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9200 LEESBURG PIKE
VIENNA, VA 22182-1699

# ANALYSIS OF

# T-5878

# IN A CELL PROLIFERATION ASSAY IN RAT LIVER CELLS

## FINAL REPORT

## AUTHOR

Maria A. Cifone, Ph.D.

## PERFORMING LABORATORY

Hazleton Washington, Inc.
9200 Leesburg Pike
Vienna, Virginia 22182

## LABORATORY PROJECT ID

HWA Study No.: 154-209

## SUBMITTED TO

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

## STUDY COMPLETION DATE

November 1, 1994

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## QUALITY ASSURANCE STATEMENT

PROJECT TITLE: Analysis of T-5878 in a Cell Proliferation Assay in Rat Liver Cells

PROJECT NO.: 20991 HWA STUDY NO.: 154-209

PROTOCOL NO.: 493 EDITION NO.: 1, Modified for 3M Corporation

Quality Assurance inspection(s) 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 |
|---|---|---|
| Dosing and pump implantation (surgery)/ 2-7-94 | 2-8-94 | B. Mullett |
| Draft report review/ 7-12,13,14-94 | 7-14-94 | B. Mullett |
| Final report review/ 11-1-94 | 11-1-94 | B. Mullett |

Brad Mullett — Quality Assurance Unit

11/1/94 — Date Released

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## COMPLIANCE AND CERTIFICATION STATEMENT

The described study was conducted in compliance with the Good Laboratory Practice Regulations as set forth in the Code of Federal Regulations (21 CFR 58, 40 CFR 792, and 40 CFR 160). 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 raw data, documentation, records, protocols, specimens and final reports generated as a result of this study will be archived by Hazleton for a period of at least one year following submission of the final report to the sponsor. After the one year period, the sponsor may elect to have these materials retained in the storage facilities of Hazleton for an additional period of time or sent to a storage facility designated by the sponsor.

SUBMITTED BY:

Andrea L. Ham
Andrea L. Ham, M.S.
Associate Scientist

11/1/94
Date

Study Director:

Maria A. Cifone
Maria A. Cifone, Ph. D.
Study Director
Genetic and Cellular Toxicology

11-1-94
Study Completion Date

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## ABSTRACT

The purpose of this study was to determine the hepatotoxicity of T-5878 by measuring cell proliferation (CP) assayed as S-phase induction in rat liver cells after in vivo treatment. The doses chosen for the study were 500, 1000, 2000, and 3000 mg/kg. Dimethylnitrosamine (DMN) at 15 mg/kg was included as a positive control.

In the cell proliferation assay, a single oral dose of the test material was administered and five animals per condition were labeled with BrdU for 72 hours using ALZET® osmotic pumps. No histomorphological alterations were observed at 500, 1000 and 2000 mg/kg but increased mitoses were observed at 3000 mg/kg. Treatment-related changes were also observed in the dimethylnitrosamine (DMN) positive control animals.

Following determination that there were no treatment-related lobular differences in the labeling indices, sections from the left lateral lobe of the livers, as well as samples from the duodenum, were processed for immunohistochemistry. Each slide was prepared with sections from both liver and duodenum. The duodenum (a rapidly proliferating organ) was used as an internal control for delivery of label and immunohistochemical staining. The percentage of nuclei incorporating label in the liver was determined microscopically. Only hepatocyte nuclei were enumerated. The control animals had a labeling index of 1.46 and treated animals had labeling indices that ranged from 6.73 to 7.83. When comparing vehicle and treated groups and there were significant increases in the labeling index at 2000 and 3000 mg/kg ($0.01 \leq p \leq 0.05$) and a positive trend was observed. The average values showed heterogeneous variance when comparing vehicle and treated groups. Significant increases in cell proliferation were also observed in the positive control animals.

T-5878 was therefore considered positive for the induction of cell proliferation in rat liver cells.

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# Analysis of T-5878 in a Cell Proliferation Assay in Rat Liver Cells

I. SPONSOR: 3M Corporation

II. MATERIAL TESTED:

A. Genetics Assay No.: 154-209

B. Identification: T-5878

C. Physical Description: waxy cream colored solid

D. Date Received: January 19, 1994

III. TYPE OF ASSAYS: Analysis of Cell Proliferation in Rat Liver Cells

IV. PROTOCOL NUMBER: 493, Edition 3, Modified for 3M Corporation

V. STUDY DATES:

A. Study Initiation Date: January 10, 1994

B. Experimental Start Date: February 7, 1994

C. Experimental Termination Date: May 26, 1994

VI. SUPERVISORY PERSONNEL:

A. Study Director: Maria A. Cifone, Ph.D.

B. Associate Scientist: Andrea Ham, M.S.

VII. OBJECTIVE:

The objective of this assay was to measure hepatotoxicity caused by T-5878 by measuring cell proliferation (CP) measured as S-phase induction induced in rat liver cells after in vivo treatment.

Cell proliferation measured the fraction of cells undergoing cell replication in rat liver using an immunohistochemical technique (1,2) to detect bromodeoxyuridine (BrdU) incorporated during DNA synthesis. Animals were given a single oral dose of the test material and the livers were isolated following administration of BrdU for 72 hours in vivo with an ALZET® osmotic pump implanted subcutaneously. Quantification of cells that have incorporated DNA precursors over the 72-hour period indicates increased cell proliferation in the liver (3).

VIII. DEFINITION:

Hepatotoxicants such as carbon tetrachloride and dinitrotoluene induce an increase in cell proliferation to replace necrotic tissue (2,4). These proliferating cells may be detected during S-phase analysis. Other chemicals may induce S-phase in the absence of hepatotoxicity. It is not apparent how cell proliferation may act in the carcinogenic process but there are numerous mechanisms which can be affected during replication (5-8). Chemically induced cell proliferation may increase the probability of spontaneous mutations as well as increase the probability of converting unrepaired DNA adducts into mutations. Unscheduled cell proliferation may also play a role in the expansion of preneoplastic populations leading to the emergence of a fully transformed clone of cells. Some of these examples act by a non-genotoxic mechanism and it is theoretically possible to detect nongenotoxic carcinogens as well as genotoxic carcinogens using this technique.

IX. MATERIALS:

A. Indicator Cells

Young adult male rats of the Sprague-Dawley strain, 10-12 weeks old at the time of dosing, were purchased from Charles River Laboratories, Raleigh, NC (Crl:CD®BR). This healthy random bred strain was selected to maximize genetic heterogeneity and assure access to a common source. Animals scheduled for this study were housed according to standard operating procedures and were fed Purina Certified® Rodent Chow (Formula 5002) and water ad libitum. Animals were quarantined a minimum of 7 days prior to random assignment to study groups and identification by ear tag for the dose rangefinding assay and by implantable microidentification device for the cell proliferation assay.

The cell proliferation assay was initiated with rats that ranged from 289 to 355 grams. Within 2 hours after dosing, the animals were anesthetized using Metofane® (methoxyflurane, Pitman-Moore, Inc.) inhalation anesthesia and one ALZET® pump per animal was aseptically inserted subcutaneously (dorsal surface). Seventy-two hours later, animals were anesthetized with $CO_2$ prior to removal of the livers and duodenum.

B. Osmotic Pumps and Label for Cell Proliferation Analysis

ALZET® osmotic pumps (ALZA Corporation, Palo Also, CA), Model 2ML1 were used. A single lot (#042301) was used throughout the study. The pump has a 2000 µl capacity with a pump rate of 10 µl/hour. The pumps were pre-filled with BrdU at a concentration of 20 mg/ml.

C. Control Articles

1. Vehicle control

A vehicle control consisting five rats was dosed by oral gavage (P.O.) with the vehicle, 0.5% high viscosity carboxymethylcellulose (CMC) (Sigma, Lot 121F-0544; CAS # 9004-32-4). Tissues from vehicle control animals were subjected to the same manipulations used for the tissues derived from treated animals. The dosing volume of the vehicle control animals did not exceed 10 ml/kg.

2. Positive control article

The positive control compound is known to induce S-phase in rat hepatocytes in vivo. The positive control, dimethylnitrosamine (DMN, CAS# 62-75-9, Sigma Chemical Co., Lot# 82H0365) was dosed at 15.0 mg/kg. Five rats were treated P.O..

D. Test Article

For the preparation of the dosing solutions of the test article, the test article was suspended in CMC at concentrations of 50, 100, 200 and 300 mg/ml prior to dosing. The maximum dosing volumes for the test article did not exceed 10 ml/kg.

X. EXPERIMENT DESIGN:

A. Dosing Procedure

Five rats per condition were treated by oral gavage with T-5878 for the cell proliferation assay. Delivery volumes were calculated on the basis of the most recent animal weight and the target dose. The maximum volume of the test article suspensions administered did not exceed 10 ml/kg. Fresh preparations of test article in vehicle were used for any testing purpose. Confirmation of the concentration of the test material under conditions of preparation and dosing of the assay was not determined in conjunction with this study.

B. Implantation of Osmotic Pumps

For the cell proliferation assay, ALZET® Model 2ML1 osmotic pumps (Lot #042301) were preloaded with 2000 µl of BrdU at a concentration of 20 mg/ml. The animals were anesthetized using Metofane® and one pump per animal was aseptically inserted subcutaneously (dorsal surface) approximately 2 hours after dosing. The incision was closed with wound clips and the animals monitored until the time of sacrifice to ensure that there were no clinical signs of infection. The osmotic pumps were implanted three days prior to sacrifice.

C. Tissue Collection and Preparation

Each animal was anesthetized prior to removal of organs for analysis. The thoracic cavity was opened and the liver removed and fixed in neutral buffered formalin. A cross section of duodenum, a tissue with high cell turnover, was also removed from each animal and fixed. The duodenum was included as an indicator that label was administered correctly to each animal. For the livers from high dose (Group 5) animals, 5 µ paraffin embedded sections were taken from the left lateral, median and right anterior lobes. Once it was determined that no treatment-related lobular differences were present, slides from the left lateral lobe were prepared from each animal. Sections of the duodenum were also made and a section of the duodenum was mounted on each slide containing a liver section. Slides were also prepared according to standard procedures for examination by a pathologist to determine if any abnormalities were present.

D. Immunohistochemical Staining

The slides were deparaffinized and rehydrated prior to staining. The slides were stained for determination of cell proliferation as measured by incorporation of BrdU into DNA using Biogenix antibodies with peroxidase-conjugated streptavidin and a 3,3-diaminobenzidine tetrahydrochloride (DAB) chromogen and hematoxylin counterstain.

E. Assessment of Cell Proliferation

The section of the duodenum was microscopically examined to ensure that the label was properly administered to the animal. Once label delivery was confirmed, slides from the different lobes of the high dose animals were examined for lobular differences. Labeling was similar among the lobes therefore cell counting was performed with sections from the left lateral lobe from all animals. The percentage of nuclei incorporating label in the liver was determined microscopically. The areas to be counted were randomly generated by computer. A 1.0 mm square indexed

ocular grid divided into 10 x 10 squares was used to define the counting area. At least 2000 nuclei were examined per animal with a minimum of 3 sections and 6 fields per section.

Any nuclei that were blue were considered unlabeled and any nuclei containing any brown chromogenic hue were considered labeled unless a clear artifact was present. Only hepatocyte nuclei were enumerated. Fields that contained areas of necrosis were not included in the evaluation. The slides were coded for (blind) evaluation as to treatment group.

S-phase nuclei labeling indices for each animal were calculated as follows:

$$\text{Labeled S-phase nuclei (LI)} = \frac{\text{no. of labeled hepatocyte nuclei}}{\text{total no. of hepatocytes counted}} \times 100$$

## XI. ASSAY EVALUATION CRITERIA

The proportions of the number of cells labeled to the number of cells counted were analyzed by repeated measures analysis of variance (ANOVA) techniques to determine any slide, and related interaction effects. The sphericity test was also utilized to test variance homogeneity. Additionally.the average value from the three slides of each animal was calculated to conduct one-way ANOVA, Dunnett's t-test, Terpsa-Jonkheere test, and regression tests for trend using both untransformed and ranked data. See Appendix C for statistical analysis of labeling indices.

For the terminal whole body weights, liver weights, and liver to terminal body weight ratios, a mean and standard deviation were calculated for each treatment group using the individual animal mean S-phase values. Statistical analysis of labeling index was performed using one-way analysis of variance techniques. Control versus treatment group comparisons were done with Dunnet's t-test and control versus positive group comparisons were done using the Student's t-test. In the case of variance heterogeneity, rank transformations of the data were performed prior to analysis of variance and Dunnet's t-test.

A labeling index, terminal body weight, terminal liver weight and liver to body weight ratio in a dose group that deviates from the values in the concurrent control group at a significance level of $p \leq 0.05$ was considered significantly different than the control group.

## XII. INTERPRETATION OF RESULTS

### A. General Observations

All animals survived treatment. No treatment-related histomorphological alterations were observed at 500, 1000 and 2000 mg/kg but increased mitoses were observed at 3000 mg/kg. Treatment-related changes were also observed in the dimethylnitrosamine (DMN) positive control animals. Details of the histopathology are in Appendix A.

Cells stained with the brown DAB chromogen were observed in the duodenum from all of the animals used in the study. The presence of label in all the animals indicated proper delivery of the BrdU label and acceptable immunohistochemical staining. There was no apparent preferential labeling in any of the lobes and the label was random within the lobes.

Two of the dose groups (Groups 3 and 5) had mean liver weights that were significantly elevated ($p \leq 0.01$) above the Group 1 control liver weights but the increases were not dose-related. The mean liver weight of the positive control was not significantly elevated even though large increases in DNA synthesis (and subsequent cell proliferation) were induced. The 500 mg/kg (Group 2) animals also had a mean terminal body weight that was less than the Group 1 control value ($0.01 \leq p \leq 0.05$). No dose-related trend in body weights or liver weights was observed. However, when the liver to body weight ratios were determined, there were significant increases in the liver to terminal body weight ratios with $p \leq 0.01$ Groups 3 through 5.

### B. Summary of Labeled Cell Counts for the Liver

A summary of the labeled cell counts for each group is shown in Table 1. Individual animal counts are shown in Appendix A. The mean labeling index (LI) for each group is presented in the third column in Table 1.

The mean background labeling index (Group 1) was 1.46 which indicates that less than 2% of the nuclei had undergone DNA synthesis during the 72-hour labeling period. Significant increases in the labeling index were induced by T-5878 in dosed Groups 4 and 5 (2000 mg/kg and 3000 mg/kg; $0.01 \leq p \leq 0.05$). The labeling indices at 400 and 800 mg/kg were 6.92% and 7.83% respectively which represent 4.7- to 5.4-fold increases over background. Large increases were observed at 500 mg/kg and 1000 mg/kg, but the heterogeneous nature of the response resulted in a lack of significance. There was also indication of a

significant positive trend due to treatment. See Appendix D for statistical analysis. The mean labeling index of the DMN positive control animals was 34.96 which is significantly elevated ($p \leq 0.01$).

These results demonstrate that T-5878 induced significant dose-related increases in the LI in the liver in male rats after a single oral dose at concentrations of 2000 mg/kg and 3000 mg/kg. Large increases in the labeling index were also observed in the DMN positive control animals. The mean labeling index in the DMN-treated positive control animals was 34.96 ($p \leq 0.01$).

## XIII. CONCLUSIONS

The test material, T-5878, induced significant changes in the number S-phase cells following a single oral dose of 2000 mg/kg and 3000 mg/kg. The animals were labeled for 72 hours and a significant dose-related trend in the mean labeling index was observed in the treated groups. T-5878 was therefore evaluated as active in the induction of DNA synthesis in rat liver cells.

## XIV. REFERENCES

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## XV. EXPERIMENTAL DATA TABLE

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Table 1

Cell Proliferation Summary

Client: 3M Corporation HWA Assay No.: 154-209

Client Code: T-5878 Trial Initiation Date: February 7, 1994

| Group/Sex[a] | Dose Level (mg/kg) | Labeling Index[b] (%) | Liver Weight (grams) | Terminal Body Weight (grams) | Liver/Body Weight (%) |
|---|---|---|---|---|---|
| 1M | 0[c] | 1.46 ± 1.23 | 12.92 ± 1.06 | 333.5 ± 14.3 | 3.87 ± 0.20 |
| 2M | 500 | 6.91 ± 7.04 | 14.56 ± 0.75 | 291.2 ± 21.0**↓ | 5.01 ± 0.25 |
| 3M | 1000 | 6.73 ± 5.11 | 16.09 ± 0.58**↑ | 307.8 ± 11.3 | 5.23 ± 0.09*↑ |
| 4M | 2000 | 6.92 ± 3.49*↑ | 14.15 ± 1.15 | 309.6 ± 16.8 | 4.57 ± 0.32**↑ |
| 5M | 3000 | 7.83 ± 5.04*↑ | 15.80 ± 1.96**↑ | 354.3 ± 22.5 | 4.44 ± 0.29**↑ |
| 6M[d] | 15[d] | 34.96 ± 14.86 | 13.13 ± 0.73 | 318.4 ± 7.7 | 4.12 ± 0.19 |

[a]Five animals per group
[b]Percentage of labeled hepatocyte nuclei per total number of hepatocytes counted (at least 2000)
[c]Vehicle control, Carboxymethylcellulose
[d]Positive control, 15 mg/kg of DMN

* Significant at $0.01 \leq p \leq 0.05$
** Significant at $p \leq 0.01$

↑ Increase in the mean
↓ Decrease in the mean

APPENDIX A Individual Animal and Slide Labeling Indices

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| Slide # | Animal ID | Group | # Labeled | # Counted | % Labeled |
|---|---|---|---|---|---|
| 16 | 47773 | 1M | 34 | 700 | 4.86 |
| 17 | 47773 | 1M | 10 | 700 | 1.43 |
| 18 | 47773 | 1M | 9 | 700 | 1.29 |
| | | | | Mean | 2.52 |
| | | | | SD | 2.02 |
| 19 | 47774 | 1M | 5 | 700 | 0.71 |
| 20 | 47774 | 1M | 7 | 700 | 1.00 |
| 21 | 47774 | 1M | 4 | 700 | 0.57 |
| | | | | Mean | 0.76 |
| | | | | SD | 0.22 |
| 22 | 47775 | 1M | 2 | 700 | 0.29 |
| 23 | 47775 | 1M | 5 | 700 | 0.71 |
| 24 | 47775 | 1M | 2 | 700 | 0.29 |
| | | | | Mean | 0.43 |
| | | | | SD | 0.25 |
| 25 | 47776 | 1M | 11 | 700 | 1.57 |
| 26 | 47776 | 1M | 3 | 700 | 0.43 |
| 27 | 47776 | 1M | 8 | 700 | 1.14 |
| | | | | Mean | 1.05 |
| | | | | SD | 0.58 |
| 28 | 47777 | 1M | 16 | 700 | 2.29 |
| 29 | 47777 | 1M | 18 | 700 | 2.57 |
| 30 | 47777 | 1M | 19 | 700 | 2.71 |
| | | | | Mean | 2.52 |
| | | | | SD | 0.22 |
| | | | GROUP | MEAN | 1.46 |
| | | | GROUP | SD | 1.23 |

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| Slide # | Animal ID | Group | # Labeled | # Counted | % Labeled |
|---|---|---|---|---|---|
| 46 | 47778 | 2M | 124 | 700 | 17.71 |
| 47 | 47778 | 2M | 159 | 700 | 22.71 |
| 48 | 47778 | 2M | 140 | 700 | 20.00 |
| | | | | Mean | 20.14 |
| | | | | SD | 2.50 |
| 49 | 47779 | 2M | 47 | 700 | 6.71 |
| 50 | 47779 | 2M | 20 | 700 | 2.86 |
| 51 | 47779 | 2M | 39 | 700 | 5.57 |
| | | | | Mean | 5.05 |
| | | | | SD | 1.98 |
| 52 | 47780 | 2M | 21 | 700 | 3.00 |
| 53 | 47780 | 2M | 35 | 700 | 5.00 |
| 54 | 47780 | 2M | 13 | 700 | 1.86 |
| | | | | Mean | 3.29 |
| | | | | SD | 1.59 |
| 55 | 47781 | 2M | 19 | 700 | 2.71 |
| 56 | 47781 | 2M | 18 | 700 | 2.57 |
| 57 | 47781 | 2M | 23 | 700 | 3.29 |
| | | | | Mean | 2.86 |
| | | | | SD | 0.38 |
| 58 | 47782 | 2M | 32 | 700 | 4.57 |
| 59 | 47782 | 2M | 22 | 700 | 3.14 |
| 60 | 47782 | 2M | 14 | 700 | 2.00 |
| | | | | Mean | 3.24 |
| | | | | SD | 1.29 |
| | | | GROUP | MEAN | 6.91 |
| | | | GROUP | SD | 7.04 |

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| Slide # | Animal ID | Group | # Labeled | # Counted | % Labeled |
|---|---|---|---|---|---|
| 61 | 47783 | 3M | 10 | 700 | 1.43 |
| 62 | 47783 | 3M | 12 | 700 | 1.71 |
| 63 | 47783 | 3M | 22 | 700 | 3.14 |
| | | | | Mean | 2.10 |
| | | | | SD | 0.92 |
| 64 | 47784 | 3M | 99 | 700 | 14.14 |
| 65 | 47784 | 3M | 98 | 700 | 14.00 |
| 66 | 47784 | 3M | 125 | 700 | 17.86 |
| | | | | Mean | 15.33 |
| | | | | SD | 2.19 |
| 67 | 47785 | 3M | 45 | 700 | 6.43 |
| 68 | 47785 | 3M | 38 | 700 | 5.43 |
| 69 | 47785 | 3M | 38 | 700 | 5.43 |
| | | | | Mean | 5.76 |
| | | | | SD | 0.58 |
| 70 | 47786 | 3M | 58 | 700 | 8.29 |
| 71 | 47786 | 3M | 47 | 700 | 6.71 |
| 72 | 47786 | 3M | 63 | 700 | 9.00 |
| | | | | Mean | 8.00 |
| | | | | SD | 1.17 |
| 73 | 47787 | 3M | 26 | 700 | 3.71 |
| 74 | 47787 | 3M | 12 | 700 | 1.71 |
| 75 | 47787 | 3M | 14 | 700 | 2.00 |
| | | | | Mean | 2.48 |
| | | | | SD | 1.08 |
| | | | GROUP | MEAN | 6.73 |
| | | | GROUP | SD | 5.11 |

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| Slide # | Animal ID | Group | # Labeled | # Counted | % Labeled |
|---|---|---|---|---|---|
| 31 | 47788 | 4M | 71 | 700 | 10.14 |
| 32 | 47788 | 4M | 73 | 700 | 10.43 |
| 33 | 47788 | 4M | 55 | 700 | 7.86 |
| | | | | Mean | 9.48 |
| | | | | SD | 1.41 |
| 34 | 47789 | 4M | 23 | 700 | 3.29 |
| 35 | 47789 | 4M | 38 | 700 | 5.43 |
| 36 | 47789 | 4M | 49 | 700 | 7.00 |
| | | | | Mean | 5.24 |
| | | | | SD | 1.86 |
| 37 | 47790 | 4M | 61 | 700 | 8.71 |
| 38 | 47790 | 4M | 89 | 700 | 12.71 |
| 39 | 47790 | 4M | 95 | 700 | 13.57 |
| | | | | Mean | 11.67 |
| | | | | SD | 2.59 |
| 40 | 47791 | 4M | 21 | 700 | 3.00 |
| 41 | 47791 | 4M | 33 | 700 | 4.71 |
| 42 | 47791 | 4M | 23 | 700 | 3.29 |
| | | | | Mean | 3.67 |
| | | | | SD | 0.92 |
| 43 | 47792 | 4M | 28 | 700 | 4.00 |
| 44 | 47792 | 4M | 31 | 700 | 4.43 |
| 45 | 47792 | 4M | 37 | 700 | 5.29 |
| | | | | Mean | 4.57 |
| | | | | SD | 0.65 |
| | | | GROUP | MEAN | 6.92 |
| | | | GROUP | SD | 3.49 |

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| Slide # | Animal ID | Group | # Labeled | # Counted | % Labeled |
|---|---|---|---|---|---|
| 1 | 47793 | 5M | 84 | 715 | 11.75 |
| 2 | 47793 | 5M | 66 | 700 | 9.43 |
| 3 | 47793 | 5M | 71 | 700 | 10.14 |
| | | | | Mean | 10.44 |
| | | | | SD | 1.19 |
| 4 | 47794 | 5M | 24 | 700 | 3.43 |
| 5 | 47794 | 5M | 32 | 700 | 4.57 |
| 6 | 47794 | 5M | 17 | 700 | 2.43 |
| | | | | Mean | 3.48 |
| | | | | SD | 1.07 |
| 7 | 47795 | 5M | 66 | 700 | 9.43 |
| 8 | 47795 | 5M | 60 | 700 | 8.57 |
| 9 | 47795 | 5M | 67 | 700 | 9.57 |
| | | | | Mean | 9.19 |
| | | | | SD | 0.54 |
| 10 | 47796 | 5M | 109 | 700 | 15.57 |
| 11 | 47796 | 5M | 112 | 700 | 16.00 |
| 12 | 47796 | 5M | 85 | 700 | 12.14 |
| | | | | Mean | 14.57 |
| | | | | SD | 2.11 |
| 13 | 47797 | 5M | 8 | 700 | 1.14 |
| 14 | 47797 | 5M | 10 | 700 | 1.43 |
| 15 | 47797 | 5M | 13 | 700 | 1.86 |
| | | | | Mean | 1.48 |
| | | | | SD | 0.36 |
| | | | GROUP | MEAN | 7.83 |
| | | | GROUP | SD | 5.04 |

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| Slide # | Animal ID | Group | # Labeled | # Counted | % Labeled |
|---|---|---|---|---|---|
| 76 | 47758 | 6M | 260 | 700 | 37.14 |
| 77 | 47758 | 6M | 364 | 700 | 52.00 |
| 78 | 47758 | 6M | 435 | 700 | 62.14 |
| | | | | Mean | 50.43 |
| | | | | SD | 12.57 |
| 79 | 47759 | 6M | 265 | 700 | 37.86 |
| 80 | 47759 | 6M | 251 | 700 | 35.86 |
| 81 | 47759 | 6M | 171 | 700 | 24.43 |
| | | | | Mean | 32.71 |
| | | | | SD | 7.24 |
| 82 | 47760 | 6M | 155 | 700 | 22.14 |
| 83 | 47760 | 6M | 246 | 700 | 35.14 |
| 84 | 47760 | 6M | 369 | 700 | 52.71 |
| | | | | Mean | 36.67 |
| | | | | SD | 15.34 |
| 85 | 47761 | 6M | 270 | 700 | 38.57 |
| 86 | 47761 | 6M | 292 | 700 | 41.71 |
| 87 | 47761 | 6M | 285 | 700 | 40.71 |
| | | | | Mean | 40.33 |
| | | | | SD | 1.61 |
| 88 | 47762 | 6M | 54 | 700 | 7.71 |
| 89 | 47762 | 6M | 165 | 700 | 23.57 |
| 90 | 47762 | 6M | 89 | 700 | 12.71 |
| | | | | Mean | 14.67 |
| | | | | SD | 8.11 |
| | | | GROUP | MEAN | 34.96 |
| | | | GROUP | SD | 14.86 |

APPENDIX B Individual Animal Body and Liver Weights and Liver to Body Weight Ratios

HAZLETON
WASHINGTON

ANALYSIS OF CELL PROLIFERATION IN RAT LIVER CELLS

*DRAFT* ABSOLUTE ORGAN WEIGHTS (g) *DRAFT*

STUDY NUMBER: 154209

ORGAN ABBREVIATION: LI - LIVER

| SEX | DOSE GROUP | ANIMAL NUMBER | TERMINAL BODY WEIGHT (g) | LI |
|---|---|---|---|---|
| M | 1 | B47773 | 327.7 | 13.25 |
| M | 1 | B47774 | 341.0 | 13.77 |
| M | 1 | B47775 | 313.0 | 11.32 |
| M | 1 | B47776 | 334.9 | 12.40 |
| M | 1 | B47777 | 351.0 | 13.84 |
| NUMBER IN GROUP: | | | 5 | 5 |
| MEAN: | | | 333.5 | 12.92 |
| STANDARD DEV: | | | 14.3 | 1.06 |
| M | 2 | B47778 | 299.0 | 15.04 |
| M | 2 | B47779 | 316.0 | 15.37 |
| M | 2 | B47780 | 273.0 | 14.68 |
| M | 2 | B47781 | 302.0 | 14.25 |
| M | 2 | B47782 | 266.0 | 13.45 |
| NUMBER IN GROUP: | | | 5 | 5 |
| MEAN: | | | 291.2 | 14.56 |
| STANDARD DEV: | | | 21.0 | 0.75 |
| M | 3 | B47783 | 294.8 | 15.31 |
| M | 3 | B47784 | 312.0 | 16.02 |
| M | 3 | B47785 | 324.0 | 16.94 |
| M | 3 | B47786 | 308.0 | 16.06 |
| M | 3 | B47787 | 300.0 | 16.14 |
| NUMBER IN GROUP: | | | 5 | 5 |
| MEAN: | | | 307.8 | 16.09 |
| STANDARD DEV: | | | 11.3 | 0.58 |



005162

HAZLETON
WASHINGTON

ANALYSIS OF CELL PROLIFERATION IN RAT LIVER CELLS

*DRAFT* ABSOLUTE ORGAN WEIGHTS (g) *DRAFT*

STUDY NUMBER: 154209

ORGAN ABBREVIATION: LI - LIVER

| SEX | DOSE GROUP | ANIMAL NUMBER | TERMINAL BODY WT (g) | LI |
|---|---|---|---|---|
| M | 4 | B47788 | 285.0 | 12.44 |
| M | 4 | B47789 | 322.0 | 14.56 |
| M | 4 | B47790 | 299.0 | 14.98 |
| M | 4 | B47791 | 322.0 | 13.58 |
| M | 4 | B47792 | 320.0 | 15.22 |
| NUMBER IN GROUP: | | | 5 | 5 |
| MEAN: | | | 309.6 | 14.15 |
| STANDARD DEV: | | | 16.8 | 1.15 |
| M | 5 | B47793 | 350.8 | 14.83 |
| M | 5 | B47794 | 379.0 | 17.67 |
| M | 5 | B47795 | 350.0 | 15.60 |
| M | 5 | B47796 | 370.9 | 17.76 |
| M | 5 | B47797 | 321.0 | 13.13 |
| NUMBER IN GROUP: | | | 5 | 5 |
| MEAN: | | | 354.3 | 15.80 |
| STANDARD DEV: | | | 22.5 | 1.96 |
| M | 6 | B47798 | 322.9 | 13.60 |
| M | 6 | B47799 | 317.0 | 12.39 |
| M | 6 | B47800 | 306.0 | 12.91 |
| M | 6 | B47801 | 320.0 | 12.58 |
| M | 6 | B47802 | 326.0 | 14.14 |
| NUMBER IN GROUP: | | | 5 | 5 |
| MEAN: | | | 318.4 | 13.13 |
| STANDARD DEV: | | | 7.7 | 0.73 |



005163

HAZLETON
WASHINGTON

ANALYSIS OF CELL PROLIFERATION IN RAT LIVER CELLS

*DRAFT* ORGAN-TO-TERMINAL BODY WEIGHT RATIOS (%) *DRAFT*

STUDY NUMBER: 154209

ORGAN ABBREVIATION: LI - LIVER

| SEX | DOSE GROUP | ANIMAL NUMBER | TERMINAL BODY WT (g) | LI |
|---|---|---|---|---|
| M | 1 | B47773 | 327.7 | 4.044 |
| M | 1 | B47774 | 341.0 | 4.039 |
| M | 1 | B47775 | 313.0 | 3.617 |
| M | 1 | B47776 | 334.9 | 3.701 |
| M | 1 | B47777 | 351.0 | 3.944 |
| NUMBER IN GROUP: | | | 5 | 5 |
| | | MEAN: | 333.5 | 3.869 |
| | | STANDARD DEV: | 14.3 | 0.198 |
| M | 2 | B47778 | 299.0 | 5.031 |
| M | 2 | B47779 | 316.0 | 4.865 |
| M | 2 | B47780 | 273.0 | 5.377 |
| M | 2 | B47781 | 302.0 | 4.717 |
| M | 2 | B47782 | 266.0 | 5.055 |
| NUMBER IN GROUP: | | | 5 | 5 |
| | | MEAN: | 291.2 | 5.009 |
| | | STANDARD DEV: | 21.0 | 0.247 |
| M | 3 | B47783 | 294.8 | 5.192 |
| M | 3 | B47784 | 312.0 | 5.136 |
| M | 3 | B47785 | 324.0 | 5.229 |
| M | 3 | B47786 | 308.0 | 5.214 |
| M | 3 | B47787 | 300.0 | 5.379 |
| NUMBER IN GROUP: | | | 5 | 5 |
| | | MEAN: | 307.8 | 5.230 |
| | | STANDARD DEV: | 11.3 | 0.091 |

HAZLETON
WASHINGTON

ANALYSIS OF CELL PROLIFERATION IN RAT LIVER CELLS

*DRAFT* ORGAN-TO-TERMINAL BODY WEIGHT RATIOS (%) *DRAFT*

STUDY NUMBER: 154209

ORGAN ABBREVIATION: LI - LIVER

| SEX | DOSE GROUP | ANIMAL NUMBER | TERMINAL BODY WT (g) | LI |
|---|---|---|---|---|
| M | 4 | B47788 | 285.0 | 4.363 |
| M | 4 | B47789 | 322.0 | 4.522 |
| M | 4 | B47790 | 299.0 | 5.009 |
| M | 4 | B47791 | 322.0 | 4.216 |
| M | 4 | B47792 | 320.0 | 4.757 |
| NUMBER IN GROUP: | | | 5 | 5 |
| | MEAN: | | 309.6 | 4.574 |
| | STANDARD DEV: | | 16.8 | 0.315 |
| M | 5 | B47793 | 350.8 | 4.226 |
| M | 5 | B47794 | 379.0 | 4.662 |
| M | 5 | B47795 | 350.0 | 4.456 |
| M | 5 | B47796 | 370.9 | 4.787 |
| M | 5 | B47797 | 321.0 | 4.090 |
| NUMBER IN GROUP: | | | 5 | 5 |
| | MEAN: | | 354.3 | 4.444 |
| | STANDARD DEV: | | 22.5 | 0.291 |
| M | 6 | B47798 | 322.9 | 4.212 |
| M | 6 | B47799 | 317.0 | 3.910 |
| M | 6 | B47800 | 306.0 | 4.219 |
| M | 6 | B47801 | 320.0 | 3.931 |
| M | 6 | B47802 | 326.0 | 4.339 |
| NUMBER IN GROUP: | | | 5 | 5 |
| | MEAN: | | 318.4 | 4.122 |
| | STANDARD DEV: | | 7.7 | 0.191 |

005165

APPENDIX C Histopathology Report

HAZLETON
WASHINGTON

Pathology Report
Analysis of Cell Proliferation in Rat Liver Cells
Project No. 154-209

General Protocol

Thirty, young adult, male Sprague-Dawley rats were placed in six groups of five rats/group. Group 1 served as the vehicle control. Groups 2, 3, 4, and 5 served as the low-, low-mid-, high-mid-, and high-dose groups, respectively, receiving 500, 1000, 2000, and 3000 mg/kg of the test material, T-5878, via oral gavage. Group 6 served as the positive control, receiving 15 mg/kg of dimethylnitrosamine (DMN) via oral gavage. After dosing, an ALZET® Model 2ML1 osmotic pump containing 20 mg/mL of bromodeoxyuridine (BrdU) was implanted subcutaneously in each rat while it was under Metofane® anesthesia.
Seventy-two hours after pump implantation, all rats were anesthetized, exsanguinated, and necropsied. Liver, duodenum, and all gross lesions from each rat were placed in 10% neutral-buffered formalin and processed as per HWA SOPs. These tissues from all rats were evaluated microscopically by a board-certified veterinary pathologist.

Histopathology

Treatment-related change in the liver consisted of minimally to slightly increased mitoses in Group 5 (3000 mg/kg) rats. Group 6 (DMN treated) rats had varying severities of centrilobular necrosis, hepatocellular hypertrophy, chronic inflammation, peliosis, and increased mitoses.
Various spontaneous disease lesions and incidental findings, including chronic inflammation of the spleen capsule in a Group 3 rat and renal pelvis dilatation in three rats, are unrelated to treatment. The chronic active inflammation with acanthosis of the nonglandular stomach of a Group 2 rat is probably an effect of gavage trauma.

Summary

The test material, T-5878, when administered to male Sprague-Dawley rats in single oral gavage doses of 500, 1000, 2000, and 3000 mg/kg, produced increased mitoses in the liver of rats dosed at 3000 mg/kg. No treatment-related histomorphologic changes were noted in the liver of rats dosed at 500, 1000, and 2000 mg/kg.

Pathologist:

Samuel V. Machotka, D.V.M., D.A.B.T.,
Diplomate, American College of Veterinary Pathologists
Department of Pathology

Date

APPENDIX D Statistical Analysis of Labeling Indices

**Methods:**

The proportions of the number of cells labeled to the number of cells counted were analyzed by repeated measures analysis of variance (ANOVA) techniques to determine any significant dose, slide, and related interaction effects. The sphericity test was also utilized to test variance homogeneity. The model used was:

$$\text{proportion} = \mu + \text{dose} + \text{slide} + \text{slide}\times\text{dose} + \varepsilon.$$

Additionally, since the study did not show any significant between-slide variation, the average value from the 3 slides of each animal was then calculated to conduct one-way ANOVA, Dunnett's t-tests, Terpstra-Jonckheere test [1], and regression tests for trend using both untransformed and ranked data.

**Results:**

Since the sphericity test rejected variance homogeneity in comparing vehicle vs positive control ($p = .0227$), the Greenhouse-Geisser probabilities were used for this significance evaluation. There was no significant heterogeneity in comparing vehicle vs treated groups ($p = .7922$). As Text Table 1 indicates, there is no significant finding in comparing vehicle with treated groups. Within- and between-group slide-to-slide variations were not significant in either case. Only the positive control showed highly significant elevation in labelling over vehicle control.

The data based on the average values showed homogeneous variance ($p = .1449$) in comparing vehicle vs treated groups for the untransformed data. However, closer examination of the individual values in each group along with the means and standard deviations (Text Table 2) indicates that the analyses based on the rank-transformed data may be necessary. As Text Tables 2 and 3 indicate, there is significant difference between vehicle and treated groups in transformed data ($p = .0314$). Dose 2000 and 3000 showed significant increase in labelling over control. Furthermore, there was a significant positive trend as indicated in Text Table 4 using Terpstra-Jonckheere test ($p = .0049$) and regression of rank-transformed data ($p = .0090$) even though the regression based on the untransformed data did not show any significance. There was no significant lack of fit for both regressions ($p = 0.252$ and $0.473$). The following notations are used to denote direction and statistical significance:

* = significant at $p \leq 0.05$
** = significant at $p \leq 0.01$
↑ = effect in the positive direction

Text Table 1 - Univariate ANOVA

| Vehicle vs Positive Control | Vehicle vs Treated Groups |
|---|---|
| Dose p 0.0005 | **0.3029 |
| Slide p 0.2792 | 0.9965 |
| Slide×Dose p | 0.2053 | 0.2266 |

Text Table 2 - The Descriptive Statistics

| Dose(mg/kg) | Untransformed Mean | Untransformed SD | Median | Rank-Transformed Mean | Rank-Transformed SD |
|---|---|---|---|---|---|
| **Vehicle vs Positive Control** | | | | | |
| Vehicle | 1.4560 | 0.9958 | 1.0500 | 3.0000 | 1.5411 |
| Positive | 34.9620 | 13.1132 | 36.6700 | 8.0000 | 1.5811 |
| **Vehicle vs Treated Groups** | | | | | |
| Vehicle | 1.4560 | 0.9958 | 1.0500 | 4.2000 | 3.0944 |
| 500 | 6.9160 | 7.4409 | 3.2900 | 14.0000 | 6.5574 |
| 1000 | 6.7340 | 5.3855 | 5.7600 | 14.0000 | 8.2158 |
| 2000 | 6.9260 | 3.4649 | 5.2400 | 17.0000 | 3.8730 |
| 3000 | 7.8320 | 5.3229 | 9.1900 | 15.8000 | 7.7910 |

Text Table 3 - One-Way ANOVA and Dunnett's T-Test

| | Vehicle vs Positive Control | | Vehicle vs Treated Groups | |
|---|---|---|---|---|
| | Untransformed | Transformed | Untransformed | Transformed |
| Treatment | .0005 ** | .0010 ** | .3022 | .0314 * |
| Vehicle vs 500 | | | | .0817 † |
| Vehicle vs 1000 | | | | .0826 † |
| Vehicle vs 2000 | | | | .0151 *† |
| Vehicle vs 3000 | | | | .0315 *† |

Text Table 4 - Test for Trend for Vehicle vs Treated Groups

| | |
|---|---|
| Terpstra-Jonckheere Test | .0049 **† |
| Regression of Untransformed Data | .1170 † |
| Regression of Rank Transformed Data | .0090 **† |

**Discussion:**

The results of the present study based on the rank-transformed data indicate that there was significant increase in cell proliferation at Dose 2000 and 3000 compared to control due to treatment by the chemical. There was also indication of a significant positive trend due to treatment.

**References:**

[1] Ajit K. Thakur, *A Fortran Program to Perform the Nonparametric Terpstra-Jonckheere Test*, Computer Programs in Biomedicine 18: 235-240, 1984.

[2] SAS (Statistical Analysis System), SAS Institute, Cary, NC, 1991.

HAZLETON
WASHINGTON

HWA Study No. 154-209
Protocol No. 493, Edition 3

# ANALYSIS OF CELL PROLIFERATION IN RAT LIVER CELLS

Hazleton Washington, Inc. (HWA) will conduct this study in compliance with EPA and FDA 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 Hazleton Washington, Inc. This study will be conducted by HWA at 9200 Leesburg Pike, Vienna, Virginia 22182.

## PART 1. SPONSOR INFORMATION AND APPROVALS

### I. SPONSOR IDENTIFICATION

Company Name: 3M Corporation

Address: Building 220-2E-02, 3M Center, St. Paul, MN 55144-1000

### II. TEST ARTICLE IDENTIFICATION: ~~T-5878~~ ~~T-5878~~ T-5878 1/5/94

### III. TEST ARTICLE ANALYSIS

Determination of the test article stability and the test article characteristics as defined in the GLP regulations of FDA (21 CFR 58.105), EPA-TSCA (40 CFR 792.105), and EPA-FIFRA (40 CFR 160.105) is the responsibility of the Sponsor.

### IV. NOTIFICATION OF REGULATORY SUBMISSION

In order to comply with U.S. federal regulation codes (FDA, 21 CFR 58.10; EPA-TSCA, 40 CFR 792.10; EPA-FIFRA, 40 CFR 160.10) and certain foreign agencies, consulting laboratories must be notified if all or part of a study is to be submitted to the agency. HWA 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: _____

Maria A. Cifone, Ph.D.

Sponsor's Authorized Representative:

_____ Date: January 5, 1994

PART 2. STUDY PROTOCOL

ANALYSIS OF CELL PROLIFERATION
IN RAT LIVER CELLS

## I. OBJECTIVE

The objective of this assay is to detect hepatotoxicity caused by the test material by measuring cell proliferation (CP) measured as S-phase induction induced in rat liver cells after in vivo treatment.

Cell proliferation is designed to measure the fraction of cells undergoing cell replication in rat liver using an immunohistochemical technique (1,2). Animals are given a single oral dose of the chemical and the livers are isolated following administration of bromodeoxyuridine (BrdU) for 72 hours in vivo with an ALZET® osmotic pump implanted subcutaneously. Quantification of cells that have incorporated DNA precursors over the 72-hour period has been shown to be useful for the evaluation of chemicals that may cause increased cell proliferation in the liver (3).

## II. DEFINITION

Hepatotoxicants such as carbon tetrachloride and dinitrotoluene induce an increase in cell proliferation to replace necrotic tissue (2,4). These proliferating cells may be detected during S-phase analysis. Other compounds may induce S-phase synthesis in the absence of hepatotoxicity. It is not apparent how cell proliferation acts in the carcinogenic process, but there are numerous processes that can be affected during replication (5-8). Chemically induced cell proliferation may increase the probability of spontaneous mutations as well as increase the probability of converting DNA adducts into mutations prior to a repair process. Unscheduled cell proliferation may also play a role in the expansion of preneoplastic cells leading to the emergence of a fully transformed clone of cells. Some of these examples act by a nongenotoxic mechanism. It is therefore possible to detect nongenotoxic carcinogens as well as genotoxic carcinogens using this technique.

## III. MATERIALS

### A. Animals

Young adult male rats of the Sprague-Dawley strain, 10-12 weeks old at the time of dosing, will be purchased from Harlan Sprague Dawley, Inc. (HSD:Sprague-Dawley®(SD®)BR) or Charles River Laboratories, Inc. (Crl:CD®BR). This healthy random bred strain has been selected to maximize genetic heterogeneity and at the same time assure access to a common source.

The animals will be housed according to standard operating procedures and will be fed Purina Certified® Rodent Chow (formula 5002) and water ad libitum. They will be quarantined a minimum of seven days prior to use and will be randomly assigned to study groups. The rats to be used for the assay will be anesthetized before surgery.

The animals will be anesthetized using Metofane® (methoxyflurane, Pitman-Moore, Inc.) inhalation anesthesia and one pump per animal will be aseptically inserted subcutaneously (dorsal surface). Seventy-two hours later, animals will be anesthetized with $CO_2$ prior to removal of the livers and duodenum (control organ).

B. Osmotic Pumps and Label for Cell Proliferation Analysis

ALZET® osmotic pumps (ALZA Corporation, Palo Alto, CA), Model 2ML1 will be used. A single lot will be used throughout the study. The ALZET® Model 2ML1 osmotic pump has a 2000 µl capacity with a pump rate of 10 µl/hr. The pumps will be filled with bromodeoxyuridine (BrdU) at a concentration of 20 mg/ml.

C. Control Articles

1. Vehicle control

A vehicle negative control consisting of a minimum of five rats for cell proliferation. They will be treated with the vehicle or solvent selected for the test material. The same dosing methods (usually oral gavage) used for the test material treatments will be employed for the vehicle control. Where possible dosing volumes for oral gavage will not exceed about 10 ml/kg body weight.

2. Positive control article

The positive control articles used are known to induce S-phase in rat hepatocytes in vivo. The positive control for cell proliferation will be 15 mg/kg of DMN. At least five rats will be treated by per os.

D. Test Article

Unless specified by the sponsor, the test article will normally be tested as supplied. Any operations performed on the test article such as grinding, extraction, or solvent-exchange must be specified by the sponsor prior to the initiation of testing. All operations performed on the test article will be described in the final report.

## IV. EXPERIMENTAL DESIGN

### A. Dosing Procedure

A preliminary test will be performed to determine vehicle/solvent selection for the test article unless a vehicle/solvent is specified by the Sponsor. Materials which may be selected include water, methylcellulose, carboxymethylcellulose, corn oil or another suitable vehicle/solvent. Rats will be treated by oral gavage with the test article in volumes that will not exceed about 10 ml/kg body weight. Alternate routes of exposure may be requested by the Sponsor. DMN will be dissolved in sterile deionized water. Fresh preparations of the test article and positive controls in the solvent or vehicle will be used for any testing purpose. Stability of the test material under conditions of preparation and dosing will be the responsibility of the Sponsor.

### B. Dose Selection

Unless specified otherwise, the highest dose selected will usually be 1 g/kg or half the $LD_{50}$, whichever is less. Four doses will be selected using approximately two-fold dilution steps. Five animals from each dose level and control group will be used to analyze cell proliferation at 72-hours.

### C. Implantation of Osmotic Pumps

ALZET® Model 2ML1 osmotic pumps will be preloaded with 2000 µl of BrdU at a concentration of 20 mg/ml. Following dosing with the test material, the animals will be anesthetized using Metofane® (methoxyflurane, Pitman-Moore, Inc.) inhalation anesthesia and one pump per animal will be aseptically inserted subcutaneously (dorsal surface). The incision will be closed with wound clips and the animals monitored until the time of sacrifice to ensure that there are no clinical signs of infection. The osmotic pumps will be kept in the rats for three days prior to sacrifice.

### D. Tissue Collection and Preparation

Each animal will be anesthetized prior to removal of organs for analysis. The thoracic cavity will be opened and the liver removed and fixed in neutral buffered formalin. A cross section of duodenum, a tissue with high cell turnover, will also be removed

from each animal and fixed. The duodenum will be included as an indicator that the label was administered correctly to the animal. For the liver, 3-5 $\mu$ paraffin embedded sections will be taken. In the high dose animals, three slides from the left lateral lobe and one slide from the median and right anterior lobes will be prepared. Three slides from the remaining animals will also be prepared following qualitative analysis of the high dose slides. If labeling is similar among the lobes, all three slides will be prepared from the left lateral lobe. If labeling is different among the lobes, a slide from each lobe will be prepared. 3-5 $\mu$ sections of the duodenum will also be made. The liver sections will be mounted on slides and a sample from the duodenum will be included on each slide. One slide each from the left lateral, median and right anterior lobes of the livers will also be prepared for analysis by a pathologist. Liver sections from all animals will be analyzed for histopathology, including gross lesions.

E. Immunohistochemical Staining

The slides will be deparaffinized and rehydrated prior to staining using 1) Biogenix primary and secondary antibodies with peroxidase-conjugated streptavidin, 3,3-diaminobenzidine tetrahydrochloride (DAB) chromogen and hematoxylin counterstain. Separate slides for histopathology will be stained with hematoxylin and eosin.

F. Assessment of Cell Proliferation Rates

The section of the intestine will be microscopically examined to ensure that the label was properly administered to the animal. If adequate labeling is not observed, slides from the particular animal will not be analyzed. Once label distribution has been confirmed, a sampling of liver slides from the different lobes from the high dose animals will be examined to determine if differences in labeling are observed. If a qualitative difference in labeling among the liver lobes is observed, all the lobes will be counted. If no differences are observed, labeled hepatocytes in the left lateral lobe will be determined. At least 2000 nuclei will be examined per animal with a minimum of 6 fields per section analyzed. Counting will be confined to hepatocyte nuclei but other cell types such as inflammatory cells may be counted (separately) if the data appears relevant. The coverslips will be coded to prevent bias in counting.

V. DATA PRESENTATION

The final report will include the following information in tabular form for each timepoint, for the negative control, positive control, and each analyzed treatment:

- The calculated %S-phase ± standard deviation among the three slides for each animal analyzed for S-phase.

VI. ASSAY EVALUATION CRITERIA

A mean and standard deviation for the percentage of S-phase cells will be calculated for each treatment group using the individual animal mean S-phase values. Statistical analysis of labeling index will be performed using one-way analysis of variance techniques (9). Control versus treatment group comparisons will be done with Dunnet's t-test (10,11). In the case of variance heterogeneity, rank transformation of the data will be performed prior to analysis of variance and Dunnet's t-test. Student's t-test will be used for comparison of the positive control versus the vehicle control. An S-phase percentage in a dose group that deviates from the S-phase percentage in the concurrent control group at a significance level of $p \leq 0.05$ will be considered significantly different than the control group.

VII. REFERENCES

1. DeFazio, A., Leary, J.A., Hedley, D.W. and Tattersall, M.H.N. (1987). Immunohistochemical detection of proliferating cell in vivo. J. Histochem. Cytochem. 35, 571-577.

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3. Butterworth, B.E., Ashby, J., Bermudez, E., Casciano, D., Mirsalis, J., Probst, G., and G. Williams: A protocol and guide for the in vivo rat hepatocyte DNA repair assay. Mutation Research, 189:123-133, 1987.

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5. Marsman, D.S., Cattley, R.C., Conway, J.G. and Popp, J.A. (1988). Relationship of hepatic peroxisome proliferation and replicative DNA synthesis to the hepatocarcinogenicity of the peroxisome proliferators di(2-ethylhexyl)phthalate and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643) in rats. Cancer Res. 48, 6739-6744.

6. Craddock, V.M. (1976). Cell proliferation and experimental liver cancer. In: "Liver Cell Cancer", Cameron,H.M., Linsell,C.A. and Warwick,G.P., Elsevier, North Holland Biomedical Press, Amsterdam.

7. Columbano, A., Rajalaksmi, S. and Sarma, D.S.R. (1981). Requirement of cell proliferation for the initiation of liver carcinogenesis as assayed by three different procedures. Cancer Res. 41, 2079-2083.

8. Glinos, A.D., Butcher, N.L.R. and Aub, J.C. (1951) The effect of liver regeneration on tumor formation in rats fed 4-diaminobenzene. J. Exp. Med. 933, 313-324.

9. Winer, B.J. (1971). Statistical Principles in Experimental Design, McGraw-Hill, New York, 2nd Edition, pp. 149-220.

10. Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Assoc. 50, 1096-1121.

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

## VIII. REPORT FORMAT

The final report will provide the following information.

- Sponsor identification.
- Test material identification and Assay Number. A physical description of the test material and date of receipt will be included in this section.
- Type of assay and protocol number.
- Dates of study initiation and completion.
- Names of Study Director, Senior Technician, Scientist
- Interpretation of results.
- Conclusions.
- Historical control data for negative and positive control cultures.
- Signatures of Study Supervisor and Study Director.
- Test results presented in tabular forms.
- Methods.
- Evaluation criteria.
- References.
- Quality Assurance statement.

IX. 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. The sponsor will be notified of any change or revisions.

X. RECORDS TO BE MAINTAINED

All raw data, documentation, records, protocols, and final reports generated as a result of this study will be archived in the storage facilities of Hazleton 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 Hazleton for an additional period of time or sent to a storage facility designated by the sponsor.

## 3M Internal Correspondence

cc: R. G. Perkins - 220-2E-10

**To:** **B. C. Copley - 53-3S-02**

**From:** **R. D. Howells - 53-3S-02**

**Subject:** **Key to FC Alcohol Tox Samples**

**Date:** **September 14, 1995**

| | | |
|---|---|---|
| **T5877** | Wide Range EtFOSE<br>FM 3924 Lot 547<br>Retain from 2 year feeding study | Analytical Request 41220<br><br>L-13203 |
| **T5710** | Narrow Range EtFOSE<br>Lot 884<br>Typical Raw Material<br>for FC-807 | Analytical Request 41220<br><br>L-10059 |
| **T5711** | Wide Range MeFOSE<br>Lot 555<br>Typical Raw Material<br>for FX-845 | Analytical Request 41220<br><br>L-1276 |
| **T5794** | Narrow Range MeFOSE<br>Notebook 97900-107-2<br>Lab Prepared Sample | Analytical Request 41343<br><br>L-13097 |
| **T5795** | Wide Range EtFOSE<br>Notebook 97900-112-2<br>Lab Prepared Sample | Analytical Request 41343<br><br>L-13098 |
| **T5878** | Wide Range MeFOSE<br>Lab Prepared from<br>Washed POSF | Analytical Request 42607 |

RDH/jld R. D. Howells

Attachments

REQ 41220 R. Howells

GC/MS analyses of these samples were accomplished using a 25 m X 0.32 mm HP-1 GC column to introduce the samples into the Finnigan SSQ-70 mass spectrometer. The sample components were ionized using chemical ionization with methane as the reagent gas. The GC column was operated from 40 to 300 C at a rate of10 degrees per minute. The results of these analyses combined with their corresponding GC/FID area percents show the following:

| Component I.D. | L-10059 N.R. N-EtFOSE Lot | W.R. N-EtFOSE Lot 547 |
|---|---|---|
| N-Ethyl-carboxamides (Rf-C(O)N(Et)H) | 0.22 % | 1.76 % |
| N-EtFOS Amide ( $C_8F_{17}SO_2N(Et)H$) | 0.04 % | 0.13 % |
| $C_2F_5SO_2N(Et)CH_2CH_2OH$ | 0.01 % | 0.12 % |
| $C_3F_7SO_2N(Et)CH_2CH_2OH$ | 0.13 % | 1.17 % |
| $C_4F_9SO_2N(Et)CH_2CH_2OH$ | 0.11 % | 1.52 % |
| $C_8F_{17}SO_2N(Et)_2$ | 0.03 % | 0.04 % |
| $C_5F_{11}SO_2N(Et)CH_2CH_2OH$ | 0.03 % | 1.34 % |
| $C_6F_{13}SO_2N(Et)CH_2CH_2OH$ | 0.51 % | 3.52 % |
| $C_8F_{17}SO_2N(Et)CH_2CH_2Cl$ | 0.08 % | 0.16 % |
| $C_7F_{15}SO_2N(Et)CH_2CH_2OH$ | 0.82 % | 1.40 % |
| N-EtFOSE $C_8F_{17}SO_2N(Et)CH_2CH_2OH$ | 96.09 % | 87.16 % |
| $C_8F_{15}SO_2N(Et)CH_2CH_2OH$ | 0.78 % | 0.50 % |
| $C_8F_{17}SO_2N(Et)(CH_2CH_2O)_2H$ | 0.17 % | ----- |
| $C_8H_{17}SO_2N(Et)H$ | 0.16 % | 0.37 % |
| $C_8F_{17}SO_2N(Et)CH_2CH_2OCO_2CH_2CH_3$ (or similar) | 0.21 % | 0.22 % |
| Other High Boilers | 0.28 % | 0.59 % |

| Component I.D. | L-1276 N-MeFOSE Lot |
|---|---|
| N-Methyl-carboxamides (Rf-C(O)N(Me)H) | 1.46 % |
| $C_2F_5SO_2N(Me)CH_2CH_2OH$ | 0.24 % |
| $C_8F_{17}SO_2N(Me)_2$ | trace |
| $C_3F_7SO_2N(Me)CH_2CH_2OH$ | 1.15 % |
| $C_8F_{17}SO_2N(Me)H$ | trace |
| $C_4F_9SO_2N(Me)CH_2CH_2OH$ | 1.62 % |
| $C_5F_{11}SO_2N(Me)CH_2CH_2OH$ | 1.34 % |
| $C_6F_{13}SO_2N(Me)CH_2CH_2OH$ | 5.05 % |
| $C_8F_{17}SO_2N(Me)CH_2CH_2Cl$ | 0.13 % |
| $C_7F_{15}SO_2N(Me)CH_2CH_2OH$ | 1.69 % |
| N-MeFOSE $C_8F_{17}SO_2N(Me)CH_2CH_2OH$ | 83.88 % |
| $C_9F_{19}SO_2N(Me)CH_2CH_2OH$ | 0.87 % |
| $C_8F_{17}SO_2N(Me)(CH_2CH_2O)_2H$ | 0.34 % |
| $C_8H_{17}SO_2N(Me)H$ | 0.54 % |
| Other High Boilers | 1.55 % |

Further work has been done by GC on these samples which involves derivatization of the alcohols with trifluoroacetic anhydride (TFAA) and again with BSA (to give the trimethylsilyl ethers). This work was designed to investigate the potential problems that could be overlooked by any one method of analysis. Preliminary results show that analyzing the EtFOSE underivatized could hide a significant amount of N-EtFOS Amide under the C-3 alcohol peak in wide range material. However, analyzing the same material that has been derivatized with TFAA shows that any EtFOSE-chloride that is present in the sample is now completely masked by the derivatized C-8 alcohol. The BSA derivative has not been evaluated yet, but similar problems are expected because of the number of different components in the sample. The same sort of problems will most likely exist with MeFOSE and will be even more complicated in the analysis of MeFOSEA.

7/2/93

REQ 41343 J. Grant

GC/MS analyses of these samples were accomplished using a 25 m X 0.32 mm HP-1 GC column to introduce the samples into the Finnigan SSQ-70 mass spectrometer. The sample components were ionized using chemical ionization with methane as the reagent gas. The GC column was operated from 40 to 300 C at a rate of10 degrees per minute. The results of these analyses combined with their corresponding GC/FID area percents show the following:

| Component I.D. | W.R. N-EtFOSE Precut 97900-112-1 | W.R. N-EtFOSE main cut 97900-112-2 |
|---|---|---|
| N-Ethyl-carboxamides (Rf-C(O)N(Et)H) | 68.34 % | 1.27 % |
| $C_6F_{13}SO_2N(Et)H$ | 1.28 % | 0.32 % |
| $C_2F_5SO_2N(Et)CH_2CH_2OH$ | trace | trace |
| $C_7F_{15}SO_2N(Et)H$ | trace | trace |
| N-EtFOS Amide ( $C_8F_{17}SO_2N(Et)H$) | 0.41 % | trace |
| $C_6F_{13}SO_2N(Et)_2$ | 1.41 % | trace |
| $C_3F_7SO_2N(Et)CH_2CH_2OH$ | 1.53 % | 0.51 % |
| $C_4F_9SO_2N(Et)CH_2CH_2OH$ | 0.62 % | 0.42 % |
| $C_5F_{11}SO_2N(Et)CH_2CH_2OH$ | 1.48 % | 2.16 % |
| $C_6F_{13}SO_2N(Et)CH_2CH_2OH$ | 20.16 % | 60.86 % |
| $C_7F_{15}SO_2N(Et)CH_2CH_2OH$ | 3.52 % | 22.55 % |
| N-EtFOSE $C_8F_{17}SO_2N(Et)CH_2CH_2OH$ | 1.14 % | 11.92 % |

| Component I.D. | N.R. N-MeFOSE B.P. 132 97900-107-2 |
|---|---|

| | |
|---|---|
| mw 137 possibly -$SO_2N(Me)CH_2CH_2O$- | 0.58 % |
| $C_7F_{15}SO_2N(Me)CH_2CH_2OH$ | 0.41 % |
| N-MeFOSE $C_8F_{17}SO_2N(Me)CH_2CH_2OH$ | 98.19 % |
| $C_9F_{19}SO_2N(Me)CH_2CH_2OH$ | 0.59 % |
| $C_8F_{17}SO_2N(Me)(CH_2CH_2O)_2H$ | trace |
| $C_8H_{17}SO_2N(Me)H$ | trace |
| Other High Boilers | 0.23 % |

| Component I.D. | W.R. N-EtFOS Amide 97900-111 |
|---|---|
| N-Ethyl-carboxamides ($R_f$-C(O)N(Et)H) | 10.23 % |
| $C_3F_7SO_2N(Et)H$ | trace |
| $C_4F_9SO_2N(Et)H$ | 0.71 % |
| $C_5F_{11}SO_2N(Et)H$ | 3.58 % |
| $C_6F_{13}SO_2N(Et)H$ | 52.50 % |
| $C_7F_{15}SO_2N(Et)H$ | 20.06 % |
| N-EtFOS Amide ( $C_8F_{17}SO_2N(Et)H$) | 12.93 % |
| $C_8F_{15}SO_2N(Et)H$ | trace |
| numerous other impurities of most homologs that include hydrides, chlorine in the backbone, and unidentified high boilers | trace |

To: I. Muggli 53-6S-02

From: R. M. Payfer 236-2B-11 (612)733-4212

Subject: SA&C Analytical Request No. **42607**

Date: Dec. 21, 1993

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GC/MS analysis of this sample was accomplished using a 25 m X 0.32 mm HP-1 GC column to introduce the sample into the Finnigan SSQ-70 mass spectrometer. The sample components were ionized using chemical ionization with methane as the reagent gas. The GC column was operated from 40 to 300 C at a rate of 10 degrees per minute. GC analysis with flame ionization detection was also done, and the area percent values from this work were applied to the peak identities from the mass spec work. The results of these analyses (which are not necessarily quantitative) show the following:

| Mol. Weight | Component I.D. | L-13202 N-MeFOSE |
|---|---|---|
| 427 | N-Methyl-carboxamides $C_7F_{15}$-C(O)N(Me)H | 0.06 % |
| 527 | $C_8F_{17}SO_2N(Me)_2$ | 0.12 % |
| 513 | $C_8F_{17}SO_2N(Me)H$ | 0.25 % |
| 357 | $C_4F_9SO_2N(Me)CH_2CH_2OH$ | 0.03 % |
| 407 | $C_5F_{11}SO_2N(Me)CH_2CH_2OH$ | 0.52 % |
| 457 | $C_6F_{13}SO_2N(Me)CH_2CH_2OH$ | 3.38 % |
| 507 | $C_7F_{15}SO_2N(Me)CH_2CH_2OH$ | 2.16 % |
| 557 | N-MeFOSE $C_8F_{17}SO_2N(Me)CH_2CH_2OH$ | 89.48 % |
| 519 | $C_8F_{15}SO_2N(Me)CH_2CH_2OH$ | 0.84 % |
| 607 | $C_9F_{19}SO_2N(Me)CH_2CH_2OH$ | 0.55 % |
| 573 | $C_8F_{16}ClSO_2N(Me)CH_2CH_2OH$ | 0.67 % |
| 665 | $C_8F_{16}SF_5$-$SO_2N(Me)CH_2CH_2OH$ | 0.29 % |

T-5878

| | | |
|---|---|---|
| 601 | $C_8F_{17}SO_2N(Me)(CH_2CH_2O)_2H$ | 0.54 % |
| | Other High Boilers | 1.05 % |