Mean of median of 150 cells scored per animal

Table 16: DNA Damage Data in Female Nasal Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	2501	41	0.10	13.78	0.54	0.08	13.63	0.42
			0.09	14.70	0.50			
			0.04	12.40	0.22			
	2502	25	0.05	15.16	0.23	0.06	13.48	0.35
			0.07	14.24	0.38			
			0.06	11.03	0.43			
[redacted] (375 ppm)	2503	21	0.12	15.16	0.62	0.09	13.02	0.48
			0.03	9.65	0.19			
			0.11	14.24	0.63			
	2504	38	0.02	9.19	0.12	0.02	5.97	0.19
			0.03	4.13	0.24			
			0.03	4.59	0.20			
	2505	25	0.08	16.08	0.40	0.07	17.30	0.37
			0.06	17.46	0.42			
			0.06	18.38	0.30			

[A] Mean of median of 150 cells scored per animal

Table 16: DNA Damage Data in Female Nasal Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	3501	47	0.04	11.49	0.24	0.17	17.46	0.98
			0.25	20.21	1.25			
			0.20	20.67	1.46			
	3502	21	0.02	11.94	0.17	0.03	14.09	0.24
			0.01	11.49	0.14			
			0.07	18.84	0.42			
[redacted] (750 ppm)	3503	39	0.07	17.00	0.39	0.05	15.77	0.32
			0.06	17.00	0.42			
			0.02	13.32	0.15			
	3504	21	0.04	12.40	0.22	0.13	19.60	0.71
			0.23	22.97	1.30			
			0.13	23.43	0.62			
	3505	34	0.06	15.16	0.47	0.09	17.46	0.78
			0.15	18.84	1.35			
			0.08	18.38	0.50			

[A] Mean of median of 150 cells scored per animal

Table 16: DNA Damage Data in Female Nasal Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post Final Exposure Completion

Treatment (6 hours of Exposure)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Test substance:	4501	42	0.04	15.62	0.30	0.04	15.47	0.23
			0.02	13.32	0.09			
			0.06	17.46	0.30			
	4502	27	0.03	15.16	0.15	0.04	16.85	0.20
			0.05	18.38	0.31			
			0.03	17.00	0.14			
	4503	27	0.02	12.86	0.08	0.02	13.78	0.13
[redacted] (1500 ppm)			0.02	11.49	0.16			
			0.03	17.00	0.15			
	4504	25	0.02	12.40	0.10	0.03	14.85	0.16
			0.02	13.32	0.11			
			0.05	18.84	0.27			
	4505	20	0.02	17.00	0.09	0.03	18.53	0.20
			0.02	17.92	0.21			
			0.05	20.67	0.30			

[A] Mean of median of 150 cells scored per animal

Table 16: DNA Damage Data in Female Nasal Cells - Phase 1 (Continued)

Samples Collected 2 to 4 hours Post-Last Dose

Treatment (10 mL/kg/treatment)	Animal	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Positive Control:	5501	0	0.01	0.00	0.21	0.02	0.15	0.29
			0.04	0.00	0.41			
			0.01	0.46	0.24			
	5502	15	0.00	0.00	0.09	0.43	3.83	3.26
			1.08	10.57	7.98			
			0.22	0.92	1.70			
EMS 200 mg/kg[B]	5503	7	0.01	0.00	0.17	0.01	0.00	0.12
			0.00	0.00	0.13			
CP 20mg/kg[C]			0.00	0.00	0.07			
	5504	0	0.10	0.46	0.75	0.04	0.31	0.28
			0.00	0.46	0.04			
			0.00	0.00	0.04			
	5505	0	0.01	0.00	0.20	0.01	0.00	0.13
			0.00	0.00	0.13			
			0.01	0.00	0.19			

[A] Mean of median of 150 cells scored per animal

[B] Ethyl methanesulfonate (EMS), positive control for Comet assay, orally administered at 10 mL/kg on Study Days 2 and 3.

[C] Cyclophosphamide monohydrate (CP), positive control for Micronucleus assay, orally administered at 10 mL/kg on days 1 and 2.

11. APPENDIX I: Historical Control

Male Rat Historical Control Data[6]
2008 to 2018
Electrophoresis performed refrigerated (2 to 12°C), protected from light
Organs harvested at ~3 hours post last dose

VEHICLE (NEGATIVE) CONTROL[1]
N = 57 animals or 11 Studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual animals			Study		
Kidney	Mean[3]	0.21	24.89	1.05	0.22	25.31	1.09
	Standard Deviation	0.15	9.73	0.72	0.11	8.74	0.42
	Range[4]	0.040	6.58	0.21	0.097	14.04	0.52
		0.71	43.12	3.30	0.43	41.73	1.76
	95% Confidence[5]	0.00	5.43	0.00	0.00	7.84	0.24
		0.52	44.35	2.48	0.45	42.79	1.93

POSITIVE CONTROL[2]
N = 51 animals or 12 Studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual animals			Study		
Kidney	Mean[3]	6.34	51.69	29.26	6.16	50.70	28.67
	Standard Deviation	3.25	11.74	10.44	2.76	9.18	9.22
	Range[4]	2.00	23.28	11.94	3.33	36.46	18.35
		18.90	83.46	60.81	11.24	72.26	45.07
	95% Confidence[5]	0.00	28.22	8.37	0.63	32.34	10.24
		12.84	75.17	50.15	11.68	69.07	47.10

[1]Negative control articles: all vehicles used; Route of administration: oral gavage (PO), intraperitoneal (IP), subcutaneous (SC), or intravenous (IV).

[2]Positive control article: Ethyl methanesulfonate (200 mg/kg).

[3]Average (mean) of the median Comet Assay parameters measured in studies.

[4]Minimum and maximum range of median Comet Assay measurements.

[5]95% Confidence is calculated by the mean of the median Comet parameter ± 2 standard deviations.

[6]Historical range includes data from nonGLP studies.

Female Rat Historical Control Data[6]
2008 to 2018
Electrophoresis performed refrigerated (2 to 12°C), protected from light
Organs harvested at ~3 hours post last dose

VEHICLE (NEGATIVE) CONTROL[1]
N = 24 animals or 4 Studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual animals			Study		
Kidney	Mean[3]	0.15	21.94	0.80	0.15	21.94	0.80
	Standard Deviation	0.12	18.70	0.58	0.12	20.50	0.49
	Range[4]	0.0088	1.92	0.056	0.072	8.41	0.45
		0.53	59.81	2.61	0.32	52.47	1.50
	95% Confidence[5]	0.00	0.00	0.00	0.00	0.00	0.00
		0.40	59.34	1.96	0.38	62.93	1.77

POSITIVE CONTROL[2]
N = 18 animals or 4 Studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual animals			Study		
Kidney	Mean[3]	4.66	48.89	23.89	4.43	45.15	23.33
	Standard Deviation	2.13	22.37	7.94	1.96	22.64	6.95
	Range[4]	1.76	24.57	10.60	2.97	30.32	17.03
		8.44	84.39	37.69	7.29	78.82	32.96
	95% Confidence[5]	0.40	4.16	8.01	0.50	0.00	9.44
		8.93	93.62	39.76	8.36	90.43	37.23

[1]Negative control articles: all vehicles used; Route of administration: oral gavage (PO), intraperitoneal (IP), subcutaneous (SC), or intravenous (IV).

[2]Positive control article: Ethyl methanesulfonate (200 mg/kg).

[3]Average (mean) of the median Comet Assay parameters measured in studies.

[4]Minimum and maximum range of median Comet Assay measurements.

[5]95% Confidence is calculated by the mean of the median Comet parameter ± 2 standard deviations.

[6]Historical range includes data from nonGLP studies.

Male Rat Historical Control Data
2016 to 2018
Electrophoresis performed refrigerated (2 to 12°C), protected from light
Organs harvested at ~3 hour post last dose

VEHICLE (NEGATIVE) CONTROL[1]
N = 396 animals or 64 studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual animals			Study		
Liver	Mean[3]	0.067	8.08	0.40	0.071	8.15	0.42
	Standard Deviation	0.138	7.72	0.82	0.127	7.28	0.75
	Range[4]	0.00	0.00	0.00	0.00	0.00	0.0051
		1.06	39.61	6.02	0.63	32.99	3.43
	95% Confidence[5]	0.00	0.00	0.00	0.00	0.00	0.00
		0.34	23.51	2.03	0.32	22.71	1.91

POSITIVE CONTROL[2]
N = 270 animals or 76 studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual animals			Study		
Liver	Mean[3]	4.68	39.66	22.47	4.12	38.03	20.63
	Standard Deviation	3.73	15.40	11.81	3.18	12.79	10.44
	Range[4]	0.280	4.13	2.01	0.44	10.45	3.35
		20.50	96.02	61.57	16.64	78.01	53.66
	95% Confidence[5]	0.00	8.85	0.00	0.00	12.46	0.00
		12.15	70.47	46.09	10.49	63.61	41.52

[1]Negative control articles: all vehicles used; Route of administration: oral gavage (PO), intraperitoneal (IP), subcutaneous (SC), inhalation, or intravenous (IV).

[2]Positive control article: Ethyl methanesulfonate (200 mg/kg).

[3]Average (mean) of the median Comet Assay parameters measured in studies.

[4]Minimum and maximum range of median Comet Assay measurements.

[5]95% Confidence is calculated by the mean of the median Comet parameter ± 2 standard deviations.

Female Rat Historical Control Data
2008 to 2018
Electrophoresis performed refrigerated (2 to 12°C), protected from light
Organs harvested at ~3 hour post last dose

VEHICLE (NEGATIVE) CONTROL[1]
N = 323 animals or 57 studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual Animals			Study		
Liver	Mean[3]	0.102	11.32	0.54	0.100	11.31	0.53
	Standard Deviation	0.24	10.36	1.26	0.21	9.53	1.15
	Range[4]	0.00	0.00	0.00	0.00044	0.00	0.0056
		2.53	47.29	11.89	1.46	32.74	8.06
	95% Confidence[5]	0.00	0.00	0.00	0.00	0.00	0.00
		0.58	32.04	3.05	0.53	30.36	2.83

POSITIVE CONTROL[2]
N = 255 animals or 61 studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual Animals			Study		
Liver	Mean[3]	4.15	40.81	20.95	4.02	39.85	20.55
	Standard Deviation	2.64	12.19	10.15	2.35	10.52	9.16
	Range[4]	0.13	15.31	0.84	1.30	22.05	7.95
		13.91	84.70	51.24	11.47	70.89	45.00
	95% Confidence[5]	0.00	16.43	0.64	0.00	18.81	2.23
		9.42	65.19	41.25	8.71	60.88	38.87

[1]Negative control articles: all vehicles used; Route of administration: oral gavage (PO), intraperitoneal (IP), subcutaneous (SC), inhalation, or intravenous (IV)

[2]Positive control article: Ethyl methanesulfonate (200 mg/kg)

[3]Average (mean) of the median Comet Assay parameters measured in studies.

[4]Minimum and maximum range of median Comet Assay measurements.

[5]95% Confidence is calculated by the mean of the median Comet parameter ± 2 standard deviations.

Male Rat Historical Control Data[6]
2008 to 2018
Electrophoresis performed refrigerated (2 to 12°C), protected from light
Organs harvested at ~3 hour post last dose

VEHICLE (NEGATIVE) CONTROL[1]
N = 123 animals or 20 studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual animals			Study		
Lung	Mean[3]	0.066	13.61	0.36	0.072	14.39	0.40
	Standard Deviation	0.08	9.64	0.47	0.074	9.52	0.41
	Range[4]	0.00	0.00	0.010	0.0026	1.10	0.027
		0.47	50.08	3.19	0.32	43.82	1.70
	95% Confidence[5]	0.00	0.00	0.00	0.00	0.00	0.00
		0.24	32.88	1.30	0.22	33.43	1.23

POSITIVE CONTROL[2]
N = 100 animals or 21 studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual animals			Study		
Lung	Mean[3]	7.43	53.02	31.23	7.36	52.27	31.11
	Standard Deviation	4.78	20.30	14.95	4.41	18.47	14.32
	Range[4]	0.41	3.33	3.11	0.81	7.56	5.82
		20.31	99.92	59.51	15.07	79.61	50.44
	95% Confidence[5]	0.00	12.41	1.33	0.00	15.33	2.47
		16.99	93.63	61.12	16.18	89.21	59.76

[1]Negative control articles: all vehicles used; Route of administration: oral gavage (PO), intraperitoneal (IP), subcutaneous (SC), inhalation, or intravenous (IV).

[2]Positive control article: Ethyl methanesulfonate (200 mg/kg).

[3]Average (mean) of the median Comet Assay parameters measured in studies.

[4]Minimum and maximum range of median Comet Assay measurements.

[5]95% Confidence is calculated by the mean of the median Comet parameter ± 2 standard deviations.

[6]Historical range includes data from nonGLP studies.

Female Rat Historical Control Data[6]
2008 to 2018
Electrophoresis performed refrigerated (2 to 12°C), protected from light
Organs harvested at ~3 hour post last dose

VEHICLE (NEGATIVE) CONTROL[1]
N = 63 animals or 10 studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual animals			Study		
Lung	Mean[3]	0.060	16.75	0.36	0.062	16.71	0.37
	Standard Deviation	0.042	9.48	0.25	0.026	9.56	0.17
	Range[4]	0.0099	6.44	0.052	0.028	11.16	0.16
		0.23	50.08	1.39	0.10	43.35	0.58
	95% Confidence[5]	0.00	0.00	0.00	0.010	0.00	0.034
		0.14	35.70	0.86	0.11	35.83	0.71

POSITIVE CONTROL[2]
N = 52 animals or 10 studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual animals			Study		
Lung	Mean[3]	5.42	49.31	23.53	5.48	48.81	23.69
	Standard Deviation	5.49	13.24	10.71	4.41	12.34	9.95
	Range[4]	1.88	24.95	8.94	2.38	32.48	14.13
		36.94	75.58	57.52	15.01	70.94	46.93
	95% Confidence[5]	0.00	22.82	2.12	0.00	24.13	3.79
		16.40	75.80	44.95	14.30	73.49	43.58

[1]Negative control articles: all vehicles used; Route of administration: oral gavage (PO), intraperitoneal (IP), subcutaneous (SC), inhalation, or intravenous (IV).

[2]Positive control article: Ethyl methanesulfonate (200 mg/kg).

[3]Average (mean) of the median Comet Assay parameters measured in studies.

[4]Minimum and maximum range of median Comet Assay measurements.

[5]95% Confidence is calculated by the mean of the median Comet parameter ± 2 standard deviations.

[6]Historical range includes data from nonGLP studies.

Male Rat Historical Control Data[6]
2015 to 2018
Electrophoresis performed refrigerated (2 to 12ºC), protected from light
Organs harvested at ~3 hour post last dose

VEHICLE (NEGATIVE) CONTROL[1]
N = 118 animals or 20 studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual animals			Study		
Nasal Cavity	Mean[3]	0.054	12.56	0.32	0.057	13.61	0.34
	Standard Deviation	0.063	5.97	0.37	0.048	6.92	0.29
	Range[4]	0.00	1.68	0.014	0.016	6.85	0.089
		0.39	40.43	2.43	0.23	39.43	1.36
	95% Confidence[5]	0.00	0.63	0.00	0.00	0.00	0.00
		0.18	24.49	1.06	0.15	27.46	0.92

POSITIVE CONTROL[2]
N = 103 animals or 21 studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual animals			Study		
Nasal Cavity	Mean[3]	5.62	48.24	24.65	5.59	47.79	24.94
	Standard Deviation	4.32	20.12	13.74	4.09	18.48	13.52
	Range[4]	0.22	10.87	1.04	1.05	19.63	5.02
		20.09	97.09	57.62	13.72	82.18	46.25
	95% Confidence[5]	0.00	8.00	0.00	0.00	10.84	0.00
		14.26	88.48	52.14	13.77	84.74	51.97

[1]Negative control articles: all vehicles used; Route of administration: oral gavage (PO) and inhalation.
[2]Positive control article: Ethyl methanesulfonate (200 mg/kg);
[3]Average (mean) of the median Comet Assay parameters measured in studies.
[4]Minimum and maximum range of median Comet Assay measurements.
[5]95% Confidence is calculated by the mean of the median Comet parameter ± 2 standard deviations.
[6]Historical range includes data from nonGLP studies.

Female Rat Historical Control Data[6]
2016 to 2018
Electrophoresis performed refrigerated (2 to 12ºC), protected from light
Organs harvested at ~3 hour post last dose

VEHICLE (NEGATIVE) CONTROL[1]
N = 56 animals or 9 studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual animals			Study		
Nasal Cavity	Mean[3]	0.15	18.40	0.91	0.15	17.91	0.93
	Standard Deviation	0.17	9.07	0.88	0.083	5.44	0.49
	Range[4]	0.0053	6.89	0.031	0.023	10.69	0.12
		0.96	57.43	3.68	0.27	28.90	1.46
	95% Confidence[5]	0.00	0.27	0.00	0.00	7.03	0.00
		0.49	36.54	2.67	0.32	28.79	1.90

POSITIVE CONTROL[2]
N = 47 animals or 9 studies

Organ	Parameter	Tail Moment	Tail Migration (µm)	% Tail DNA	Tail Moment	Tail Migration (µm)	% Tail DNA
		Individual animals			Study		
Nasal Cavity	Mean[3]	3.23	42.73	17.66	3.27	42.70	17.80
	Standard Deviation	2.10	11.02	8.75	2.08	9.56	8.58
	Range[4]	0.76	23.03	5.14	1.23	31.36	7.48
		9.10	71.06	39.21	7.68	58.68	34.81
	95% Confidence[5]	0.00	20.69	0.17	0.00	23.58	0.65
		7.43	64.78	35.15	7.42	61.81	34.96

[1]Negative control articles: all vehicles used; Route of administration: oral gavage (PO) and inhalation.
[2]Positive control article: Ethyl methanesulfonate (200 mg/kg).
[3]Average (mean) of the median Comet Assay parameters measured in studies.
[4]Minimum and maximum range of median Comet Assay measurements.
[5]95% Confidence is calculated by the mean of the median Comet parameter ± 2 standard deviations.
[6]Historical range includes data from nonGLP studies.