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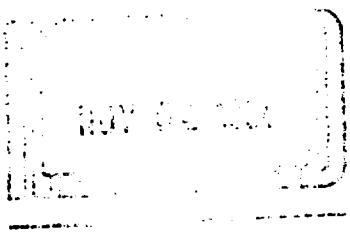
ANALYSIS OF

T-5877

IN A CELL PROLIFERATION ASSAY IN RAT LIVER CELLS



FINAL REPORT

AUTHOR

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LABORATORY PROJECT ID

HWA Study No.: 154-208

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-5877 in a Cell Proliferation Assay in Rat Liver Cells

PROJECT NO.: 20991 HWA STUDY NO.: 154-208

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	B. Mullett
Draft report review/ 7-25,26-94	7-26-94	B. Mullett
Final report review/ 11-1-94	11-1-94	B. Mullett

Brad T. 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-5877 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 100, 200, 400 and 800 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 100 and 200 mg/kg but minimal to moderate vacuolization was observed at 400 and 800 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 1.34 and treated animals had labeling indices that ranged from 1.40 to 4.42. When comparing vehicle and treated groups, there were no significant increases in the labeling index in any of the treated groups and no positive trend was observed. The high dose (Group 5) animals did have an elevated mean labeling index but it was not significant because the average value showed heterogeneous variance. Significant increases in cell proliferation were observed in the positive control animals.

T-5877 was therefore considered negative for the induction of cell proliferation in rat liver cells.

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Analysis of T-5877 in a Cell Proliferation Assay in Rat Liver Cells

I. SPONSOR: 3M Corporation

II. MATERIAL TESTED:

A. Genetics Assay No.: 154-208

B. Identification: T-5877

C. Physical Description: waxy amber 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: June 14, 1994

VI. SUPERVISORY PERSONNEL:

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

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

VII. OBJECTIVE:

The objective of this assay was to measure hepatotoxicity caused by T-5877 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 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.

The cell proliferation assay was initiated with rats that ranged from 306 to 355 grams. Approximately 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, corn oil (Duke's Corn Oil, Lot 2L290833). 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 corn oil at concentrations of 10, 20, 40 and 80 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-5877 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® according to standard procedures 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 histomorphological alterations were observed at 100 and 200 mg/kg but minimal to moderate vacuolization was observed at 400 and 800 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.

None of the livers of the treated animals showed a dose-related increase in weight compared to control animals. 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. However, the 400 mg/kg (Group 4) and 800 mg/kg (Group 5) animals had mean terminal body weights that were less than the Group 1 control value ($p \leq 0.01$). When the liver to body weight ratios were determined, there were significant increases in the liver to terminal body weight ratios with a p value of between 0.01 and 0.05 for Group 3 (200 mg/kg) and increases at 400 mg/kg (Group 4) and 800 mg/kg (Group 5)($p \leq 0.01$).

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 1.34 which indicates that less than 2% of the nuclei had undergone DNA synthesis during the 72-hour labeling period. The labeling indices of the test article-treated cells ranged from 1.40 to 4.42. None were considered significantly elevated and there was

no indication of a significant positive trend due to treatment. The high dose (800 mg/kg) had the highest labeling index of 4.42, but because of a heterogeneous response, there was a lack of significance. If the results from individual animals are compared (see Appendix A), two of the five animals responded, with labeling indices of 8.00 and 9.05, while the remaining three animals had labeling indices close to background levels. This may indicate a weak response, but also may be the result of animal variability. 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-5877 did not induce significant dose-related increases in the LI in the liver in male rats after treatment with single oral doses at concentrations of 100 mg/kg to 800 mg/kg. Large increases in the labeling index were 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-5877, did not induce significant changes in the number S-phase cells following a single oral dose of 100 mg/kg to 800 mg/kg. The animals were labeled for 72 hours and no significant dose-related trend in the mean labeling index was observed in the treated groups. T-5877 was therefore evaluated as negative for 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.: 154-208

Client Code: T-5877 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]	1.34 ± 0.66	13.58 ± 1.09	344.2 ± 12.9	3.94 ± 0.20
2M	100	2.98 ± 1.61	14.32 ± 1.81	348.1 ± 17.3	4.11 ± 0.36
3M	200	1.40 ± 0.92	15.00 ± 2.24	335.6 ± 20.5	4.45 ± 0.38*↑
4M	400	2.26 ± 1.26	14.76 ± 1.02	303.6 ± 13.0**↓	4.86 ± 0.25**↑
5M	800	4.42 ± 3.60	15.38 ± 1.05	300.0 ± 15.0**↓	5.13 ± 0.19**↑
6M[d]	15[d]	34.96 ± 14.86**↑	12.73 ± 1.61	319.6 ± 22.5	3.97 ± 0.29

[a]Five animals per group
[b]Percentage of labeled hepatocyte nuclei per total number of hepatocytes counted (at least 2000)
[c]Vehicle control, Corn oil
[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	47733	1M	10	700	1.43
17	47733	1M	21	700	3.00
18	47733	1M	5	700	0.71
				Mean	1.71
				SD	1.17
19	47734	1M	8	700	1.14
20	47734	1M	9	700	1.29
21	47734	1M	7	700	1.00
				Mean	1.14
				SD	0.14
22	47735	1M	5	700	0.71
23	47735	1M	4	700	0.57
24	47735	1M	4	700	0.57
				Mean	0.62
				SD	0.08
25	47736	1M	11	700	1.57
26	47736	1M	10	700	1.43
27	47736	1M	15	700	2.14
				Mean	1.71
				SD	0.38
28	47737	1M	7	700	1.00
29	47737	1M	13	700	1.86
30	47737	1M	12	700	1.71
				Mean	1.52
				SD	0.46
			GROUP	MEAN	1.34
			GROUP	SD	0.66

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Slide #	Animal ID	Group	# Labeled	# Counted	% Labeled
46	47738	2M	21	700	3.00
47	47738	2M	16	700	2.29
48	47738	2M	29	700	4.14
				Mean	3.14
				SD	0.94
49	47739	2M	22	700	3.14
50	47739	2M	19	700	2.71
51	47739	2M	16	700	2.29
				Mean	2.71
				SD	0.43
52	47740	2M	14	700	2.00
53	47740	2M	26	700	3.71
54	47740	2M	8	700	1.14
				Mean	2.29
				SD	1.31
55	47741	2M	37	700	5.29
56	47741	2M	22	700	3.14
57	47741	2M	49	700	7.00
				Mean	5.14
				SD	1.93
58	47742	2M	12	700	1.71
59	47742	2M	4	700	0.57
60	47742	2M	18	700	2.57
				Mean	1.62
				SD	1.00
			GROUP	MEAN	2.98
			GROUP	SD	1.61

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Slide #	Animal ID	Group	# Labeled	# Counted	% Labeled
61	47743	3M	6	700	0.86
62	47743	3M	2	700	0.29
63	47743	3M	6	700	0.86
				Mean	0.67
				SD	0.33
64	47744	3M	14	700	2.00
65	47744	3M	9	700	1.29
66	47744	3M	11	700	1.57
				Mean	1.62
				SD	0.36
67	47745	3M	16	700	2.29
68	47745	3M	11	700	1.57
69	47745	3M	27	700	3.86
				Mean	2.57
				SD	1.17
70	47746	3M	4	700	0.57
71	47746	3M	6	700	0.86
72	47746	3M	7	700	1.00
				Mean	0.81
				SD	0.22
73	47747	3M	9	700	1.29
74	47747	3M	3	700	0.43
75	47747	3M	16	700	2.29
				Mean	1.33
				SD	0.93
			GROUP	MEAN	1.40
			GROUP	SD	0.92

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Slide #	Animal ID	Group	# Labeled	# Counted	% Labeled
31	47748	4M	11	700	1.57
32	47748	4M	14	700	2.00
33	47748	4M	15	700	2.14
				Mean	1.90
				SD	0.30
34	47749	4M	7	700	1.00
35	47749	4M	19	700	2.71
36	47749	4M	10	700	1.43
				Mean	1.71
				SD	0.89
37	47750	4M	19	700	2.71
38	47750	4M	39	700	5.57
39	47750	4M	16	700	2.29
				Mean	3.52
				SD	1.79
40	47751	4M	4	700	0.57
41	47751	4M	13	700	1.86
42	47751	4M	9	700	1.29
				Mean	1.24
				SD	0.64
43	47752	4M	14	700	2.00
44	47752	4M	17	700	2.43
45	47752	4M	30	700	4.29
				Mean	2.90
				SD	1.21
			GROUP	MEAN	2.26
			GROUP	SD	1.26

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Slide #	Animal ID	Group	# Labeled	# Counted	% Labeled
1	47753	5M	23	700	3.29
2	47753	5M	8	700	1.14
3	47753	5M	18	700	2.57
				Mean	2.33
				SD	1.09
4	47754	5M	62	700	8.86
5	47754	5M	60	700	8.57
6	47754	5M	46	700	6.57
				Mean	8.00
				SD	1.25
7	47755	5M	12	700	1.71
8	47755	5M	5	700	0.71
9	47755	5M	5	700	0.71
				Mean	1.05
				SD	0.58
10	47756	5M	63	700	9.00
11	47756	5M	56	700	8.00
12	47756	5M	71	700	10.14
				Mean	9.05
				SD	1.07
13	47757	5M	15	700	2.14
14	47757	5M	10	700	1.43
15	47757	5M	10	700	1.43
				Mean	1.67
				SD	0.41
				GROUP MEAN	4.42
				GROUP SD	3.60

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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

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APPENDIX B

ANALYSIS OF CELL PROLIFERATION IN RAT LIVER CELLS

DRAFT ABSOLUTE ORGAN WEIGHTS (g) *DRAFT*
STUDY NUMBER: 154208

ORGAN ABBREVIATION: LI - LIVER

SEX	DOSE GROUP	ANIMAL NUMBER	TERMINAL BODY WT (g)	LI
M	1	B47733	344.0	14.24
M	1	B47734	350.0	13.77
M	1	B47735	322.0	11.66
M	1	B47736	352.0	14.19
M	1	B47737	353.0	14.11
		NUMBER IN GROUP:	5	5
		MEAN:	344.2	13.59
		STANDARD DEV:	12.9	1.09
M	2	B47738	371.0	16.35
M	2	B47739	350.0	14.31
M	2	B47740	340.0	12.12
M	2	B47741	355.0	15.87
M	2	B47742	324.6	12.98
		NUMBER IN GROUP:	5	5
		MEAN:	348.1	14.32
		STANDARD DEV:	17.3	1.81
M	3	B47743	368.0	18.79
M	3	B47744	330.0	14.28
M	3	B47745	327.0	14.33
M	3	B47746	313.0	12.87
M	3	B47747	340.0	14.70
		NUMBER IN GROUP:	5	5
		MEAN:	335.6	15.00
		STANDARD DEV:	20.5	2.24

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APPENDIX B

ANALYSIS OF CELL PROLIFERATION IN RAT LIVER CELLS

DRAFT ABSOLUTE ORGAN WEIGHTS (g) *DRAFT*
STUDY NUMBER: 154208

ORGAN ABBREVIATION: LI - LIVER

SEX	DOSE GROUP	ANIMAL NUMBER	TERMINAL BODY WT (g)	LI
M	4	B47748	300.0	15.02
M	4	B47749	308.0	13.74
M	4	B47750	324.0	16.31
M	4	B47751	291.9	13.96
M	4	B47752	294.0	14.77
		NUMBER IN GROUP:	5	5
		MEAN:	303.6	14.76
		STANDARD DEV:	13.0	1.02
M	5	B47753	314.0	16.26
M	5	B47754	293.0	14.30
M	5	B47755	278.6	14.19
M	5	B47756	314.0	15.89
M	5	B47757	300.2	16.25
		NUMBER IN GROUP:	5	5
		MEAN:	300.0	15.38
		STANDARD DEV:	15.0	1.05
M	6	B47758	336.0	14.50
M	6	B47759	294.0	11.08
M	6	B47760	334.0	14.20
M	6	B47761	296.0	11.21
M	6	B47762	338.0	12.64
		NUMBER IN GROUP:	5	5
		MEAN:	319.6	12.73
		STANDARD DEV:	22.5	1.61

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APPENDIX B

ANALYSIS OF CELL PROLIFERATION IN RAT LIVER CELLS

DRAFT ORGAN-TO-TERMINAL BODY WEIGHT RATIOS (%) *DRAFT*
STUDY NUMBER: 154208

ORGAN ABBREVIATION: LI - LIVER

SEX	DOSE GROUP	ANIMAL NUMBER	TERMINAL BODY WT (g)	RATIO
M	1	B47733	344.0	4.138
M	1	B47734	350.0	3.933
M	1	B47735	322.0	3.622
M	1	B47736	352.0	4.030
M	1	B47737	353.0	3.998
		NUMBER IN GROUP:	5	5
		MEAN:	344.2	3.944
		STANDARD DEV:	12.9	0.195
M	2	B47738	371.0	4.407
M	2	B47739	350.0	4.087
M	2	B47740	340.0	3.564
M	2	B47741	355.0	4.470
M	2	B47742	324.6	3.999
		NUMBER IN GROUP:	5	5
		MEAN:	348.1	4.106
		STANDARD DEV:	17.3	0.363
M	3	B47743	368.0	5.107
M	3	B47744	330.0	4.326
M	3	B47745	327.0	4.382
M	3	B47746	313.0	4.112
M	3	B47747	340.0	4.325
		NUMBER IN GROUP:	5	5
		MEAN:	335.6	4.450
		STANDARD DEV:	20.5	0.381

APPENDIX B

ANALYSIS OF CELL PROLIFERATION IN RAT LIVER CELLS

DRAFT ORGAN-TO-TERMINAL BODY WEIGHT RATIOS (%) *DRAFT*
STUDY NUMBER: 154208

ORGAN ABBREVIATION: LI - LIVER

SEX	DOSE GROUP	ANIMAL NUMBER	TERMINAL BODY WT (g)	RATIO
M	4	B47748	300.0	5.006
M	4	B47749	308.0	4.460
M	4	B47750	324.0	5.033
M	4	B47751	291.9	4.782
M	4	B47752	294.0	5.024
	NUMBER IN GROUP:		5	5
	MEAN:		303.6	4.861
	STANDARD DEV:		13.0	0.247
M	5	B47753	314.0	5.179
M	5	B47754	293.0	4.879
M	5	B47755	278.6	5.093
M	5	B47756	314.0	5.060
M	5	B47757	300.2	5.412
	NUMBER IN GROUP:		5	5
	MEAN:		300.0	5.125
	STANDARD DEV:		15.0	0.194
M	6	B47758	336.0	4.315
M	6	B47759	294.0	3.768
M	6	B47760	334.0	4.251
M	6	B47761	296.0	3.789
M	6	B47762	338.0	3.739
	NUMBER IN GROUP:		5	5
	MEAN:		319.6	3.972
	STANDARD DEV:		22.5	0.285

APPENDIX C Histopathology Report

HAZLETON
WASHINGTON

Pathology Report
Analysis of Cell Proliferation in Rat Liver Cells
Project No. 154-208

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 100, 200, 400, and 800 mg/kg of the test material, T-5877, 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 minimal to moderate vacuolization in Group 4 (400 mg/kg) and Group 5 (800 mg/kg) rats. Group 6 (DMN treated) rats had varying severities of centrilobular necrosis, hepatocellular hypertrophy, chronic inflammation, peliosis, and increased mitoses. The single cases of pelvic dilatation in the kidney and degeneration with mineralization of the testis in Group 5 are considered to be spontaneous and without relation to treatment.

Summary

The test material, T-5877, when administered to male Sprague-Dawley rats in single oral gavage doses of 100, 200, 400, and 800 mg/kg, produced vacuolization in the liver of rats dosed at 400 and 800 mg/kg. No treatment-related histomorphologic changes were noted in the liver of rats dosed at 100 and 200 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, Terpstra-Jonckheere test [1], and regression tests for trend using both untransformed and ranked data.

Results:

Since the sphericity tests rejected variance homogeneity for both vehicle vs positive control and vehicle vs treated groups ($p = 0.0215$ and 0.0000, respectively), the Greenhouse-Geisser probabilities were used for the significance evaluation. 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 extremely heterogeneous variance ($p = 0.0000$) in comparing vehicle vs treated groups so that the rank transformation was used to conduct one-way ANOVA. As Text Tables 2 and 3 indicate, there are no significant differences between vehicle and treated groups for both untransformed and transformed cases ($p = 0.0953$ and $p = 0.0692$, respectively). Furthermore, Terpstra-Jonckheere test and regression of rank-transformed data did not show any significant positive trend as indicated in Text Table 4 even though the regression based on the untransformed data showed marginally significant trend over doses ($p = 0.0240$). There was no significant lack of fit for both regressions ($p = 0.075$ and 0.378). 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	0.0004 **	0.0963
Slide p	0.2269	0.4920
SlidexDose p	0.2499	0.1360

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	1.3400	0.4649	1.5200	3.0000	1.5411
Positive	34.9620	13.1132	36.6700	8.0000	1.5811
Vehicle vs Treated Groups					
Vehicle	1.3400	0.4649	1.5200	8.0000	5.1962
100	2.9800	1.3313	2.7100	17.7000	5.2631
200	1.4000	0.7588	1.3300	7.9000	6.4070
400	2.2540	0.9313	1.9000	15.2000	6.3008
800	4.4200	3.7928	2.3300	16.2000	8.8713

Text Table 3 - One-Way ANOVA

	Vehicle vs Positive Control		Vehicle vs Treated Groups	
	Untransformed	Transformed	Untransformed	Transformed
Treatment	.0004 **	.0010 **	.0953	.0692

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

Terpstra-Jonckheere Test	.0865 †
Regression of Untransformed Data	.0240 *†
Regression of Rank Transformed Data	.1870 †

Discussion:

The results of the present study indicate that there was no statistically significant increase in cell proliferation over control due to treatment by the chemical. Moreover, there was no 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. 154208
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-5877

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:

Roger D. Perkins 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 μ 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 μ 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.

2. Lanier, T.L., Berger, E.K. and Eacho, P.I. (1989) Comparison of 5-bromodeoxyuridine and ^{3}H-thymidine in rodent hepatocellular proliferation studies. Toxicologist 9, 64.

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.

4. Mirsalis, J.C. and Butterworth, B.E.: Induction of unscheduled DNA synthesis in rat hepatocytes following in vivo treatment with dinitrotoluene. Carcinogenesis, 3:241-245, 1982.

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.

HAZLETON
WASHINGTON

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: ANALYSIS OF CELL PROLIFERATION IN RAT LIVER CELLS

HWA PROTOCOL: 493, EDITION 4 ASSAY NO.: 154-208

EFFECTIVE DATE: FEBRUARY 8, 1994 AMENDMENT NO.: 1

The following changes are made to the study protocol:

The second paragraph of Section III. A. Animals is changed from:

"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 surgery will be anesthetized before surgery."

to:

"The animals will be housed according to standard operating procedures and will be fed Purina Certified® Rodent Chow (formula 5002) and water ad libitum. No contaminants are known to be present in the diet or water at levels which might interfere with the study. The animals will be quarantined a minimum of seven days prior to use and will be randomly assigned to study groups. Randomization of the animals and animal identification will be performed according to standard operating procedures of the Mammalian Toxicology Section. The rats to be used for surgery will be anesthetized before surgery."

The first two sentences of Section IV. D. Tissue Collection and Preparation is changed from:

"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."

to:

"Each animal will be weighed and anesthetized prior to removal of organs for analysis. The thoracic cavity will be opened and the liver removed, weighed and fixed in neutral buffered formalin."

Reason: Information inadvertently left out of protocol.

HAZLETON
WASHINGTON

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: ANALYSIS OF CELL PROLIFERATION IN RAT LIVER CELLS

AMENDMENT NO.: 1 ASSAY NO.: 154-208

STUDY DIRECTOR'S SIGNATURE: Maria Cifone DATE: 2-8-94

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
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

file

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 (Rf-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 %