**CORNING** Hazleton

# MUTAGENICITY TEST ON

# T-6358

# IN AN *IN VIVO* MOUSE MICRONUCLEUS ASSAY

## FINAL REPORT

## AUTHOR

Hemalatha Murli, Ph.D.

## PERFORMING LABORATORY

Corning Hazleton Inc. (CHV)
9200 Leesburg Pike
Vienna, Virginia 22182

## LABORATORY PROJECT IDENTIFICATION

CHV Study No.: 17388-0-455

## SUBMITTED TO

3M
3M Center, Building 220-2E-02
St. Paul, Minnesota 55144-1000

## STUDY COMPLETION DATE

May 14, 1996

# QUALITY ASSURANCE STATEMENT

Project Title: *In Vivo* Mouse Micronucleus Assay

Project No.: 20996 Assay No.: 17388

Protocol No.: 455 Edition No.: 17

Quality Assurance inspections of the study and review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

| Inspection/Date | Findings Reported | Auditor |
|---|---|---|
| Test Article Dilution/03/05/96 | 03/05/96 | C. Smith |
| Dosing/03/05/96 | 03/05/96 | C. Smith |
| Draft Report Review/05/01,02/1996 | 05/02/1996 | S. Ballenger |
| Final Report Review/05/14/1996 | 05/14/1996 | S. Ballenger |

Quality Assurance Unit — Date Released: 5/14/96

## STUDY COMPLIANCE AND CERTIFICATION

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Food and Drug Administration (FDA) Title 21 of the U.S. Code of Federal Regulations Part 58, issued December 22, 1978, (effective June 20, 1979) with any applicable amendments. There were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results in this report are supported by an experimental data record and this record has been reviewed by the Study Director. All raw data, documentation, records, protocol and a copy of the final report generated as a result of this study will be archived in the storage facilities of Corning Hazleton Inc. for at least one year following submission of the final report to the Sponsor. After the one year period, the Sponsor may elect to have the aforementioned materials retained in the storage facilities of Corning Hazleton Inc. for an additional period of time, or sent to a storage facility designated by the Sponsor.

Submitted By:

Study Director:

Hemalatha Murli

Hemalatha Murli, Ph.D.
Mammalian Cytogenetics
Department of Genetic and Cellular Toxicology

5/14/96

Study Completion
Date

TABLE OF CONTENTS

## SUMMARY

Mutagenicity Test on T-6358 in an *In Vivo* Mouse Micronucleus Assay

The objective of this *in vivo* assay was to evaluate the ability of the test article, T-6358, to induce micronuclei in bone marrow polychromatic erythrocytes of Crl:CD-1®(ICR) BR mice.

In the dose selection study, the test article was solubilized in deionized water and dosed by oral gavage at 200, 400, 600, 800, and 1000 mg/kg. Six animals (three males and three females) were assigned to each dose group. Animals were observed for three days after dosing for toxic signs and/or mortality.

Based on the results of the dose selection study, the maximum tolerated dose was estimated as 800 mg/kg. In the micronucleus assay, the test article was solubilized in deionized water and dosed by oral gavage at 200, 400, and 800 mg/kg. Ten animals (five males and five females) were randomly assigned to each dose/harvest time group. Vehicle and positive control groups, euthanatized approximately 24 hours after dosing, were included in the assay. The animals dosed with the test article were euthanatized approximately 24, 48 and 72 hours after dosing for extraction of the bone marrow.

The test material, T-6358, did not induce a significant increase in micronuclei in bone marrow polychromatic erythrocytes under the conditions of this assay and is considered negative in the mouse bone marrow micronucleus test.

Mutagenicity Test on T-6358 in an *in vivo* Mouse Micronucleus Assay

1.0 SPONSOR: 3M

2.0 MATERIAL (Test Article)

2.1 Client's Identification: T-6358

2.2 Date Received: January 16, 1996

2.3 Physical Description: White powder

2.4 Genetics Assay No.: 17388

3.0 TYPE OF ASSAY: *In Vivo* Mouse Micronucleus Assay

4.0 PROTOCOL NO.: 455, Edition 17

5.0 STUDY DATES

5.1 Initiation Date: January 18, 1996

5.2 Experimental Start Date: February 20, 1996

5.3 Experimental Termination Date: April 16, 1996

6.0 SUPERVISORY PERSONNEL

6.1 Study Director: Hemalatha Murli, Ph.D.

6.2 Laboratory Supervisor: Monica Vegarra, B.S.

7.0 OBJECTIVE

The objective of this *in vivo* assay was to evaluate the ability of the test article, T-6358, to induce micronuclei in bone marrow polychromatic erythrocytes of Crl:CD-1®(ICR) BR mice. This study was conducted using modifications of the procedures suggested by Heddle *et al.* (1983).

## 8.0 MATERIALS

Adult male and female mice, strain Crl:CD-1®(ICR) BR, were purchased from Charles River Laboratories, Portage MI. This healthy, random bred strain was selected to maximize genetic heterogeneity and at the same time assure access to a common source. The protocol for this study was approved by the CHV-ACUC prior to the initiation of dosing.

Animals were housed up to seven per cage during quarantine, and housed up to five at randomization. The temperature and relative humidity were maintained at 72±6°F and 55±15%, respectively, except on February 17, 18, 19, 24 and 25, 1996, for the dose selection study, when the relative humidity was recorded as 39.1%, 37.3%, 34.5%, 32.5%, 34.4%, and 20.8%, respectively, and on March 2, 1996, for the micronucleus study, when the relative humidity was recorded as 35.5%. A 12-hour light/12-hour dark cycle was maintained. A commercial diet (Purina® Certified Laboratory Pellets ® # 5002) and water were available ad libitum for the duration of the study. The feed was analyzed by the manufacturer for concentrations of specified heavy metals, aflatoxin, chlorinated hydrocarbons, organophosphates, and specified nutrients. The water was analyzed on a retrospective basis for specified microorganisms, pesticides, alkalinity, heavy metals, and halogens. Sanitized caging was used for housing the animals. Personnel handling animals or working within the animal facilities were required to wear suitable protective garments and equipment.

Animals were quarantined for seven days before being placed on study. Animals were randomly assigned to study groups and were individually weighed prior to dosing. All animals were dosed based upon the individual body weights. Animals were uniquely identified by ear tag. Dose or treatment groups were identified by cage card/label.

At the termination of the study all surviving animals were euthanatized by $CO_2$ followed by penetration of the thorax. Any extra animals not used for the study were euthanatized by $CO_2$ followed by penetration of the thorax.

## 9.0 SOLUBILITY AND STABILITY:

The test article, T-6358, was supplied as a white powder. The solubility of the test article was evaluated in deionized water. A clear, colorless solution was obtained at a concentration of about 445.2 mg/ml. The stability of the test material under the dosing conditions of this assay is the responsibility of the sponsor.

## 10.0 DOSE SELECTION STUDY

### 10.1 Dose Selection

Dose levels of 200, 400, 600, 800, and 1,000 mg/kg were administered by oral gavage for the dose selection study.

### 10.2 Dosing Information

The animals used in the dose selection assay were dosed on February 20, 1996. The weight range of the animals used in the dose range finding assay was 27.7 - 34.6 and 21.6 - 25.7 grams, for the males and females, respectively. Dosing solutions were prepared just prior to dosing and were prepared by making a 100 mg/ml stock for the high dose (1000 mg/kg). This was prepared by adding 11.5 ml of deionized water (Lot # 19, prepared at CHV) to1.20 g of T-6358, resulting in a clear colorless solution. Dilutions of this stock were prepared for the 200, 400, 600, and 800 mg/kg dose levels.

Dosing was achieved using a 10 ml/kg dosing volume. All animals were eight weeks and one day old at the time of dosing. An outline of the dosing scheme is found in the following table.

A total of 30 animals was used in this assay.

| | DOSE GROUPS | |
|---|---|---|
| TREATMENT | M | F |
| T-6358 | | |
| 200 mg/kg | 3 | 3 |
| 400 mg/kg | 3 | 3 |
| 600 mg/kg | 3 | 3 |
| 800 mg/kg | 3 | 3 |
| 1000 mg/kg | 3 | 3 |

All doses given were on an acute (one-time only) basis.

### 10.3 Results and Interpretation

All animals were examined after dosing and daily throughout the duration of the study (three days) for toxic effects and/or mortalities. All animals appeared normal immediately after dosing.

Approximately one hour after dosing, all animals at the 200 and 400 mg/kg dose levels appeared normal. All females and one male at the 600 mg/kg dose level appeared hypoactive with the male also showing lacrimation in both eyes. The remaining males appeared normal. The males at the 800 mg/kg dose level appeared normal. The females at the 800 mg/kg dose level and all animals at the 1000 mg/kg dose level appeared hypoactive.

Approximately 21 hours after dosing, all animals at the 200, 400, 600 and 800 mg/kg dose levels appeared normal. At the 1000 mg/kg dose level, one male (# 6485) and one female (#6504) were found dead. The remaining males appeared hypoactive, with one male (# 6486) also showing eyes glued shut with chromodacryorrhea. The remaining females appeared hypoactive with hunched postures.

Approximately 45 hours after dosing, one male (# 6486) at the 1000 mg/kg dose level was found dead. All remaining animals at all dose levels appeared normal. The mortality data for this assay are summarized in the following table:

**Summary of Mortalities Within 3 Days in Mice Dosed Acutely with T-6358**

| Treatment | Male | Female |
|---|---|---|
| 200 mg/kg | 0/3 | 0/3 |
| 400 mg/kg | 0/3 | 0/3 |
| 600 mg/kg | 0/3 | 0/3 |
| 800 mg/kg | 0/3 | 0/3 |
| 1000 mg/kg | 2/3 | 1/3 |

10.4 Conclusion

Based on these results, the maximum tolerated dose was estimated to be 800 mg/kg.

## 11.0 MICRONUCLEUS STUDY

### 11.1 Dose Selection

Based on results from the dose selection study, dose levels of 200, 400, and 800 mg/kg were selected for testing in this study.

### 11.2 Micronucleus Assay Dosing Information

The animals used in the micronucleus assay were dosed on March 5, 1996. Cyclophosphamide (CAS # 6055-19-2; Sigma, Lot # 44H0486), the positive

control, was solubilized in sterile deionized water (Lot # 19, prepared at CHV) and was administered by oral gavage at 80.0 mg/kg. The vehicle control, deionized water (Lot # 19, prepared at CHV), was administered concurrently with the test article at a volume of 10.0 ml/kg. The weight range of the animals used in the micronucleus assay was 28.1 - 35.2 and 22.0 - 29.1 grams for the males and females, respectively. The dosing solutions for the assay were prepared by making a 80.0 mg/ml stock for the high dose (800 mg/kg). This was prepared by adding the vehicle to 2.1200 g of T-6358 up to a volume of 26.5 ml. A clear colorless solution was obtained. Dilutions of this stock were prepared for the remaining dose levels. A second group of animals (designated Secondary Dose Group) was also assigned to the study and was dosed with the high dose of the test article. These animals were only used in the assay as replacements for any which died in the primary dose group.

Ten animals (five males and five females) were randomly assigned to each dose/-harvest time group. Vehicle and positive control groups, euthanatized approximately 24 hours after dosing, were included in the assay. The animals dosed with the test article were euthanatized approximately 24, 48 and 72 hours after dosing for extraction of the bone marrow. An outline of the dosing scheme is found in the following table:

**Dosing Scheme for Micronucleus Assay**

| | Number of Animals Assigned | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Primary Dose Groups | | | | | | Secondary Dose Groups[a] | |
| | 24 Hr | | 48 Hr | | 72 Hr | | | |
| Treatment | M | F | M | F | M | F | Male | Female |
| T-6358 | | | | | | | | |
| 200 mg/kg | 5 | 5 | 5 | 5 | 5 | 5 | - | - |
| 400 mg/kg | 5 | 5 | 5 | 5 | 5 | 5 | - | - |
| 800 mg/kg | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Vehicle Control, deionized water, 10.0 ml/kg | 5 | 5 | - | - | - | - | - | - |
| Positive Control, Cyclophosphamide, 80.0 mg/kg | 5 | 5 | - | - | - | - | - | - |

[a] The animals assigned to the secondary dose groups were dosed and were only used to replace animals which died in the primary dose group at the high dose level. All extra animals not used as replacements were euthanatized at the completion of the trial.

The age of the animals at the time of dosing was eight weeks and one day. A total of 120 animals was used in this assay.

Volumes dosed were 10.0 ml/kg and were based upon individual animal weights.

## 12.0 BONE MARROW HARVEST, SLIDE PREPARATION AND ANALYSIS

At the appropriate harvest time, the animals were euthanatized with $CO_2$ followed by penetration of the thorax. The adhering soft tissue and epiphyses of both femora were removed. The marrow was flushed from the bone and transferred to centrifuge tubes containing 3 - 5 ml bovine serum (one tube for each animal). Following centrifugation to pellet the tissue, the supernatant was removed by aspiration and portions of the pellet were spread on slides and air dried. The slides were fixed in methanol, and stained in May-Grunwald solution followed by Giemsa (Schmid, 1975). The air-dried slides were coverslipped using Depex® mounting medium.

The slides were coded for analysis, and scored for micronuclei and the polychromatic erythrocyte (PCE) to normochromatic erythrocyte (NCE) cell ratio. Standard forms were used to record these data. One thousand PCEs per animal were scored. The frequency of micronucleated cells was expressed as percent micronucleated cells based on the total PCEs present in the scored optic field. The normal frequency of micronuclei in this Crl:CD-1®(ICR) BR strain is about 0.0-0.4%.

The frequency of PCEs versus NCEs was determined by scoring the number of PCEs and NCEs observed in the optic fields while scoring the first 1000 erythrocytes.

## 13.0 EVALUATION CRITERIA:

### 13.1 General

The criteria for the identification of micronuclei were those of Schmid (1976). Micronuclei were darkly stained and generally round, although almond and ringshaped micronuclei occasionally occurred. Micronuclei had sharp borders and were generally between 1/20 and 1/5 the size of the PCE. The unit of scoring was the micronucleated cell, not the micronucleus; thus the occasional cell with more than one micronucleus was counted as one micronucleated PCE, not two (or more) micronuclei. The staining procedure permitted the differentiation by color of PCEs and NCEs (bluish-grey and red, respectively).

### 13.2 Data Presentation and Interpretation

Data are summarized by sex and dose groups for the different time points. Individual animal data are also presented. The analysis of these data was performed using an analysis of variance (Winer, 1971) on either untransformed (when variances are homogeneous) and rank transformed (when variances are heterogeneous) proportions of cells with micronuclei per animal. If the analysis of variance was significant ($p<0.05$), a Dunnett's t-test (Dunnett, 1955; 1964) was used to determine which dose groups, if any, were significantly different from the

negative control. Analyses were performed separately for each harvest time and sex combination. The criteria for determining a positive response involved a statistically significant dose-related increase in micronucleated PCEs, or the detection of a reproducible and statistically significant positive response for at least one dose level. A test article that induced neither a statistically significant dose response nor a statistically significant and reproducible increase at one dose level was considered negative. In either case, the final decision was based on scientific judgment.

## 14.0 RESULTS AND INTERPRETATION:

All animals were observed immediately after dosing and periodically throughout the duration of the assay for toxic symptoms and/or mortalities. All animals in the vehicle and positive control groups appeared normal after dosing and remained healthy until the appropriate harvest times.

All test article dosed groups appeared normal immediately and approximately 1.5 hours after dosing.

Approximately 22 hours after dosing, at the 800 mg/kg dose level, one male (# 6748) and one female (#6764) from the 24 hour harvest group and one male (# 6753) from the 48 hour harvest group were found dead. All remaining animals at all dose levels appeared normal.

Approximately 23.5 hours after dosing, at the 800 mg/kg dose level, one female (# 6803) from the secondary group and one female (# 6766) from the 72 hour harvest group were found dead. One female (# 6790) from the secondary dose group appeared hypoactive.

Approximately 46 hours after dosing, at the 800 mg/kg dose level, three females (#'s 6761, 48 hour harvest group; 6784, 72 hour harvest group; 6790, secondary dose group) were found dead. All remaining animals at all dose levels appeared normal.

Approximately 70 hours after dosing, all remaining animals at all dose levels appeared normal.

The test article, T-6358, induced no significant increases in micronucleated polychromatic erythrocytes over the levels observed in the vehicle controls in either sex or at any of the harvest times, except for the females from the 48 hour harvest of the 800 mg/kg dose group. This is a statistical anomaly, for the mean of this group, 0.30%, is very close to the historical control data (maximum of 0.24%) and within the published historical data (Salamone and Mavournin, 1994). The positive control, CP, induced significant increases in micronucleated PCEs in both sexes as compared to the vehicle controls, with means and standard errors of 3.06% ± 0.50% and 4.24% ± 0.47% for the

males and females, respectively. The data summarized by dose group are presented in Table 1 and individual animal data are found in Tables 2 through 7. Historical control data are presented in Table 8.

15.0 CONCLUSION:

The test material, T-6358, did not induce a significant increase in micronuclei in bone marrow polychromatic erythrocytes under the conditions of this assay and is considered negative in the mouse micronucleus assay.

16.0 REFERENCES:

Dunnett, C.W.: A multiple comparisons procedure for comparing several treatments with a control. J. Am. Statist. Assoc., 50:1096-1121, 1955.

Dunnett, C.W.: New tables for multiple comparisons with a control. Biometrics, 20:482-491, 1964.

Heddle, J.A., Hite, M., Kirkhart, B., Larsen, K., MacGregor, J.T., Newell, G.W. and Salamone, M.F.: The induction of micronuclei as a measure of genotoxicity. Mutation Res., 123:61-118, 1983.

Salamone, M.F., and Mavournin, K.H. (1994), Bone marrow micronucleus assay: a review of the mouse stocks used and their published mean spontaneous micronucleus frequencies, Env. and Mol. Mutagen., 23, 239-273.

Schmid, W.: The micronucleus test. Mutation Res., 31:9-15, 1975.

Schmid, W.: The micronucleus test for cytogenetic analysis. Chemical Mutagens: Principles and Methods for Their Detection, Vol. 4 (A. Hollaender, ed.). Plenum, pp. 31-53, 1976.

Winer, B.J.: Statistical Principles in Experimental Design, McGraw-Hill, New York, Second Edition, 1971.

## 17.0 DEVIATIONS FROM THE SIGNED PROTOCOL

1. Due to unknown reasons, on February 17, 18, 19, 24 and 25, 1996, for the dose selection study, the relative humidity was recorded as 39.1%, 37.3%, 34.5%, 32.5%, 34.4%, and 20.8%, respectively; on March 2, 1996, for the micronucleus study, the relative humidity was recorded as 35.5%. This had no impact on the animals or the integrity of the study.

2. Due to a technical error, the water system was not checked on February 24 and 25, 1996. Although the system was not checked, water was available and hence there was no adverse effetcs on the animals, and there was no impact on the integrity of the study.

# 18.0 EXPERIMENT DATA TABLES

## TABLE I

MICRONUCLEUS DATA SUMMARY TABLE

SPONSOR: 3M

TEST ARTICLE: T-6358

ASSAY: 17388

| TREATMENT | DOSE | HARVEST TIME (HR) | % MICRONUCLEATED PCEs MEAN OF 1000 PER ANIMAL ± S.E. MALES | FEMALES | TOTAL | RATIO PCE:NCE MEAN ± S.E. MALES | FEMALES |
|---|---|---|---|---|---|---|---|
| CONTROLS | | | | | | | |
| VEHICLE | Water | 24 hr | 0.08 ± 0.04 | 0.08 ± 0.04 | 0.08 ± 0.02 | 0.64 ± 0.11 | 0.60 ± 0.07 |
| POSITIVE | CP 80.0 mg/kg | 24 hr | 3.06 ± 0.50* | 4.24 ± 0.47* | 3.65 ± 0.38* | 0.63 ± 0.10 | 0.81 ± 0.07 |
| TEST ARTICLE | 200 mg/kg | 24 hr | 0.14 ± 0.05 | 0.04 ± 0.02 | 0.09 ± 0.03 | 0.59 ± 0.05 | 0.66 ± 0.06 |
| | | 48 hr | 0.16 ± 0.04 | 0.08 ± 0.04 | 0.12 ± 0.03 | 0.56 ± 0.08 | 0.52 ± 0.07 |
| | | 72 hr | 0.10 ± 0.05 | 0.02 ± 0.02 | 0.06 ± 0.03 | 0.44 ± 0.06 | 0.61 ± 0.06 |
| | 400 mg/kg | 24 hr | 0.16 ± 0.05 | 0.06 ± 0.04 | 0.11 ± 0.03 | 0.72 ± 0.08 | 0.65 ± 0.09 |
| | | 48 hr | 0.08 ± 0.04 | 0.10 ± 0.04 | 0.09 ± 0.03 | 0.66 ± 0.13 | 0.53 ± 0.11 |
| | | 72 hr | 0.10 ± 0.03 | 0.26 ± 0.21 | 0.18 ± 0.11 | 0.45 ± 0.12 | 0.65 ± 0.06 |
| | 800 mg/kg | 24 hr | 0.12 ± 0.06 | 0.04 ± 0.02 | 0.08 ± 0.03 | 0.59 ± 0.13 | 0.72 ± 0.17 |
| | | 48 hr | 0.16 ± 0.05 | 0.30 ± 0.04* | 0.22 ± 0.04* | 0.49 ± 0.08 | 0.35 ± 0.09 |
| | | 72 hr | 0.08 ± 0.02 | 0.12 ± 0.06 | 0.10 ± 0.03 | 0.31 ± 0.08 | 0.56 ± 0.20 |

* Significantly greater than the corresponding vehicle control, $p<0.05$. CP = Cyclophosphamide

**TABLE 2**

MICRONUCLEUS TEST - INDIVIDUAL ANIMAL DATA

SPONSOR: 3M

TEST ARTICLE: T-6358

ASSAY NO.: 17388

| TREATMENT | | ANIMAL NUMBER | # MN PCEs/ 1000 PCEs | RATIO PCE:NCE |
|---|---|---|---|---|
| **24 HOUR HARVEST** | MALE | | | |
| VEHICLE CONTROL | Water | 6697 | 0 | 0.51 |
| | | 6741 | 1 | 0.50 |
| | | 6744 | 1 | 0.45 |
| | | 6745 | 0 | 0.72 |
| | | 6752 | 2 | 1.02 |
| POSITIVE CONTROL | CP 80.0 mg/kg | 6704 | 23 | 0.34 |
| | | 6716 | 16 | 0.66 |
| | | 6720 | 32 | 0.93 |
| | | 6724 | 42 | 0.53 |
| | | 6730 | 40 | 0.66 |
| TEST ARTICLE | 200 mg/kg | 6698 | 3 | 0.66 |
| | | 6701 | 0 | 0.75 |
| | | 6729 | 1 | 0.48 |
| | | 6737 | 2 | 0.58 |
| | | 6749 | 1 | 0.47 |
| | 400 mg/kg | 6699 | 3 | 0.75 |
| | | 6717 | 0 | 1.00 |
| | | 6721 | 1 | 0.69 |
| | | 6726 | 2 | 0.61 |
| | | 6728 | 2 | 0.56 |
| | 800 mg/kg | 6702 | 3 | 0.64 |
| | | 6703 | 0 | 0.38 |
| | | 6707 | 0 | 0.99 |
| | | 6722 | 1 | 0.70 |
| | | 6746 | 2 | 0.26 |

CP = Cyclophosphamide
MN = Micronucleus
PCE = Polychromatic erythrocyte
# MN PCEs = Micronucleated PCEs
NCE = Normochromatic erythrocyte

TABLE 3

MICRONUCLEUS TEST - INDIVIDUAL ANIMAL DATA

SPONSOR: 3M

TEST ARTICLE: T-6358

ASSAY NO.: 17388

| TREATMENT | | ANIMAL NUMBER | # MN PCEs/ 1000 PCEs | RATIO PCE:NCE |
|---|---|---|---|---|
| **24 HOUR HARVEST** | FEMALE | | | |
| VEHICLE CONTROL | Water | 6759 | 1 | 0.71 |
| | | 6762 | 0 | 0.63 |
| | | 6798 | 1 | 0.80 |
| | | 6810 | 2 | 0.45 |
| | | 6812 | 0 | 0.43 |
| POSITIVE CONTROL | CP 80.0 mg/kg | 6757 | 61 | 0.95 |
| | | 6782 | 37 | 0.63 |
| | | 6787 | 41 | 0.81 |
| | | 6794 | 36 | 0.66 |
| | | 6809 | 37 | 1.00 |
| TEST ARTICLE | 200 mg/kg | 6760 | 0 | 0.81 |
| | | 6765 | 1 | 0.61 |
| | | 6783 | 1 | 0.54 |
| | | 6796 | 0 | 0.80 |
| | | 6799 | 0 | 0.52 |
| | 400 mg/kg | 6767 | 2 | 0.62 |
| | | 6780 | 1 | 0.37 |
| | | 6807 | 0 | 0.75 |
| | | 6808 | 0 | 0.93 |
| | | 6813 | 0 | 0.57 |
| | 800 mg/kg | 6758 | 0 | 0.67 |
| | | 6772 | 1 | 1.39 |
| | | 6786 | 1 | 0.38 |
| | | 6801 | 0 | 0.55 |
| | | 6806 | 0 | 0.61 |

CP = Cyclophosphamide
MN = Micronucleus
PCE = Polychromatic erythrocyte
# MN PCEs = Micronucleated PCEs
NCE = Normochromatic erythrocyte

**TABLE 4**

MICRONUCLEUS TEST - INDIVIDUAL ANIMAL DATA

SPONSOR: 3M

TEST ARTICLE: T-6358

ASSAY NO.: 17388

| TREATMENT | | ANIMAL NUMBER | # MN PCEs/ 1000 PCEs | RATIO PCE:NCE |
|---|---|---|---|---|
| **48 HOUR HARVEST** | MALE | | | |
| TEST ARTICLE | 200 mg/kg | 6708 | 1 | 0.58 |
| | | 6709 | 2 | 0.38 |
| | | 6713 | 3 | 0.45 |
| | | 6742 | 1 | 0.58 |
| | | 6751 | 1 | 0.82 |
| | 400 mg/kg | 6719 | 1 | 0.43 |
| | | 6732 | 2 | 0.42 |
| | | 6739 | 0 | 0.66 |
| | | 6747 | 0 | 0.66 |
| | | 6754 | 1 | 1.12 |
| | 800 mg/kg | 6705 | 3 | 0.39 |
| | | 6711 | 0 | 0.30 |
| | | 6714 | 2 | 0.54 |
| | | 6740 | 2 | 0.43 |
| | | 6756 | 1 | 0.77 |

MN = Micronucleus
PCE = Polychromatic erythrocyte
# MN PCEs = Micronucleated PCEs
NCE = Normochromatic erythrocyte

**TABLE 5**

MICRONUCLEUS TEST - INDIVIDUAL ANIMAL DATA

SPONSOR: 3M

TEST ARTICLE: T-6358

ASSAY NO.: 17388

| TREATMENT | | ANIMAL NUMBER | # MN PCEs/ 1000 PCEs | RATIO PCE:NCE |
|---|---|---|---|---|
| **48 HOUR HARVEST** | FEMALE | | | |
| TEST ARTICLE | 200 mg/kg | 6763 | 1 | 0.62 |
| | | 6791 | 2 | 0.43 |
| | | 6795 | 1 | 0.36 |
| | | 6804 | 0 | 0.45 |
| | | 6805 | 0 | 0.75 |
| | 400 mg/kg | 6771 | 0 | 0.44 |
| | | 6776 | 2 | 0.93 |
| | | 6789 | 0 | 0.54 |
| | | 6797 | 1 | 0.49 |
| | | 6814 | 2 | 0.24 |
| | 800 mg/kg | 6774 | 2 | 0.59 |
| | | 6781 | 3 | 0.34 |
| | | 6785 | 3 | 0.34 |
| | | 6815 | 4 | 0.14 |

* Animal found dead
MN = Micronucleus
PCE = Polychromatic erythrocyte
# MN PCEs = Micronucleated PCEs
NCE = Normochromatic erythrocyte

**TABLE 6**

MICRONUCLEUS TEST - INDIVIDUAL ANIMAL DATA

SPONSOR: 3M

TEST ARTICLE: T-6358

ASSAY NO.: 17388

| TREATMENT | | ANIMAL NUMBER | # MN PCEs/ 1000 PCEs | RATIO PCE:NCE |
|---|---|---|---|---|
| **72 HOUR HARVEST** | MALE | | | |
| TEST ARTICLE | 200 mg/kg | 6715 | 3 | 0.55 |
| | | 6725 | 1 | 0.43 |
| | | 6727 | 1 | 0.28 |
| | | 6738 | 0 | 0.33 |
| | | 6755 | 0 | 0.61 |
| | 400 mg/kg | 6710 | 0 | 0.85 |
| | | 6718 | 1 | 0.50 |
| | | 6734 | 1 | 0.30 |
| | | 6735 | 1 | 0.48 |
| | | 6743 | 2 | 0.13 |
| | 800 mg/kg | 6700 | 0 | 0.07 |
| | | 6712 | 1 | 0.33 |
| | | 6731 | 1 | 0.57 |
| | | 6733 | 1 | 0.22 |
| | | 6736 | 1 | 0.36 |

MN = Micronucleus
PCE = Polychromatic erythrocyte
# MN PCEs = Micronucleated PCEs
NCE = Normochromatic erythrocyte

**TABLE 7**

MICRONUCLEUS TEST - INDIVIDUAL ANIMAL DATA

SPONSOR: 3M

TEST ARTICLE: T-6358

ASSAY NO.: 17388

| TREATMENT | | ANIMAL NUMBER | # MN PCEs/ 1000 PCEs | RATIO PCE:NCE |
|---|---|---|---|---|
| **72 HOUR HARVEST** | FEMALE | | | |
| TEST ARTICLE | 200 mg/kg | 6768 | 0 | 0.62 |
| | | 6770 | 0 | 0.55 |
| | | 6779 | 1 | 0.57 |
| | | 6800 | 0 | 0.85 |
| | | 6811 | 0 | 0.48 |
| | 400 mg/kg | 6769 | 0 | 0.69 |
| | | 6773 | 11 | 0.84 |
| | | 6775 | 0 | 0.62 |
| | | 6792 | 0 | 0.47 |
| | | 6816 | 2 | 0.62 |
| | 800 mg/kg | 6777 | 3 | 1.31 |
| | | 6778 | 1 | 0.47 |
| | | 6788 | 2 | 0.25 |
| | | 6793 | 0 | 0.57 |
| | | 6802 | 0 | 0.22 |

MN = Micronucleus
PCE = Polychromatic erythrocyte
# MN PCEs = Micronucleated PCEs
NCE = Normochromatic erythrocyte

**TABLE 8**

MOUSE MICRONUCLEUS HISTORICAL CONTROL DATA 7 95 THROUGH 12/95

| | | % MICRONUCLEATED PCEs PER 1000 PCE MEAN OF 1000 PER ANIMAL ± S.E. | | | RATIO PCE NCE MEAN ± S E | |
|---|---|---|---|---|---|---|
| | | MALES | FEMALES | TOTAL | MALES | FEMALES |
| POOLED VEHICLE CONTROLS | | | | | | |
| | MIN | 0 00 | 0 00 | 0 01 | 0 31 | 0 24 |
| | MAX | 0 22 | 0 24 | 0 17 | 0 85 | 1 03 |
| | AVG | 0 087 ± 0 007 | 0 081 ± 0 008 | 0 084 ± 0 005 | 0 550 ± 0 021 | 0 587 ± 0 025 |
| | N | 47 | 47 | 47 | 47 | 47 |
| POSITIVE CONTROLS Cyclophosphamide, 80 0 mg/kg | | | | | | |
| | MIN | 2 00 | 1 50 | 2 41 | 0 41 | 0 40 |
| | MAX | 5 68 | 6 36 | 5 38 | 0 72 | 0 79 |
| | AVG | 3 682 ± 0 240 | 3 170 ± 0 245 | 3 426 ± 0 184 | 0 577 ± 0 020 | 0 588 ± 0 026 |
| | N | 19 | 19 | 19 | 19 | 19 |

PCE = Polychromatic erythrocyte
NCE = Normochromatic erythrocyte

CHV STUDY NO. _____
PROTOCOL NO. 455, EDITION 17

# IN VIVO MOUSE MICRONUCLEUS ASSAY

Corning Hazleton Inc. (CHV) will conduct this study in compliance with Good Laboratory Practice (GLP) Regulations. This protocol, critical phase(s) of the work in progress and the final report will be subject to audit by Quality Assurance in accordance with SOPs at Corining Hazleton Inc. The study will be conducted by CHV at 9200 Leesburg Pike, Vienna, Virginia 22182.

## PART 1. SPONSOR INFORMATION AND APPROVALS

### I. SPONSOR IDENTIFICATION

Company Name: 3M

Address: St. Paul, MN

### II. TEST ARTICLE IDENTIFICATION: T-6358

### III. TEST ARTICLE ANALYSIS

Determination of the test article stability and the test article characteristics as defined in the GLP regulations is the responsibility of the Sponsor.

### IV. NOTIFICATION OF REGULATORY SUBMISSION

In order to comply with the GLP regulations, consulting laboratories must be notified if all or part of a study is intended for regulatory submission. CHV maintains a master schedule of studies which fall under regulatory review. Please indicate which agency, if any, might receive the results of this study:

☑ Undetermined ☐ FDA ☐ EPA-TSCA ☐ EPA-FIFRA

☐ MAFF ☐ MOHW ☐ OECD ☐ OTHER _____

V. STUDY DATES

Proposed Experimental Start Date: _____

Proposed Experimental Termination Date: _____

VI. APPROVAL OF STUDY PROTOCOL

Study Director:

_____ Date: _____

Hemalatha Murli, Ph.D.

Sponsor's Authorized Representative:

St. Paul, MN Date: 3/12/95

PART 2 - STUDY PROTOCOL

IN VIVO MOUSE MICRONUCLEUS ASSAY

## I. OBJECTIVE

The objective of this study is to evaluate a test article for clastogenic activity and disruption of the mitotic apparatus in polychromatic erythrocyte stem cells in mouse bone marrow in vivo.

## II. DEFINITIONS

Micronucleus: a small chromatin body, consisting of entire chromosome(s) and/or of acentric chromosome fragment(s), which lags behind at mitotic anaphase. After telophase, these chromosome(s) and fragment(s) may not be included in the daughter nuclei, and may form single or multiple micronuclei in the cytoplasm.

## III. RATIONALE

The micronucleus test can serve as a rapid screen for clastogenic agents and test articles which interfere with normal mitotic cell division (Schmid, 1975; Heddle et al., 1983). Micronuclei are formed from chromosomes or chromosome fragments left behind during anaphase and can be scored during interphase because they persist (Schmid, 1975). In this assay, polychromatic erythrocytes (PCEs) in the bone marrow are scored for the presence of micronuclei. During maturation from erythroblast to erythrocyte the nucleus is extruded, while micronuclei, if present, remain in the cytoplasm. Detection of micronuclei in non-nucleated cells is thus facilitated, and time involved in searching for metaphase spreads in treated cell populations is eliminated. Test articles affecting spindle-fiber function or formation as well as clastogenic agents can be detected through micronucleus induction (Schmid, 1975).

## IV. MATERIALS

### A. Animals

Young adult male and female mice of the ICR strain, 8-10 weeks old at the time of dosing, will be purchased from Charles River Laboratories, Inc., or Harlan Sprague-Dawley, Inc. This strain has been selected to

maximize genetic heterogeneity and at the same time ensure access to a common source.

B. Control Articles

Cyclophosphamide (CP, 80 mg/kg; dosing volume of 10 ml/kg) will be used as the positive control article and will be administered by oral gavage. The vehicle control article will consist of the solvent or vehicle used for the test article and will be administered by the same route as, and concurrently with, the test article and in amounts equal to the maximum volumes administered to the experimental animals. The dosing volume will not exceed 20 ml/kg for oral gavage and IP administrations. The vehicles generally used in the assay are water, 0.5% aqueous carboxymethylcellulose solution, or corn oil.

## V. EXPERIMENTAL DESIGN

A. Animal Husbandry

All applicable CHV SOPs will be followed. Animals will be isolated by sex. Animals will be housed up to seven per cage during quarantine, and will be housed up to five prior to experiment initiation. Animals are housed under the following climatic conditions: temperature, 72°F ± 6°F; humidity, 55% ± 15%; light cycle, 12 hours light/dark. A commercial diet (Purina® Certified Laboratory Chow® #5002) and tap water will be available ad libitum. The feed is analyzed by the manufacturer for concentrations of specified heavy metals, aflatoxin, chlorinated hydrocarbons, organophosphates, and specified nutrients. The water is analyzed biannually on a retrospective basis for specified microorganisms, pesticides, heavy metals, alkalinity, and halogens. Animals will be quarantined for at least 7 days before being placed on study.

Animals will be assigned to study groups at random according to Coning Hazleton Standard Operating Procedures. Animals will be weighed prior to dosing. They will be dosed based upon the individual animal weights. Animals will be uniquely identified by ear tag. Treatment groups will be identified by cage label/card.

Sanitary cages will be used. Personnel handling animals or working within the animal facilities will be required to wear suitable protective garments and equipment.

B. Dose Selection

The high dose generally will be selected as 80% of the maximum tolerated dose. The high dose should produce some indication of toxicity (e.g., death, depression of ratio of PCEs to normochromatic erythrocytes (NCEs). One-half and one-quarter of this high dose will normally be used as the intermediate and low dose levels, respectively. Use of a high dose increases the likelihood that a weak clastogen will be detected, and is therefore recommended.

If no appropriate range finding data are available, a range finding study can be performed. The top dose tested in the dose rangefinding study will be 5000 mg/kg. The dose levels tested will be issued as an amendment.

DOSE RANGEFINDING STUDY

The dose rangefinding study will be conducted using five treatment groups. Each of the five groups will consist of 3 male and 3 female mice.

Group Designation and Treatment Regimens

| Group No. | Number of Mice Male | Female | Route | Duration (Days) |
|---|---|---|---|---|
| 1 | 3 | 3 | PO | 3 |
| 2 | 3 | 3 | PO | 3 |
| 3 | 3 | 3 | PO | 3 |
| 4 | 3 | 3 | PO | 3 |
| 5 | 3 | 3 | PO | 3 |

The route of administration will be oral gavage. In the event that test article characteristics preclude oral gavage, IP injection will be employed. These routes of administration have been selected because they are the most common routes of administration for this test procedure. The dosing volume will not exceed 20 ml/kg for oral gavage and IP administrations. Other routes of administration that may be used are intravenous, intramuscular, sub-cutaneous administrations or by feed. The test material will generally be solubilized in one of the following solvents: water, 0.9% saline, 0.5% aqueous carboxymethylcellulose solution, or corn oil. All animals will be dosed based upon individual body weights. Dose levels will be assigned by a protocol amendment.

Body weights will be taken prior to dosing. Dosing formulation will be prepared just prior to dosing. Dosing solutions will be prepared and held at ambient temperatures until dosing (0-2 hours). All animals will be euthanized 3 days after receiving a single dose.

The animals will be observed daily for toxic signs and mortality for the duration of the study. Animals will be euthanized by $CO_2$ inhalation followed by penetration of the thorax.

The daily observations of toxic symptoms and/or mortalities data will be used to estimate the Maximum Tolerated Dose (MTD). Doses will then be assigned for the subsequent cytogenetics assay.

MICRONUCLEUS STUDY

C. Dosing Schedule and Route of Administration

Normally an acute dosing regimen (single administration) will be used (see Table below). Harvest will be approximately 24, 48, 72 hours after administration of the test article, and at approximately 24 hours after administration of the control articles. A total of 110 animals will be used. Equal numbers of males and females will be used at each treatment group. An additional group of animals consisting of 3-10 males and 3-10 females may be dosed as a secondary dose group with the high dose of the test material. This group will be dosed if toxicity is expected at the high dose and the animals in this group will only be used as replacements for any which die prior to euthanasia. The use of the secondary dose group will be determined by the study director. Freshly prepared solutions will be

employed. The animals will be observed daily for toxic signs and mortality.

NUMBER OF ANIMALS USED FOR MICRONUCLEUS ASSAY

| Group No. | Treatment | Harvest Times After Treatment (Males and Females) 24 Hours | 48 Hours | 72 Hours | Total |
|---|---|---|---|---|---|
| 1 | Positive Control | 5 + 5 | --- | --- | 5 + 5 |
| 2 | Vehicle Control | 5 + 5 | --- | --- | 5 + 5 |
| 3 | Low Dose | 5 + 5 | 5 + 5 | 5 + 5 | 15 + 15 |
| 4 | Medium Dose | 5 + 5 | 5 + 5 | 5 + 5 | 15 + 15 |
| 5 | High Dose | 5 + 5 | 5 + 5 | 5 + 5 | 15 + 15 |
| | TOTAL | 25 + 25 | 15 + 15 | 15 + 15 | 55 + 55 |

The route of administration will be oral gavage. In the event that test article characteristics preclude oral gavage, IP injection will be employed. The dosing volume will not exceed 20 ml/kg. These routes of administration have been selected because they are the most common routes of administration for this test procedure. Other routes of administration that may be used are intravenous, intramuscular, sub-cutaneous administrations or by feed.

D. Extraction of Bone Marrow

Euthanasia will be with $CO_2$, followed by penetration of the thorax, and hind limb bones will be removed for marrow extraction. The marrow will be flushed from the bone and transferred to centrifuge tubes containing 3-5 ml bovine serum (one tube for each animal).

E. Preparation of Slides

Following centrifugation to pellet the tissue, the supernatant will be removed by aspiration and portions of the pellet will be spread on slides and air-dried.

The slides will then be fixed in methanol, stained in May-Grunwald Solution and Giemsa, and protected by mounting with coverslips. For control of bias, all slides are coded for analysis.

F. Scoring the Slides

An attempt will be made to score one-thousand PCEs per animal. The frequency of micronucleated cells will be expressed as percent micronucleated cells based on the number of PCEs analyzed. The normal background frequency of micronuclei in the ICR mouse strain is around 0.0-0.4%.

The frequency of PCEs versus mature erythrocytes (NCEs) will be determined by scoring the number of PCEs and NCEs observed in the optic fields while scoring the first 1000 erythrocytes on the slide.

## VI. DATA

The criteria for the identification of micronuclei are those of Schmid (1976). Micronuclei are darkly stained and generally round, although almond and ring-shaped micronuclei occasionally occur. Micronuclei have sharp borders and are generally between 1/20 and 1/5 the size of The PCE. The unit of scoring is the micronucleated cell, not the micronucleus; thus the occasional cell with more than one micronucleus is counted as one micronucleated PCE, not two (or more) micronuclei.

The staining procedure permits the differentiation by color of polychromatic and normochromatic erythrocytes (bluish-grey and red, respectively).

### Data Presentation

The data reported will include the number of PCEs scored, the number of micronucleated PCEs, the percentage of micronucleated PCEs, and the ratio of polychromatic to normochromatic erythrocytes for each experimental animal.

### Evaluation Criteria

The criteria for a positive response is a statistically significant dose-related increase in micronucleated PCEs, or the detection of a reproducible and statistically significant positive response for at least one dose level. A test article that induces neither a statistically significant dose response nor a statistically significant and reproducible increase at one dose level is considered negative. In either case, the final decision is based upon scientific judgement.

## VII. TEST INTERPRETATION

The analysis of this data will be performed using an analysis of variance (Winer, 1971) on either untransformed (when variances are homogeneous) or rank transformed (when variances are heterogeneous) proportions of cells with micronuclei per animal. If the analysis of variance is significant ($p<0.05$), a Dunnett's t-test (Dunnett, 1955; 1964) will be used to determine which dose groups, if any, are significantly different from the negative control. Analyses will be performed separately for each harvest time and sex combination.

## VIII. REFERENCES

Dunnett, C.W.: A multiple comparisons procedure for comparing several treatments with a control. J. Am. Statist. Assoc., 50:1096-1121, 1955.

Dunnett, C.W.: New tables for multiple comparisons with a control. Biometrics, 20:482-491, 1964.

Heddle, J.A., Hite, M., Kirkhart, B., Larsen, K., MacGregor, J.T., Newell, G.W. and Salamone, M.F.: The induction of micronuclei as a measure of genotoxicity. Mutation Res., 123:61-118, 1983.

Schmid, W.: The micronucleus test. Mutation Res., 31:9-15, 1975.

Schmid, W.: The micronucleus test for cytogenetic analysis. In, Chemical Mutagens: Principles and Methods for Their Detection, Vol. 4 (A. Hollaender, ed.). Plenum, pp. 31-53, 1976.

Winer, B.J.: Statistical Principles in Experimental Design, McGraw-Hill, New York, Second Edition, 1971.

## IX. REPORT FORMAT

CHV employs a standard report format for each assay design. The final report will provide the following information.

- Sponsor identification.
- Quality Assurance statement.
- Statement of GLP Compliance.
- Signature of study director.
- Test article identification and CHV Study Number. A physical description of the test article and date of receipt will be included in this section.
- Type of assay and protocol number.
- Dates of study initiation and completion.
- Study director and senior technician.
- Methods.

- Evaluation criteria.
- Interpretation of results.
- Conclusions.
- References.
- Test results presented in tabular form.

## X. CHANGES OR REVISIONS

Any changes or revisions of this approved protocol will be documented, signed by the Study Director, dated, and maintained with this protocol.

## XI. ANIMAL CARE AND USE STATEMENT

In the opinion of the Study Director, no alternative testing methods are appropriate, the study does not duplicate any previous work with this material, and the number and species selected are appropriate. This protocol will be reviewed by the CHV-IACUC for compliance with regulatory guidelines concerning the care and use of animals. If not in compliance, a modification will be required. Any changes or revisions of this approved protocol will be sent to the CHV-IACUC for their review.

## XII. RECORDS TO BE MAINTAINED

All raw data, documentation, records, protocols, and the final report generated as a result of this study will be archived in the storage facilities of Coning Hazleton Inc. for at least one year following submission of the final report to the sponsor. After the one year period, the sponsor may elect to have the aforementioned materials retained in the storage facilities of Corning Hazleton Inc. for an additional period of time or sent to a storage facility designated by the sponsor.

# AMENDMENT TO THE STUDY PROTOCOL



STUDY TITLE: *IN VIVO* MOUSE MICRONUCLEUS ASSAY

PROTOCOL NO.: 455, Edition 17

STUDY NO.: 17388-0-455

Amendment #2

Section 2, Part V.C. Based on the mortality data in the dose selection study, the mouse micronucleus assay will be conducted testing aqueous preparations with dose groups of 200, 400, and 800 mg/kg. A secondary dose group will be used.

STUDY DIRECTOR

Hemalatha Murli 2/28/96

Hemalatha Murli, Ph.D. Date

Mammalian Cytogenetics

Department of Genetic and Cellular Toxicology

# AMENDMENT TO THE STUDY PROTOCOL



STUDY TITLE: *IN VIVO* MOUSE MICRONUCLEUS ASSAY

PROTOCOL NO.: 455, Edition 17

STUDY NO.: 17388-0-455

Amendment #1

Section 2, Part V.B. The Sponsor has $LD_{50}$ data in rats of 440 mg/kg and in mice of 457 mg/kg solubilized in acetone/corn oil mixture. Based on this information, the dose selection study will be conducted testing aqueous preparations (since the test article is soluble in water) with dose groups of 200, 400, 600, 800, and 1000 mg/kg.

STUDY DIRECTOR

Hemalatha Murli

Hemalatha Murli, Ph.D.
Mammalian Cytogenetics
Department of Genetic and Cellular Toxicology

2/19/96
Date