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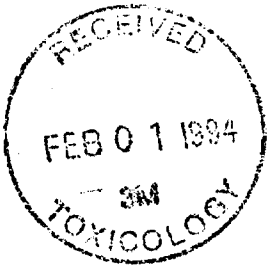

ANALYSIS OF

T-5794

IN A CELL PROLIFERATION ASSAY IN RAT LIVER CELLS

FINAL REPORT

AUTHOR

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LABORATORY PROJECT ID

HWA Study No.: 154-207

SUBMITTED TO

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

STUDY COMPLETION DATE

January 26, 1994

005101

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QUALITY ASSURANCE STATEMENT

PROJECT TITLE: Analysis of T-5794 in a Cell Proliferation Assay in Rat Liver Cells

PROJECT NO.: 20991 HWA STUDY NO.: 154-207

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

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
Necropsy/Tissue Preservation/8-20-93	8-20-93	M.J. Robertson
Draft Report Review/ 11-29,30-93,12-1,6,7-93	12-7-93	B. Mullett
Final report review/ 1-10,11-94	1-11-94	B. Mullett

Brad Mullett 1/26/94
Quality Assurance Unit 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 — January 26, 1994
Andrea L. Ham, B.S. — Date
Associate Scientist

Study Director:

Maria A. Cifone — January 26, 1994
Maria A. Cifone, Ph. D. — Study Completion Date
Study Director
Genetic and Cellular Toxicology

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ABSTRACT

The purpose of this study was to determine the hepatotoxicity of T-5794 by measuring cell proliferation (CP) assayed as S-phase induction in rat liver cells after in vivo treatment. A dose rangefinding study with T-5794 was performed prior to initiation of the cell proliferation assay. In the dose rangefinding assay, T-5794 was administered to rats at doses of approximately 500, 1000, and 2000 mg/kg body weight. All rats remained healthy and appeared normal throughout the four-day observation period. Based on the lack of observable toxicity in the dose range-finding assay, a high dose of 3000 mg/kg was chosen for the assay. The cell proliferation assay was initiated at dose levels of 500, 1000, 2000, and 3000 mg/kg.

Following a single oral dose of the test material for the cell proliferation assay, five animals per condition were labeled with BrdU for 72 hours using ALZET® osmotic pumps. Two animals at 3000 mg/kg and one animal at 2000 mg/kg were found dead during the labeling period. Evidence for histomorphological alterations were observed at 2000 mg/kg and 3000 mg/kg as well as in the DMN positive control animals and in some animals treated with 5710.1.

For the cell proliferation assay, sections from the left lateral, median and right lateral lobes of the livers as well as samples from the duodenum were fixed in 10% neutral Formalin, embedded in paraffin, and 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. Significant increases in cell proliferation were observed following treatment with the test material at the two lowest dose levels. However, the increases were small ($0.05>p>0.01$) and higher dose levels where toxicity was observed and cell proliferative likely to occur, were not significantly elevated. Therefore, no dose-related increase in cell proliferation was induced.

T-5794 was therefore considered negative for the induction of cell proliferation in rat liver cells.

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Analysis of T-5794 in a Cell Proliferation Assay in Rat Liver Cells

I. SPONSOR: 3M Corporation

II. MATERIAL TESTED:

A. Genetics Assay No.: 154-207

B. Identification: T-5794 (L-13097)

C. Physical Description: light amber solid

D. Date Received: July 19, 1993

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

IV. PROTOCOL NUMBER: 493, Edition 1, Modified for 3M Corporation

V. STUDY DATES:

A. Study Initiation Date: July 29, 1993

B. Experimental Start Date: August 2, 1993

C. Experimental Termination Date: September 29, 1993

VI. SUPERVISORY PERSONNEL:

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

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

VII. OBJECTIVE:

The objective of this assay was to measure hepatotoxicity caused by T-5794 by measuring cell proliferation (CP) measured as S-phase induction 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 Harlan Sprague Dawley, Frederick, MD (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 for at least 7 days prior to random assignment to study groups and identification with an ear tag for the dose range-finding assay and by implantable microidentification device for the cell proliferation assay. Animals were anesthetized prior to surgery for preparation of cell cultures, using about 60 mg/kg sodium pentobarbital, and were exsanguinated during the harvest procedure.

For the cell proliferation assay, rats were used that ranged from 255-334 grams. Approximately 24 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 (control organ).

B. Osmotic Pumps and Label for Cell Proliferation Analysis

ALZET® osmotic pumps (ALZA Corporation, Palo Also, CA), Model 2ML1 were used. A single lot (#042207) 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, CMC (high viscosity carboxymethylcellulose, 9004-32-4, Sigma Lot # 121F0644). 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# 29F0679) was dosed at 15.0 mg/kg. Five rats were treated P.O..

3. 3M Corporation cell proliferation control

Two additional controls were included consisting of five animals for each control substance. 5710.1 was included as a negative control for cell proliferation. Animals were dosed with approximately 810 mg/kg of 5710.1. 5711.1 has been shown to be positive in a single dose cell proliferation assay. Animals were dosed with approximately 1000 mg/kg of 5711.1.

D. Test Article

For the preparation of the dosing solutions of the test article, the test article was suspended in 0.5% CMC at concentrations of 50.0, 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 were treated by oral gavage with the test article for the cell proliferation assay and three rats per group were treated by oral gavage for the dose range-finding study. 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 and controls 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. Dose Range-Finding Study

The dose range-finding study was performed using three treatment groups, each group consisting of three male rats. T-5794 was solubilized in 0.5% carboxymethylcellulose solution (CMC). The rats were dosed with a single treatment of T-5794 by oral gavage (PO) at 500, 1000, and 2000 mg/kg body weight. Daily observations for toxic signs and mortality were performed for four days. At the end of the four days, all animals were euthanized using CO_2 inhalation, followed by penetration of the thorax.

C. Implantation of Osmotic Pumps

For the cell proliferation assay, ALZET® Model 2ML1 osmotic pumps were preloaded with 2000 μl of BrdU at a concentration of 20 mg/ml. The animals were anesthetized using Metofane® according to standard procedures and one pump per animal was aseptically inserted subcutaneously (dorsal surface) approximately 24 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.

D. 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 liver, 5 μ paraffin embedded sections were taken from the left lateral, median and right anterior lobes. Similarly prepared sections of the duodenum were also made and a section of the

duodenum was mounted on each slide. Slides were also prepared according to standard procedures for examination by a pathologist to determine if any abnormalities were present.

E. Immunohistochemical Staining

The slides were deparaffinized and rehydrated prior to staining. Slides of the liver were stained for determination of cell proliferation as measured by incorporation of BrdU into DNA using the Biogenex primary and secondary antibodies with peroxidase-conjugated streptavidin and a 3,3-diaminobenzidine tetrahydrochloride (DAB) chromogen and hematoxylin counterstain. Parallel slides were stained with hematoxylin and eosin for analysis by a pathologist.

F. Assessment of Cell Proliferation Rates

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 were examined for lobular differences. In this study, labeling was similar among the lobes. Cell counting was therefore performed with sections from the left lateral lobes. 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

A mean and standard deviation for the percentage of S-phase cells 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 (12). Control versus treatment group comparisons were done with Dunnet's t-test (13, 14)and

control versus positive and 3M Corporation control 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. 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 $p \leq 0.05$ was considered significantly different than the control group. The same procedures were used to analyze terminal body and liver weights.

XII. INTERPRETATION OF RESULTS

A. Dose Rangefinding Study

Three groups, each containing three male rats were dosed P.O. with a single dose of T-5794. The dose levels were approximately 500, 1000, and 2000 mg/kg body weight. For four consecutive days, the rats were observed for mortality and toxic signs. All rats remained healthy and appeared normal throughout the four-day observation period. Since, no overt toxicity was observed, it was decided that the cell proliferation assay would be initiated with four treatments (500, 1000, 2000 and 3000 mg/kg).

B. General Observations

Treatment-related histomorphologic alterations were observed in some animals which received T-5794 at levels of 2000 and 3000 mg/kg, 5710.1 at a level of 810 mg/kg, and DMN at 15 mg/kg. Affected animals had evidence of hepatocellular injury (hepatotoxicity) and this was characterized microscopically by scattered foci or centrilobular areas of necrosis which in some animals extended to portal areas, or necrosis of individual hepatocytes, chronic-active inflammation and, in some animals, vacuolization. Animals which received T-5794 at 2000 mg/kg had minimal to slight changes whereas at the 3000 mg/kg level these changes were all slight. Details of the histopathology are in Appendix A.

Two animals dosed with 3000 mg/kg and one animal dosed with 2000 mg/kg of T-5794 were found dead prior to termination of the BrdU labeling period. The presence of dead animals at 2000 mg/kg in the definitive assay and not in the dose range-finding study may be explained by the additional trama from the pump implantation that occurred in the cell proliferation assay. 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.

The livers of the treated animals did not show a dose-related increase in mean weight compared to control animals. The mean liver weight of the positive control was also not significantly elevated even though large increases in DNA synthesis (and subsequent cell proliferation) were induced. However, the 5510.1 treated animals had liver weights that were significantly lower than the control animals. Mean terminal body weights of treatments at 1000, 2000, and 3000 mg/kg and the DMN positive control mean animal weights also were significantly less than control values confirming the presence of toxicity.

C. 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 Table 2. The mean labeling index (LI) for each group is presented in the third column in Table 1. There was no apparent preferential labeling in any of the lobes and the label was random within the lobes.

The mean background labeling index (Group 1) was 1.61 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-5794 in the dosed Groups 2 and 3 (500 and 1000 mg/kg). The labeling indices at 500 and 1000 mg/kg were 4.55% and 4.43%, respectively. These represent 2.8-fold increases over background ($p \leq 0.05$). While these increases are considered significant, higher dose levels (2000 and 3000 mg/kg) did not show increases in cell proliferation. Toxicity was observed in animals at 2000 mg/kg and 3000 mg/kg as represented by animal death and histopathological changes in the liver. Since the increases in cell proliferation that were observed were not dose-related and not associated with the toxic doses, T-5794 was not considered positive for cell proliferation.

The mean labeling index of the DMN positive control animals was 44.95 which is significantly elevated ($p \leq 0.01$). The 3M control compounds behaved as expected, with 5710.1 being negative for cell proliferation and 5711.1 inducing a positive response ($p \leq 0.01$).

XVI. CONCLUSIONS

The test material, T-5794, did not induce significant dose-related changes in the number S-phase cells following a single oral dose of T-5794. The animals were labeled for 72 hours and increases were observed at the two lowest dose levels, but higher doses were negative. T-5794 was therefore evaluated as negative in the induction of cell proliferation in rat liver cells.

XVII. REFERENCES

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XVIII. EXPERIMENTAL DATA TABLES

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Table 1

Cell Proliferation Summary

Client: 3M Corporation

HWA Assay No.: 154-207

Client Code: T-5794

Trial Initiation Date: August 16, 1993

Group/Sex[a]	Dose Level (mg/kg)	Labeling Index[b] (%)	Liver Weight (grams)	Terminal Body Weight (grams)
1M	0[e]	1.61 ± 0.71	12.99 ± 1.12	322.5 ± 15.6
2M	500	4.55 ± 1.23*↑	13.62 ± 1.24	309.6 ± 20.9
3M	1000	4.43 ± 2.83*↑	14.07 ± 1.04	307.0 ± 38.5
4M[c]	2000	2.71 ± 1.09	12.01 ± 1.84	251.0 ± 30.1**↓
5M[d]	3000	1.19 ± 0.72	13.07 ± 1.28	263.7 ± 21.8*↓
6M[f]	810	1.80 ± 1.22	12.26 ± 0.45**↓	255.2 ± 14.5**↓
7M[g]	1000	6.62 ± 1.24**↑	15.02 ± 2.03	311.6 ± 35.3
8M[h]	15	44.95 ± 8.75**↑	12.10 ± 0.84	306.6 ± 23.7*↓

[a]Five animals per group unless indicated
[b]Percentage of labeled hepatocyte nuclei per total number of hepatocytes counted (at least 2000)
[c]Four animals per group
[d]Three animals per group
[e]Vehicle control, 10 ml/kg of CMC
[f]5710.1 Control, 810 mg/kg
[g]5711.1 Control, 1000 mg/kg
[h]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

Table 2

Cell Proliferation Assay
Individual Animal Data

Animal Number	Group /Sex	Mean Labeling Index (%)	Terminal Body Weight (g)	Terminal Liver Weight (g)
B41170	1M	2.05	303.0	11.46
B41171	1M	1.90	314.0	12.97
B41172	1M	2.33	342.0	13.29
B41173	1M	1.14	334.0	14.56
B41174	1M	0.62	319.4	12.69
Group Mean		1.61	322.5	12.99
Group SD		0.71	15.6	1.12
N		5	5	5
B41180	2M	6.48	346.0	15.76
B41181	2M	3.57	297.0	12.86
B41182	2M	3.76	299.0	13.21
B41183	2M	3.86	309.0	13.57
B41184	2M	5.10	297.0	12.71
Group Mean		4.55	309.6	13.62
Group SD		1.23	20.9	1.24
N		5	5	5
B41190	3M	3.57	260.0	13.38
B41191	3M	1.95	362.0	13.18
B41192	3M	9.29	314.0	15.24
B41193	3M	4.10	316.0	15.17
B41194	3M	3.24	283.0	13.40
Group Mean		4.43	307.0	14.07
Group SD		2.83	38.5	1.04
N		5	5	5

Table 6 (Con't)

Cell Proliferation Assay
Individual Animal Data

Animal Number	Group /Sex	Mean Labeling Index (%)	Terminal Body Weight (g)	Terminal Liver Weight (g)
B41200	4M	2.38	288.0	13.27
B41201	4M	4.33	263.0	13.48
B41203	4M	1.95	227.0	9.48
B41204	4M	2.19	226.0	11.79
Group Mean		2.71	251.0	12.01
Group SD		1.09	30.1	1.84
N		4	4	4
B41210	5M	0.95	283.0	14.48
B41212	5M	2.00	268.0	12.75
B41213	5M	0.62	240.0	11.99
Group Mean		1.19	263.7	13.07
Group SD		0.72	21.8	1.28
N		3	3	3
B41220	6M	3.05	249.0	12.05
B41221	6M	0.81	243.0	12.14
B41222	6M	0.57	280.0	13.02
B41223	6M	3.14	255.0	12.25
B41224	6M	1.43	249.0	11.85
Group Mean		1.80	255.2	12.26
Group SD		1.22	14.5	0.45
N		5	5	5
B41225	7M	5.57	325.0	15.21
B41226	7M	8.67	315.0	15.81
B41227	7M	6.10	250.0	11.62
B41228	7M	5.90	336.0	17.07
B41229	7M	6.86	332.0	15.37
Group Mean		6.62	311.6	15.02
Group SD		1.24	35.3	2.03
N		5	5	5

Table 6 (Con't)

Cell Proliferation Assay
Individual Animal Data

Animal Number	Group /Sex	Mean Labeling Index (%)	Terminal Body Weight (g)	Terminal Liver Weight (g)
B41230	8M	46.48	295.0	11.46
B41231	8M	57.38	339.0	12.76
B41232	8M	44.86	281.0	11.14
B41233	8M	43.19	323.0	13.11
B41234	8M	32.86	295.0	12.02
Group Mean		44.95	306.6	12.10
Group SD		8.75	23.7	0.84

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Appendix A Histopathology Report

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Pathology Report

Analysis of Cell Proliferation
in Rat Liver Cells
Project No. 154-207
Test Material - T-5794

General Protocol

The objective of this study was to detect hepatotoxicity caused by the test material by measuring cell proliferation as S-phase induction induced in rat liver cells after in vivo treatment. The pathology portion of this study consisted of microscopic examination of duplicate sections, stained by HE methods to detect any treatment-related histomorphologic alterations. Male rats were divided into 8 groups and assigned to study as follows: Group 1 animals received carboxymethylcellulose; Group 2 received T-5794 at 500 mg/kg; Group 3 received T-5794 at 1000 mg/kg; Group 4 received T-5794 at 2000 mg/kg; Group 5 received T-5794 at 3000 mg/kg; Group 6 received 5710.1 at 810 mg/kg; Group 7 received 5711.1 at 1000 mg/kg and Group 8 received DMN at 15 mg/kg. The latter three groups were common to a companion study. The following is a summary of histomorphologic findings.

Results and Discussion

Treatment-related histomorphologic alterations were observed in some animals which received T-5794 at levels of 2000 and 3000 mg/kg, 5710.1 at a level of 810 mg/kg, and DMN at 15 mg/kg. Affected animals had evidence of hepatocellular injury (hepatotoxicity) and this was characterized microscopically by scattered foci or centrilobular areas of necrosis which in some animals extended to portal areas, or necrosis of individual hepatocytes, chronic-active inflammation and, in some animals, vacuolization. Animals which received T-5794 at 2000 mg/kg had minimal to slight changes whereas at the 3000 mg/kg level these changes were all slight. Three of five animals which received 5710.1, a positive control, at a level of 810 mg/kg demonstrated evidence of slight to moderate hepatocellular injury. Five animals receiving T5711.1 at a dose of 1000 mg/kg had no evidence of hepatocellular injury. All animals which received DMN at a level of 15 mg/kg

showed evidence of minimal to moderate hepatocellular injury. The Individual Histopathology Findings table details all lesions, severity grades and distribution in individual animals from all groups.

Conclusions

Hepatocellular injury occurred in groups of animals receiving T-5794 at levels of 2000 and 3000 mg/kg, 5710.1 at a level of 810 mg/kg and DMN at 15 mg/kg.

Pathologist:

Borge M. Ulland, D.V.M.,
Diplomate, American College of Veterinary Pathologists
Department of Pathology

1-25-94
Date

154-207.94

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HWA STUDY NO. _____
ADDENDUM TO PROTOCOL NO. 493

DOSE RANGEFINDING STUDY FOR
A CELL PROLIFERATION ASSAY

ORAL GAVAGE ADMINISTRATION

Duplicate of Copy returned by fax.

Hazleton Washington, Inc. (HWA) will conduct this study in compliance with Good Laboratory Practice (GLP) Regulations. This protocol, critical phase(s) of the work in progress and 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-021 3M Center, St. Paul MN 55144-1000

II. TEST ARTICLE IDENTIFICATION:

T-5794 AND T-5795

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 BGA

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:

Roger D. Perkins Date: July 20, 1993

DOSE RANGEFINDING STUDY FOR
A CELL PROLIFERATION ASSAY

ORAL GAVAGE ADMINISTRATION

1.	Purpose	Define a dose range for cell proliferation studies to be conducted in rats.
2.	HWA Study Coordinator	Andrea Ham, B.S.S.
3.	Experimental Design	
	A. Animals	
	(1) Species	Rat
	(2) Strain/Source	Young adult males of the Sprague-Dawley strain purchased from Harlan Sprague-Dawley, Inc. (HSD:Harlan Sprague-Dawley®(SD®BR) or Charles River Laboratories, Inc. (Crl:CD®BR).
	(3) Age at Initiation	10 - 12 weeks
	(4) Number/Sex	9 males
	(5) Number/Group	3 males per group
	(6) Identification	Ear tags
	(7) Husbandry	
	(a) Housing	All applicable HWA SOPs will be followed. Animals will be isolated by sex. Animals will be housed two per cage during quarantine, but will be housed singly prior to experiment initiation. Sanitary cages will be used. Personnel handling the animals within the animal facility will be required to wear suitable protective garments and equipment.

(b)	Food	Purina® Certified Rodent Chow® 5002, ad libitum. Feed is analyzed by the manufacturer for concentrations of specified heavy metals, aflatoxin, chlorinated hydrocarbons, organophosphates, and specified nutrients. This information is on file with the manufacturer.
(c)	Water	Tap water, ad libitum. The water is analyzed biannually on a retrospective basis for specified microorganisms, pesticides, heavy metals, alkalinity, and halogens.
(d)	Environment	Every attempt will be made to maintain temperatures within 72°F±6°F with a relative humidity of 55±15%. A 12-hour light/12-hour dark cycle will be maintained.
(e)	Quarantine	All animals will be quarantined for at least seven days after receipt from the supplier. A veterinarian will release all animals prior to the assay initiation.
(8)	Randomization	Using computer-generated random numbers. Animals will be uniquely identified by ear tag. Treatment groups will be identified by cage label.
(9)	Justification	Rats historically have been used for cell proliferation studies.
B.	Study Design	The study will be conducted using three treatment groups. Each of the three groups will consist of 3 male rats.

C. Group Designation and Treatment Regimens

Group No.	Number of Rats Male	Route	Duration (Days)
1	3	PO	4
2	3	PO	4
3	3	PO	4

D. Dosing Procedures — The route of administration will be oral gavage (PO). The test material will solubilized in one of the following solvents: water, 0.5% aqueous carboxymethylcellulose solution, or corn oil.

(1) Dose Volume — Not to exceed 10 ml/kg body weight. All animals will be dosed based upon individual body weights.

(2) Dose Levels — Assigned by Protocol Amendment

(3) Duration of Treatment — All animals will be euthanized 4 days after receiving a single dose.

E. Dosing Formulations

(1) Preparation of Dosing Formulations — Dosing formulation will be prepared just prior to dosing.

(2) Storage of Dosing Formulations — Solutions will be prepared at ambient temperatures and held until dosing (0–2 hours).

F. Animal Observations

(1) Clinical Examinations — Daily observations, for toxic signs and mortality for the duration of the study.

	(2) Body Weights	Body weights will be taken prior to dosing.
G.	Method of Euthanasia	CO_2 inhalation, followed by penetration of the thorax.
H.	Data Evaluation	The daily observations toxic symptoms and/or mortalities data will be used to estimate the Maximum Tolerated Dose (MTD). Doses will then be assigned for the subsequent cell proliferation assay.
I.	Records and Reports	
	(1) Records 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.
	(2) Final Report	Hazleton Washington, Inc. employs a standard format for each assay design. Each final report will provide the following information: • Sponsor identification. • Quality Assurance statement. • Statement of GLP compliance. • Signatures of Study Coordinator and Study Director. • Test article identification and HWA 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.
- Methods.
- Evaluation criteria.
- Interpretation of results.
- Conclusions.
- Test results presented in tabular form.
- References.

J. 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 HWA-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 HWA-IACUC for their review.

K. 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 revision.

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

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 %

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