HAZLETON
WASHINGTON
9200 LEESBURG PIKE
VIENNA, VA. 22182-1699

GENOTOXICITY TEST ON

T-5711.1

IN THE IN VIVO/IN VITRO UNSCHEDULED DNA SYNTHESIS AND CELL PROLIFERATION ASSAYS IN RAT LIVER CELLS

FINAL REPORT

AUTHOR

Maria A. Cifone, Ph. D.

PERFORMING LABORATORY

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

LABORATORY PROJECT ID

HWA Study No.: 15515-0-494

SUBMITTED TO

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

STUDY COMPLETION DATE

September 14, 1993

HAZLETON
WASHINGTON

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: Genotoxicity Test on T-5711.1 in In Vivo/In Vitro Unscheduled DNA Synthesis and Cell Proliferation Assays in Rat Liver Cells

PROJECT NO.: 20991 HWA STUDY NO.: 15515

PROTOCOL NO.: 494 EDITION NO.: Modified for 3M Corporation

Quality Assurance inspections of the study and/or 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
Scoring of slides/ 4-28-93	4-28-93	K. Newland
Draft report review/ 7-13→16,26,27-93	7-27-93	B. Mullett
Final report review/ 9-14-93	9-14-93	B. Mullett

Brad Mullett — Quality Assurance Unit

9/14/93 — Date Released

HAZLETON
WASHINGTON

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). To the best of the signers' knowledge, 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, B. S.
Supervisor

9/14/93
Date

Study Director:

Maria Cifone

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

9-14-93
Study Completion Date

HAZLETON
WASHINGTON

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

ABSTRACT

The purpose of this study was to determine the hepatotoxicity and/or genotoxicity of the test material by measuring DNA repair as unscheduled DNA synthesis (UDS) and cell proliferation (CP) measured as S-phase induction in rat liver cells after in vivo treatment. The test material, T-5711.1 did not induce significant changes in UDS in rat hepatocytes but increases in cell proliferation were observed. The test material was administered to rats at doses of approximately 125, 250, 500, and 1000 mg/kg. Primary hepatocyte cultures for analysis of UDS were prepared at two sacrifice times, approximately 2-4 hours and 15-16 hours after administration of a single oral dose. For each perfusion timepoint, three male rats were treated test material suspended in CMC.

The hepatocyte cultures for UDS were incubated with 10 μCi/ml ^{3}HTdr for about 4 hours and autoradiography was performed on the ^{3}HTdr-treated cultures. After autoradiography, three treatment groups from each timepoint were selected for analysis of nuclear labeling beginning with the highest dose where cellular morphology was adequate for analysis and proceeding to successively lower doses. None of the criteria used to indicate UDS was approached by the treatments and no dose-related response was observed. The test material, T-5711.1 was therefore evaluated as inactive for the induction of UDS in rat hepatocytes after in vivo treatment and in vitro culture.

In the cell proliferation assay, dose-related increases in cell proliferation were observed following treatment with the test material. Five animals per condition were labeled with BrdU for 72 hours using ALZET® osmotic pumps. Samples of the liver and duodenum were fixed in 10% neutral Formalin and later embedded in paraffin. Samples were also processed for analysis by a pathologist.

Sections from the left lateral, median and right lateral lobes of the livers as well as samples from the duodenum were taken 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. A dose-related increase in cell proliferation was induced and statistically significant increases in cell proliferation were observed at 500 mg/kg and 1000 mg/mg.

T-5711.1 was therefore considered negative for the induction of UDS and positive for the induction of cell proliferation in rat liver cells.

HAZLETON
WASHINGTON

Genotoxicity Test on T-5711.1 in
In Vivo/*In Vitro* Unscheduled DNA Synthesis and Cell Proliferation
in Rat Liver Cells

I. SPONSOR: 3M Corporation

II. MATERIAL TESTED:

A. Genetics Assay No.: 15515

B. Identification: T-5711.1, L-1276

C. Physical Description: cream-colored granular material

D. Date Received: February 24, 1993

III. TYPE OF ASSAYS: *In Vivo/In Vitro* Rat Primary Hepatocyte Unscheduled DNA Synthesis Assay with Two Timepoints and Cell Proliferation

IV. PROTOCOL NUMBER: 494, Modified for 3M Corporation

V. STUDY DATES:

A. Study Initiation Date: February 24, 1993

B. Experimental Start Date: March 11, 1993

C. Experimental Termination Date: April 29, 1993

VI. SUPERVISORY PERSONNEL:

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

B. Scientist: Marie E. McKeon, M. Phil.

C. Study Supervisor: Andrea L. Ham, B.S.

VII. OBJECTIVE:

The objective of this assay was to detect DNA damage and/or hepatotoxicity caused by the test material by measuring DNA repair as unscheduled DNA synthesis (UDS) and cell proliferation (CP) measured as

S-phase induction induced in rat liver cells after in vivo treatment. The existence and degree of DNA damage were inferred from an increase in net nuclear grain counts in hepatocytes obtained from treated animals when compared to those from untreated animals. The types of DNA damage are unspecified but must be recognizable by the cellular repair system and result in the incorporation of new bases (including ^{3}H-thymidine) into DNA during a short (4 hours) in vitro culture period (1).

Cell proliferation measured the fraction of cells undergoing cell replication in rat liver using an immunohistochemical technique (2,3) 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 (4).

VIII. DEFINITION:

The UDS assay is designed to measure unscheduled DNA synthesis (UDS) in rat liver cells (hepatocytes) using the autoradiographic technique described by Williams, 1980 (5). Hepatocytes were isolated from the livers of rats exposed in vivo to the test article. Only a small percentage of the cells enter S-phase (replicative DNA synthesis) during the brief exposure period, so the incorporation of ^{3}HTdr into DNA during in vitro culturing, as analyzed by autoradiography, may be used as a measure of the repair of DNA damage caused by treatment with the test article. This UDS measurement of DNA repair appears to correlate well with known mutagenic or carcinogenic activities of chemicals.

Hepatotoxicants such as carbon tetrachloride and dinitrotoluene induce an increase in cell proliferation to replace necrotic tissue (3,6). 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 (7-10). 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, Inc. (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 tail tattoo. 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 liver cells for the UDS assay were obtained from rats weighing 313.0-389.6 grams for the early timepoint and 324.4-404.6 grams for the later timepoint. The cells were obtained by perfusion of the livers in situ with a collagenase solution (see Section X.C. UDS Assay). Monolayer cultures were established in culture dishes and were used the same day for analysis of the UDS activity. All cultures were maintained as monolayers at about 37°C in a humidified atmosphere containing approximately 5% CO_2.

For the cell proliferation assay, rats were used that ranged from 408.4-483.2 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. Media For UDS Assay

The cell cultures were established in Williams' Medium E supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 µg/ml streptomycin sulfate, and 150 µg/ml gentamicin (WME+). WME+ without serum is referred to as WMEI. After the establishment period, cultures were refed with WMEI containing 10 µCi/ml ^{3}HTdr, 47 Ci/mMole (WME-treat).

C. Osmotic Pumps and Label for Cell Proliferation Analysis

ALZET® osmotic pumps (ALZA Corporation, Palo Also, CA), Model 2ML1 were used. A single lot (#042205) 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.

D. Control Articles

1. Vehicle control

A vehicle negative control consisting of three rats for the UDS assay and five rats for cell proliferation treated by oral gavage with the vehicle, CMC (high viscosity carboxymethylcellulose, 9004-32-4, Sigma Lot # 121F0644), was performed at all timepoints. Vehicle control hepatocytes or tissues were subjected to all of the manipulations used for those derived from treated animals. 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 UDS or S-phase in rat hepatocytes in vivo. The positive control for the UDS timepoints, Dimethylnitrosamine (DMN, CAS# 62-75-9, Sigma Chemical Co., Lot# 29F0679) was dosed at 10 mg/kg for the 2-3 hours timepoint and 15 mg/kg for the 15-16 hours timepoint. For cell proliferation, 15.0 mg/kg of DMN was used as the positive control. Three rats for UDS and five rats for cell proliferation were treated by intraperitoneal injection for each timepoint.

E. Test Article

For the preparation of the dosing solutions of the test article, 0.5% CMC was made by adding CMC powder to deionized water with stirring. The test article was suspended in 0.5% CMC at concentrations of 12.5, 25.0, 50.0 and 100 mg/ml prior to dosing for each timepoint. The maximum dosing volumes for the test article did not exceed 10 ml/kg.

X. EXPERIMENT DESIGN (UDS ASSAY):

A. Dosing Procedure

For each timepoint, three rats were treated by oral gavage with the test article. 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 Selection and Perfusion Time

The highest dose of 1000 mg/kg was selected according to protocol, for both timepoints based on information supplied by the Sponsor. Three additional doses of test material were prepared using 2-fold dilution steps and a minimum of 3 animals per dose. Two timepoints were employed for sacrifice; 2-4 hours and 15-16 hours after the administration of a single dose of the test article by oral gavage.

C. UDS Assay

This assay was based on the procedures in rats described by Williams (1980), Mirsalis, Tyson and Butterworth (1982) and Butterworth et al. (1987). The hepatocytes were obtained by perfusion of livers in situ for about four minutes with Hanks' balanced salts (Ca^{++} - Mg^{++}-free) containing 0.5 mM ethyleneglycol-bis (β-aminoethyl ether)-N, N-tetraacetic acid (EGTA), and HEPES buffer at pH 7.2. Then WMEI containing 50-100 units/ml of collagenase (WMEC) was perfused through the liver for 10 - 11 minutes. The hepatocytes were obtained by mechanical dispersion of excised liver tissue in a culture dish containing WMEC. The suspended tissue and cells were allowed to settle to remove cell clumps and debris. The cell suspension was centrifuged and the cell pellet resuspended in WME+. After obtaining a viable cell count, a series of 35-mm culture dishes (at least 6 per animal containing a 25-mm round, plastic coverslip and at least 2 per animal to assess attachment efficiency) was inoculated for each animal with approximately 0.5×10^6 viable cells in 3 ml of WME+ per dish. Individual cultures were identified with the animal eartag number.

An attachment period of 1.7-2.0 hours at about 37°C in a humidified atmosphere containing 5% CO_2 was used to establish the cell cultures for the earlier timepoint. For the latter timepoint, an attachment period of 1.7-2.0 hours was used. After the attachment period, unattached cells were removed and the cultures were refed with 2.5 ml WME-treat. Three of the replicate cultures from each animal were used for the UDS assay; two of the replicates were used to assess attachment. Any remaining cultures were kept for analysis in the event of technical problems with autoradiography. Attachment efficiency was determined for two cultures from each animal by in situ microscopic analysis, using trypan blue dye exclusion to determine the viability of the attached cultures.

After a labeling period of about 4 hours labeled cell cultures were refed with WMEI containing 0.25 mM thymidine and returned to the incubator for 18.7-19.2 hours (2-4 hours timepoint) or 17.8-18.2 hours (15-16 hours timepoint). Nuclei were then swollen by

addition of 1% sodium citrate to the coverslips (containing the cell monolayers) for 10 minutes minutes. The cells were next fixed in three changes of acetic acid:ethanol (1:3) and dried for at least 24 hours. The fixed coverslips were mounted on glass slides, dipped in Kodak NTB2 emulsion, and dried. The emulsion coated slides were stored for 7 days at 4°C in light-tight boxes containing Drierite®. The emulsions were then developed in D19, fixed, and stained with Williams' modification of a hematoxylin and eosin procedure.

D. UDS Analysis

The cells were examined microscopically at approximately 1500x magnification under oil immersion and the field was displayed on the video screen of an automatic counter. UDS was measured by counting nuclear grains and subtracting the average number of grains in three nuclear-sized areas adjacent to each nucleus (cytoplasmic count). This value is referred to as the net nuclear grain count. The coverslips were coded to prevent bias in grain counting.

The net nuclear grain count was determined for fifty randomly selected cells on each coverslip (three coverslips per animal) unless otherwise indicated. Only nuclei with normal morphologies were scored, and any occasional nuclei blackened by grains too numerous to count were excluded as cells in which replicative DNA synthesis occurred rather than repair synthesis. The average mean net nuclear grain count (± standard deviation) was determined from the triplicate coverslips (150 total nuclei) for each animal and averaged for each treatment condition.

XI. EXPERIMENT DESIGN: CELL PROLIFERATION ANALYSIS

A. Treatment and Dose Levels

All animals were dosed as described in the UDS section. Five animals gram each dose level and control group were used to analyze cell proliferation after a single oral dose.

B. Implantation of Osmotic Pumps

ALZET® Model 2ML1 osmotic pumps (Lot #042205) 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.

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 liver, 5 μ paraffin embedded sections were taken from the left lateral, right 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.

D. Immunohistochemical Staining

The slides were deparaffinized and rehydrated prior to staining using first the BIOGENIX Supersensitive Kit using DAB stain and hematoxylin counterstain. Parallel slides were stained with hematoxylin and eosin for analysis by a pathologist.

E. 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. Labeling was similar among the lobes therefore cell counting was performed with sections from the left lateral lobe. 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:

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

XII. ASSAY ACCEPTANCE AND EVALUATION CRITERIA: UDS

An assay normally will be considered acceptable for evaluation of the test results only if all of the criteria listed below are satisfied. This listing may not encompass all test situations, so the study director must exercise scientific judgment in modifying the criteria or considering other causes that might affect assay reliability and acceptance.

1. The viability of the hepatocytes collected from the perfusion process normally exceeds 70%. A variety of factors can affect cell yield and viability, so values below 70% are not uncommon nor necessarily detrimental. Toxicity of treatment with test article may be reflected in perfusion viability, therefore no lower limit will be set.

2. The viability of the monolayer cell cultures used for the assay treatments must be 70% or greater. Normally, the viability of attached cells is about 85%.

3. The positive control is used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS. For test materials causing weak or no UDS activity, the average response to the positive control treatments must exceed both criteria used to indicate UDS. For test materials clearly causing a dose-related UDS activity, an assay will be acceptable in the absence of a positive control lost for technical reasons.

4. Grain count data obtained per animal is acceptable as part of the evaluation if obtained from two replicate cultures and at least 50 nuclei per culture. Grain count data should be available from 2 of the 3 animals treated.

5. A minimum of 3 dose levels will be analyzed at each timepoint. Repeat trials need only augment the number of analyzed dose levels in the first trial to achieve a total of 3 dose levels but must include at least one dose previously assayed as acceptable.

Several criteria have been established which, if met, provide a basis for evaluation of a test material as active in the UDS assay. The criteria for a positive response are based on a

statistical analysis of the historical data and calculation of the minimum increase required for a significant UDS response as described by Casciano and Gaylor (11).

The test material is considered active in the UDS assay at doses that cause:

1. An increase in the mean net nuclear grain count to at least five grains per nucleus above the concurrent negative control average, leading to a positive number and/or

2. The percent of nuclei with five or more net grains to increase at least 10% above the average of the concurrent negative control animals.

Generally, if the first condition is satisfied, the second will also be met. However, satisfaction of only one condition can also indicate UDS activity. Different DNA-damaging agents can give a variety of nuclear labeling patterns, and weak agents may strongly affect only a minority of the cells. Therefore, both of the above conditions are considered in an evaluation. In cases where increases are not observed in all three animals, the test material will be considered active for that condition if cells from two of the three animals show increases. If the negative control animals show an average less than -5.00 or more than 1.00 grains per nucleus, the assay will normally be considered invalid.

The test material is considered inactive in this assay if none of the above conditions are met in any of the treated animals.

When results are neither clearly positive nor clearly negative, the presence of a dose response, the frequency distribution of cellular responses, and the reproducibility of data among slides is considered; the test article is then classified as "negative", "weak positive" or "equivocal". A group in which one of three animals shows increases in nuclear labeling will be decided on a case by case basis depending on the level of activity in cells from the active animal, the level of activity in cells from the inactive animals and the presence or absence of activity in surrounding groups.

The positive control nuclear labeling is not used as a reference point to estimate mutagenic or carcinogenic risk associated with the UDS activity of the test material. UDS elicited by test agents in this assay is probably more dependent on the type of DNA damage inflicted and the available repair mechanisms than on the potency of the test agent as a mutagen or carcinogen. Some forms

of DNA damage are repaired without the incorporation of new nucleic acids. Thus, the positive controls are used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS.

XIII. ASSAY EVALUATION CRITERIA: CELL PROLIFERATION

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). 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.

XIV. INTERPRETATION OF UDS RESULTS:

At the request of the Sponsor, the test material, T-5711.1 was suspended in 0.5% CMC at a concentration of 100 mg/ml and serial two-fold dilutions were used to prepare suspensions of 50.0, 25.0 and 12.5 mg/ml. The test material appeared to form a uniform suspension in the vehicle. Individual dosing stocks were prepared for each timepoint just prior to dosing . Three rats per dose level per treatment were treated with 125, 250, 500, and 1000 mg/kg with volumes which did not exceed 10 ml/kg.

For the early timepoint, perfusions were initiated 2.3-2.6 hours after administration of a single dose of the test material. The hepatocytes collected for the UDS assay ranged in viability (determined by trypan blue exclusion) from 68.3%-94.2% of the total cells collected in the perfusate (Table 1). The attachment efficiency varied from 39.2%-87.1% and the viability of the attached cells was very good, ranging from 74.3%-95.9%.

The minimum criteria for a UDS response at this timepoint were determined by comparison to the averages of the concurrent negative control treatments (Table 2). The average mean net nuclear grain count for the negative control animals was -0.37 and the average percent of cells containing five or more net nuclear grains was 3.6%. A positive response consisted of average mean net nuclear grain counts exceeding 4.63 (5 net grains above the control value) or at least 13.6% of the nuclei containing five or more grains (10% above the average negative control value). None of the treatments with the test material samples caused nuclear labeling significantly different from the negative control. Furthermore, no dose-related trend was evident. In contrast, the DMN treatments induced large increases in nuclear labeling that

greatly exceeded criteria used to indicate UDS. Since the positive control animals were responsive, the test results were considered to provide conclusive evidence for the lack of UDS induction by the test material samples at the 2-4 hours timepoint under these test conditions.

Heavily-labeled nuclei (blackened with numerous grains) represent cells undergoing DNA replication (S-phase) as opposed to DNA repair. For each slide analyzed, at least 500 cells were scanned and the incidence of S-phase calculated and reported for each animal on the basis of at least 1500 cells observed (Table 2). The number present in the 2-4 hours study was low and did not interfere with the assay.

For the later timepoint, perfusions were initiated 14.9-15.5 hours after administration of a single dose of the test material. The hepatocytes collected for the UDS assay ranged in viability (determined by trypan blue exclusion) from 73.4%-96.9%% of the total cells collected in the perfusate (Table 3). The attachment efficiency varied from 60.2%-98.9%% and the viability of the attached cells was very good, ranging from 85.0%-97.0%%.

The minimum criteria for a UDS response at the later timepoint was calculated based upon the average of the negative control animals for the 15-16 hours timepoint (Table 4; average mean net nuclear grains = -0.48, average percent of cells containing five or more net nuclear grains = 0.67%). The criteria were mean net nuclear grain counts exceeding 4.52 or at least 10.8% of the nuclei containing five or more grains. None of the treatments with the test material samples caused nuclear labeling significantly different from the negative control and no dose-related trend was evident. The positive control treatments induced increases in nuclear labeling exceeding the criteria used to indicate UDS. Since the positive control animals responded, the test results were considered to provide conclusive evidence for the lack of UDS induction by the test material samples at the 15-16 hours timepoint under these test conditions.

Heavily-labeled nuclei (blackened with numerous grains) represent cells undergoing DNA replication as opposed to DNA repair. The number observed for each animal in the 15-16 hours study (Table 4) was low and did not interfere with the detection of UDS.

XV. INTERPRETATION OF CELL PROLIFERATION RESULTS:

A. General Observations

Cells stained with the brown DAB chromogen were observed in the duodenum from all of the animals used in the study. No

unscheduled deaths occurred. The presence of label in all the animals indicated proper delivery of the BrdU label and acceptable immunohistochemical staining. One animal from Group 6 (positive control) was eliminated from the study because the results demonstrated a lack of response by the animal. Since DMN is known to induce large increases in cell proliferation (15), this may have represented a dosing error.

The livers of the treated animals showed a dose-related increase in weight compared to control animals. The 5000 mg/kg dose induced a significant increase ($p \leq 0.01$). The mean liver weight of the positive control was not significantly elevated even though large increases in DNA synthesis (and subsequent cell proliferation) were induced. The high dose animals (Group 5) had a mean terminal body weight that was less than control value ($p \leq 0.01$) indicating some toxicity. Lower doses showed dose-related increases that did not reach significant levels.

Slides from treated and control animals were also examined and gross findings at the time of sacrifice were generally sporadic and/or incidental with no apparent relationship to treatment.

B. Summary of Labeled Cell Counts for the Liver

A summary of the labeled cell counts for each group is shown in Table 5. Individual animal counts are shown in Table 6. The mean labeling index (LI) for each group is presented in the third column. 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 0.95 which indicates that less than 1% of the nuclei had undergone DNA synthesis during the 72-hour labeling period. Dose-related increases in the labeling index were induced by T-5711.1 with significant increases ($p \leq 0.01$) observed in the dosed Groups 4 and 5 (500 and 1000 mg/kg). The labeling indices at 500 and 1000 mg/kg were 9.06% and 8.13% respectively which represents an 8- to 9-fold increase over background. The mean labeling index of the positive control animals was 31.51 which is significantly elevated ($p \leq 0.01$).

These results demonstrate that T-5711.1 induced significant increases in the LI in the liver in male rats after a single oral dose at concentrations of 2500 mg/kg and 5000 mg/kg. Large increases in the labeling index were also observed in the positive control animals. The mean labeling index of the positive control animals was 31.51.

XVI. CONCLUSIONS

The test material, T-5711.1 did not induce significant changes in the nuclear labeling of rat primary hepatocytes at either the 2-4 hours timepoint or the 15-16 hours timepoint when doses of 250, 500 and 1000 mg/kg were delivered by oral gavage. T-5711.1 was therefore evaluated as inactive in the induction of UDS in rat liver cells.

In contrast, the test material induced significant changes in the number S-phase cells following a single oral dose of T-5711.1. The animals were labeled for 72 hours and dose-related increases in the mean labeling index were observed in the treated groups. T-5711.1 was therefore evaluated as active in the induction of DNA synthesis in rat liver cells.

XVII. REFERENCES

1. Mirsalis, J.C., Tyson, C.K., and B.E. Butterworth: Detection of genotoxic carcinogens in the in vivo - in vitro hepatocyte DNA repair assay. Environ. Mutagenesis, 4:553-562, 1982.

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

3. 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.

4. 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 Res., 189:123-133, 1987.

5. Williams, G.M.: The detection of chemical mutagens-carcinogens by DNA repair and mutagenesis in liver cultures, In: Chemical Mutagens, Vol. 6. F. De Serres and A. Hollaender, (Eds.), Plenum Press, NY, pp. 61-79, 1980.

6. 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.

7. 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.

8. 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.

9. 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.

10. 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.

11. Casciano, D.A. and D.W. Gaylor (1983). Statistical criteria for evaluating chemicals as positive or negative in the hepatocytes DNA repair assay. Mutation Research, 122 81-86.

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

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

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

15. Ham, A. and Cifone, M.A. (1991). Use of cell proliferation to study liver effects induced by a single dose of DMN. Environmental and Molecular Mutagenesis 17(19), 16.

XVIII. EXPERIMENTAL DATA TABLES

HAZLETON
WASHINGTON

TABLE 1

SUMMARY OF CULTURE DATA FROM IN VIVO/IN VITRO RAT HEPATOCYTE UDS ASSAY
2-4-HOUR TIMEPOINT

Client: 3M Corporation
Client Code: T-5711

HWA Study No.: 15515-0-494
Initiation Date: 16-Mar-93

Test Condition	Animal Number	Target Dose Level(1)	Perfusion Viability	Attachment Efficiency(2)	Attachment Viability(2)
Vehicle Control - CMC (ml/kg)					
	34389	10.0	84.8%	70.4%	90.8%
	34388	10.0	69.1%	82.2%	94.8%
	34387	10.0	94.2%	82.3%	95.9%
Positive Control - DMN (mg/kg)					
	34443	10.0	87.6%	72.5%	92.3%
	34442	10.0	77.2%	76.5%	92.6%
	34441	10.0	93.3%	78.1%	93.0%
Test Material - (mg/kg)					
	34431	1000	68.7%	75.8%	92.0%
	34430	1000	72.2%	65.3%	90.5%
	34429	1000	83.2%	63.6%	89.1%
	34419	500	87.9%	69.5%	91.1%
	34418	500	81.9%	72.4%	95.0%
	34417	500	79.3%	39.2%	74.3%
	34407	250	88.6%	50.5%	85.6%
	34406	250	68.3%	70.0%	92.6%
	34405	250	92.2%	87.1%	94.3%
	34395	100	87.5%	78.1%	91.3%
	34394	100	85.3%	63.5%	87.0%
	34393	100	81.2%	51.1%	89.8%

Notes:

(1) Three animals per dose level were treated.
(2) Results based on viable counts (trypan blue dye exclusion) of randomly selected areas on two plates.
DMN = Dimethylnitrosamine
CMC = 0.5% High Viscosity Carboxymethylcellulose

HAZLETON
WASHINGTON

TABLE 2

SUMMARY OF UDS DATA FROM IN VIVO/IN VITRO RAT HEPATOCYTE UDS ASSAY
2-4-HOUR TIMEPOINT

Client: 3M Corporation
Client Code: T-5711

HWA Study No.: 15515-0-494
Initiation Date: 16-Mar-93

Test Cond.	Animal Number	Target Dose [1]	Mean Net Nuclear Grains (MNNG) ± SD [2]	% Cells w/ ≥ 5 NNG [3]	Mean Cyto. Grains [4]	Percent S-Phase Cells [5]
Vehicle Control - CMC (ml/kg)						
	34389	10.0	-0.92 ± 0.41	4.00	7.45	1.07
	34388	10.0	0.11 ± 0.20	4.00	4.94	0.73
	34387	10.0	-0.29 ± 0.53	2.67	5.68	0.87
Positive Control 1 - DMN (mg/kg)						
	34443	10.0	17.98 ± 2.06	95.33	6.27	0.13
	34442	10.0	8.09 ± 7.98	56.00	4.57	0.33
	34441	10.0	10.45 ± 0.60	89.33	5.52	0.00
Test Material - (mg/kg)						
	34431	1000	-0.15 ± 0.35	2.67	4.19	0.27
	34430	1000	0.03 ± 0.35	3.33	4.55	0.47
(a)	34429	1000	0.04 ± 0.20	1.00	3.35	1.00
	34419	500	0.23 ± 0.20	3.33	4.03	0.47
	34418	500	-0.61 ± 0.58	2.00	4.51	0.40
(a)	34417	500	0.06 ± 0.45	3.00	3.40	0.10
(a)	34407	250	-0.36 ± 0.51	2.00	3.59	0.10
(a)	34406	250	0.06 ± 0.23	1.00	3.40	0.60
	34405	250	-0.49 ± 0.39	0.67	4.49	0.67

Notes:

(1) Three animals per dose level were treated.
(2) Average of net nuclear grain counts on triplicate coverslips (150 total cells) with standard deviation (SD) between coverslips. Net nuclear grains (NNG) = Nuclear grain count - Average cytoplasmic grain count.
(3) Average percentage of cells with greater than or equal to 5 net nuclear grains on triplicate coverslips (150 total cells).
(4) Average of cytoplasmic grain counts on triplicate coverslips (150 total cells).
(5) Determined on triplicate coverslips as the percentage of heavily labeled cells observed when 500 cells per slide (1500 total cells) were examined.
(a) 1 slide not analyzed; UDS = average of mean NNG counts on 2 coverslips (100 total cells).
DMN = Dimethylnitrosamine
CMC = 0.5% High Viscosity Carboxymethylcellulose

HAZLETON WASHINGTON

TABLE 3

SUMMARY OF CULTURE DATA FROM IN VIVO/IN VITRO RAT HEPATOCYTE UDS ASSAY
15 to 16-HOUR TIMEPOINT

Client: 3M Corporation
Client Code: T-5711

HWA Study No.: 15515-1-494
Initiation Date: 18-Mar-93

Test Condition	Animal Number	Target Dose Level(1)	Perfusion Viability	Attachment Efficiency(2)	Attachment Viability(2)
Vehicle Control - CMC (ml/kg)					
	34392	10.0	94.4%	75.7%	90.6%
	34391	10.0	88.8%	66.1%	97.1%
	34390	10.0	73.4%	67.6%	87.0%
Positive Control 1 - DMN (mg/kg)					
	34446	15.0	80.3%	78.4%	88.4
	34445	15.0	86.2%	60.5%	92.5%
	34444	15.0	89.6%	68.2%	93.1%
Test Material - (mg/kg)					
	34434	1000	82.1%	98.9%	90.0%
	34433	1000	79.8%	60.2%	85.0%
	34432	1000	96.9%	78.1%	95.2%
	34422	500	85.6%	78.2%	94.0%
	34421	500	88.7%	87.5%	95.6%
	34420	500	91.1%	79.0%	95.8%
	34410	250	84.1%	92.5%	94.1%
	34409	250	79.7%	73.3%	89.9%
	34408	250	92.5%	81.6%	92.0%
	34398	125	89.1%	91.7%	89.8%
	34397	125	85.2%	96.8%	90.6%
	34396	125	84.8%	78.0%	93.5%

Notes:

(1) Three animals per dose level were treated.
(2) Results based on viable counts (trypan blue dye exclusion) of randomly selected areas on two plates.
DMN = Dimethylnitrosamine
CMC = 0.5% High Viscosity Carboxymethylcellulose

HAZLETON
WASHINGTON

TABLE 4

SUMMARY OF UDS DATA FROM IN VIVO/IN VITRO RAT HEPATOCYTE UDS ASSAY
15 to 16-HOUR TIMEPOINT

Client: 3M Corporation HWA Study No.: 15515-1-494

Client Code: T-5711 Initiation Date: 18-Mar-93

Test Cond.	Animal Number	Target Dose [1]	Mean Net Nuclear Grains (MNNG) ± SD [2]	% Cells w/ ≥ 5 NNG [3]	Mean Cyto. Grains [4]	Percent S-Phase Cells [5]
Vehicle Control - CMC (ml/kg)						
	34392	10.0	-0.17 ± 0.15	1.34	2.79	0.33
	34391	10.0	-0.79 ± 0.57	0.67	2.53	0.20
(a)	34390	10.0	-0.49 ± 0.41	0.00	2.06	0.40
Positive Control 1 - DMN (mg/kg)						
	34446	15.0	3.95 ± 2.86	38.67	2.59	0.00
	34445	15.0	4.05 ± 3.38	38.00	4.58	0.07
	34444	15.0	5.01 ± 2.62	48.00	3.32	0.07
Test Material - (mg/kg)						
	34434	1000	-0.17 ± 0.15	1.33	2.47	0.33
	34433	1000	-0.89 ± 0.01	0.00	2.18	0.13
	34432	1000	-0.37 ± 0.48	0.67	2.85	0.47
	34422	500	-0.52 ± 0.05	0.00	3.33	0.27
	34421	500	-0.91 ± 0.22	0.00	2.07	0.07
	34420	500	-1.21 ± 0.10	0.00	2.33	0.20
	34410	250	-0.58 ± 0.36	0.00	2.22	0.33
(a)	34409	250	0.02 ± 0.14	1.00	2.30	0.00
	34408	250	-0.98 ± 0.64	0.00	2.56	0.13

Notes:

(1) Three animals per dose level were treated.
(2) Average of net nuclear grain counts on triplicate coverslips (150 total cells) with standard deviation (SD) between coverslips. Net nuclear grains (NNG) = Nuclear grain count - Average cytoplasmic grain count.
(3) Average percentage of cells with greater than or equal to 5 net nuclear grains on triplicate coverslips (150 total cells).
(4) Average of cytoplasmic grain counts on triplicate coverslips (150 total cells).
(5) Determined on triplicate coverslips as the percentage of heavily labeled cells observed when 500 cells per slide (1500 total cells) were examined.
(a) 1 slide not analyzed; UDS = average of mean NNG counts on 2 coverslips (100 total cells).
DMN = Dimethylnitrosamine
CMC = 0.5% High Viscosity Carboxymethylcellulose

HAZLETON WASHINGTON

Table 5

Cell Proliferation Summary

Client: 3M Corporation

Client Code: T-5711.1

HWA Assay No.: 15515-0-494

Trial Initiation Date: -Mar-93

Group/Sex[a]	Dose Level (mg/kg)	Labeling Index[b] (%)	Liver Weight (g)	Terminal Body Weight (g)
1M	0[d]	0.95 ± 0.73	18.95 ± 2.64	491.8 ± 41.8
2M	625	1.06 ± 0.56	19.70 ± 4.49	458.4 ± 22.1
3M	1250	2.39 ± 2.25	21.82 ± 1.55	477.9 ± 16.8
4M	2500	9.06 ± 3.13**↑	23.75 ± 2.49	455.1 ± 23.1
5M[c]	5000	8.13 ± 3.15**↑	25.73 ± 2.93**↑	425.4 ± 23.9**↓
6M[c]	15[e]	31.51 ± 15.86**↑	19.48 ± 2.06	459.8 ± 30.1

[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]Vehicle control, 10 ml/kg of corn oil
[e]Positive control, 15 mg/kg of DMN
**Significant at $p \leq 0.01$
↑Increase in the mean
↓Decrease in the mean

HAZLETON
WASHINGTON

Table 6

Cell Proliferation Assay
Individual Animal Data

Animal Number	Group /Sex	Mean Labeling Index (%)	Terminal Body Weight (g)	Terminal Liver Weight (g)
34489	1M	0.95	521.3	19.17
34490	1M	0.76	509.7	21.25
34491	1M	0.14	419.6	15.12
34492	1M	0.76	492.3	17.74
34493	1M	2.14	516.1	21.47
Group Mean		0.95	491.8	18.95
Group SD		0.73	41.8	2.64
N		5	5	5
34494	2M	1.14	492.2	27.31
34495	2M	1.67	468.8	19.84
34496	2M	1.24	440.9	15.80
34497	2M	0.14	450.0	17.77
34498	2M	1.10	440.3	17.76
Group Mean		1.06	458.4	19.70
Group SD		0.56	22.1	4.49
N		5	5	5
34499	3M	6.24	456.2	21.98
34500	3M	2.19	500.7	24.28
34501	3M	1.62	486.3	20.22
34502	3M	0.38	476.4	20.84
34503	3M	1.52	470.1	21.79
Group Mean		2.39	477.9	21.82
Group SD		2.25	16.8	1.55
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)
34504	4M	11.81	437.5	24.69
34505	4M	9.24	432.4	26.99
34506	4M	10.86	446.6	22.45
34507	4M	3.76	484.3	20.35
34508	4M	9.62	474.5	24.26
Group Mean		9.06	455.1	23.75
Group SD		3.13	23.1	2.49
N		5	5	5
34509	5M	6.86	427.5	28.37
34510	5M	12.14	455.8	28.17
34512	5M	8.81	397.9	23.22
34513	5M	4.71	420.5	23.16
Group Mean		8.13	425.4	25.73
Group SD		3.15	23.9	2.93
N		4	4	4
34514	6M	22.14	499.3	22.09
34515	6M	47.43	448.5	19.45
34516	6M	42.24	428.0	17.06
34517	6M	14.24	463.3	19.31
Group Mean		31.51	459.8	19.48
Group SD		15.86	30.1	2.06
N		4	4	4

XIX. APPENDIX A: HISTORICAL CONTROLS (UDS)

HAZLETON
WASHINGTON

HISTORICAL NEGATIVE CONTROLS

IN VIVO/IN VITRO UNSCHEDULED DNA SYNTHESIS ASSAY

Number of data points is 20

Data Point	UDS Grains/ Nucleus ± SD *	% of Nuclei with ≥5 Net Nuclear Grains **	Average Cyto Grains **
1	-0.23 ±0.33	2.7	6.17
2	-1.39 ±0.49	0.0	7.76
3	-0.99 ±0.47	1.3	8.19
4	0.12 ±0.89	4.7	7.69
5	-1.43 ±0.76	0.7	10.61
6	-0.55 ±0.45	0.0	3.55
7	-0.67 ±0.32	0.0	3.93
8	0.11 ±0.63	0.0	2.66
9	-0.11 ±0.25	0.7	2.66
10	-0.05 ±0.39	0.0	4.25
11	-1.05 ±0.31	0.0	2.79
12	-0.01 ±0.89	6.7	7.55
13	-0.38 ±0.30	1.3	8.55
14	-0.27 ±0.22	2.7	6.58
15	-1.38 ±0.31	0.7	9.89
16	-0.18 ±0.33	4.0	8.18
17	-0.57 ±0.34	3.3	8.97
18	-0.21 ±0.47	2.0	7.94
19	-0.59 ±0.38	0.0	7.75
20	-0.62 ±0.52	0.0	7.20
Average:	-0.52	1.5	6.64
SD[a]	±0.50	±1.9	± 2.47
Range:			
Low	-1.43	0.0	2.66
High	0.12	6.7	10.61

* UDS = Average of net nuclear grain counts ± standard deviation from triplicate or duplicate coverslips (150 cells) analyzed for a single animal.

** Average values for triplicate or duplicate coverslips for a single animal.

a SD = Standard Deviation

HAZLETON
WASHINGTON

HISTORICAL POSITIVE CONTROLS

IN VIVO/IN VITRO UNSCHEDULED DNA SYNTHESIS ASSAY

4-Hour Timepoint

Number of data points is 9

Data Point	UDS Grains/ Nucleus ±SD *	% of Nuclei with ≥5 Net Nuclear Grains **	Average Cyto Grains **
1	22.27 ±2.36	98.0	7.46
2	23.25 ±1.08	98.7	7.01
3	22.25 ±1.96	95.3	5.84
4	19.44 ±0.75	100.0	8.43
5	24.03 ±4.22	98.7	4.99
6	29.75 ±7.98	96.0	5.26
7	19.05 ±1.76	98.0	6.56
8	17.47 ±1.78	98.0	6.65
9	16.83 ±4.00	97.3	5.74
Average:	21.59	97.8	6.44
SD[a]	±3.98	±1.4	±1.10
Range:			
Low	16.83	95.3	5.26
High	29.75	100.0	8.43

* UDS = Average of net nuclear grain counts ± standard deviation from triplicate or duplicate coverslips (150 cells) analyzed for a single animal.

** Average values for triplicate or duplicate coverslips for a single animal.

a SD = Standard Deviation

HAZLETON
WASHINGTON

HISTORICAL POSITIVE CONTROLS

IN VIVO/IN VITRO UNSCHEDULED DNA SYNTHESIS ASSAY

15-Hour Timepoint

Number of data points is 12

Data Point	UDS Grains/ Nucleus ±SD *	% of Nuclei with ≥5 Net Nuclear Grains **	Average Cyto Grains **
1	13.78 ±1.52	80.7	9.94
2	12.65 ±2.30	76.7	6.05
3	12.23 ±2.84	76.7	7.78
4	7.77 ±1.73	62.0	5.71
5	9.26 ±1.15	69.4	5.00
6	8.87 ±1.15	66.0	5.52
7	9.14 ±1.48	80.7	4.17
8	6.01 ±2.70	50.0	2.73
9	5.66 ±1.99	46.0	2.51
10	10.15 ±2.13	68.7	7.15
11	11.02 ±1.17	71.3	6.09
12	15.40 ±1.07	84.7	5.61
Average:	10.16	69.4	5.69
SD[a]	±2.99	±12.0	± 2.06
Range:			
Low	5.66	50.0	2.51
High	15.40	84.7	9.94

* UDS = Average of net nuclear grain counts ± standard deviation from triplicate or duplicate coverslips (150 cells) analyzed for a single animal.

** Average values for triplicate or duplicate coverslips for a single animal.

a SD = Standard Deviation

HAZLETON
WASHINGTON

HWA Study No._____
Protocol No. 494
Modified for 3M Corporation

IN VIVO/IN VITRO UNSCHEDULED DNA SYNTHESIS AND CELL PROLIFERATION IN RAT LIVER CELLS

Hazleton Washington, Inc. (HWA) will conduct this study in compliance with EPA and FDA Good Laboratory Practice (GLP) Guidelines. This protocol, critical phases 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-021 3M Center, St. Paul MN 55144-1000

II. TEST ARTICLE IDENTIFICATION: _____

_____

III. TEST ARTICLE ANALYSIS

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

IV. NOTIFICATION OF REGULATORY SUBMISSION

In order to comply with U.S. federal regulation codes (FDA, 21 CFR 58.10; EPA-TSCA, 40 CFR 792.10; EPA-FIFRA, 40 CFR 160.10) and certain foreign agencies, consulting laboratories must be notified if all or part of a study is to be submitted to the agency. HWA maintains a master schedule of studies which fall under regulatory review. Please indicate which agency, if any, might receive the results of this study:

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

☐ MAFF ☐ MOHW ☐ OECD ☐ OTHER _____

V. STUDY DATES

Proposed Experimental Start Date: _____

Proposed Experimental Termination Date: _____

VI. APPROVAL OF STUDY PROTOCOL

Study Director:

_____ Date: _____
Maria A. Cifone, Ph.D.

Sponsor's Authorized Representative:

_____ Date: _____

PART 2. STUDY PROTOCOL

IN VIVO/IN VITRO UNSCHEDULED DNA SYNTHESIS AND CELL PROLIFERATION IN RAT LIVER CELLS

I. OBJECTIVE

The objective of this assay is to detect DNA damage and/or hepatotoxicity caused by the test material by measuring DNA repair as unscheduled DNA synthesis (UDS) and cell proliferation (CP) measured as S-phase induction induced in rat liver cells after in vivo treatment.

The existence and degree of DNA damage will be inferred from an increase in net nuclear grain counts (UDS) compared to untreated rats. The types of detectable DNA damage are unspecified but must be recognizable by the cellular repair system and result in the incorporation of new bases (including ^{3}H-TdR) into the DNA, during a short (4 hours) in vitro culture period (1).

Cell proliferation is designed to measure the fraction of cells undergoing cell replication in rat liver using an immunohistochemical technique (2,3). 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 (4).

II. DEFINITION

The UDS assay is designed to measure unscheduled DNA synthesis (UDS) in rat liver cells (hepatocytes) using the autoradiographic technique described by Williams, 1980 (5). Hepatocytes will be isolated from the livers of rats exposed in vivo to the test article. The incorporation of ^{3}H-TdR into DNA during in vitro culturing, as analyzed by autoradiography, will be used as a measure of the repair of DNA damage caused by treatment with the test article. This UDS measurement of DNA repair appears to correlate well with known mutagenic or carcinogenic activities of chemicals.

Hepatotoxicants such as carbon tetrachloride and dinitrotoluene induce an increase in cell proliferation to replace necrotic tissue (3,6). 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 (7-10). 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. Indicator Cells

The indicator cells for this assay will be liver cells obtained from adult, male, Fischer 344 rats, (weighing 150 to 300 g), purchased from Harlan Sprague Dawley Incorporated or another reputable dealer. 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 identified by ear tag prior to random assignment to study groups. The rats to be used for the assay will be anesthetized before surgery. For the UDS assay, sodium pentobarbital at about 60 mg/kg, will be used to anesthetize the rats in order to obtain the hepatocytes prior to exsanguination during the harvest procedure.

The cells will be obtained by perfusion of the liver in situ with a collagenase solution (see Section IV, Experimental Design: UDS Assay). Monolayer cultures will be established on plastic coverslips in culture dishes and used the same day for analysis of UDS. All cultures will be maintained as monolayers at approximately 37°C in a humidified atmosphere containing about 5% CO_2.

For the cell proliferation assay, 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. Medium for UDS Assay

Cell cultures will be established in Williams' Medium E supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 µg/ml streptomycin and 150 µg/ml gentamicin (WME+). WME+ without serum is referred to as WMEI. After the establishment period, cultures will be refed with WMEI containing 10 µCi/ml ^{3}H-Tdr (40-60 Ci/mMole) (WME-treat).

C. 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.

D. Control Articles

1. Vehicle control

A vehicle negative control consisting of a minimum of three rats for the UDS assay and 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 UDS or S-phase in rat hepatocytes in vivo. The positive control for the UDS timepoints (2-4 hours and 15 to 16 hours), dimethylnitrosamine (DMN) will be dosed at 10 to 20 mg/kg. For cell proliferation, 20 to 25 mg/kg of DMN will be used as the positive control. At least three rats for UDS and five rats for cell proliferation will be treated by intraperitoneal injection for each endpoint.

E. 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 :UDS ASSAY

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 and will be dosed by intraperitoneal injection. 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 and Perfusion Time

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 and a minimum of three animals per dose per timepoint. Two timepoints will be performed for UDS, one approximately 15 to 16 hours after a single dose of the test material for the UDS timepoint and another 2-4 hours after dosing.

C. Preparation of Hepatocytes

This assay is based on the procedures described by Williams, 1980 (5), Mirsalis, Tyson and Butterworth, 1982 (1) and Butterworth et al, 1987 (4). The hepatocytes will be obtained by perfusion of livers in situ for about 4 minutes with Hanks' balanced salts (Ca++- and Mg++-free) containing 0.5 mM ethyleneglycol-bis(ß-aminoethyl ether)-N, N-tetra-acetic acid (EGTA) and 50 mM HEPES buffer at pH 7.2. WMEI with 50 to 100 units/ml of collagenase will be perfused through the liver for about 10 - 11 minutes. Depending on the condition of the liver, this time may be altered by ± 2 minutes. The hepatocytes will be obtained by mechanical dispersion of excised liver tissue in a culture dish containing the WMEI culture medium and collagenase. The suspended tissue and cells will then be allowed to settle to remove cell clumps and debris. The cell suspension will be centrifuged and the cell pellet resuspended in WME+. After obtaining a viable cell count, a series of culture dishes will be inoculated with approximately 0.5×10^6 viable cells in 3 ml of WME+. Culture dishes that will be used for the UDS assay will contain round plastic coverslips. Dishes used to assess attachment efficiency will have no coverslips. Cultures will be identified with the animal eartag number.

An attachment period of 1.5 to 2 hours at 37°C in a humidified atmosphere containing 5% CO_2 will be used to establish cell cultures. Unattached cells will then be removed and cultures will be refed with 2.5 ml WME-treat. Three of the replicate cultures from each animal will be used for the UDS assay; two replicates will be used to assess attachment. Any remaining cultures will be kept for analysis in the event of technical problems with autoradiography.

Attachment efficiency will be determined for two cultures from each animal using trypan blue dye exclusion and in situ analysis.

After a labeling period of about 4 hours, labeled cell cultures will be refed with WMEI containing 0.25 mM thymidine and returned to the incubator for 16 to 20 hours. The nuclei will then be swollen by addition of 1% sodium citrate to the cultures (containing cell monolayers) for 8 - 12 minutes. Next the cells will be fixed in acetic acid:ethanol (1:3) and dried for at least 24 hours. The coverslips will be mounted on glass slides, dipped in an emulsion of Kodak NTB2 and water and dried. The emulsion-coated slides will be stored for 7 to 10 days at 4°C in light-tight boxes containing Drierite. The emulsions will then be developed in D19, fixed, and stained with Williams' modified hematoxylin and eosin procedure.

D. UDS Analysis

For the UDS analysis, the cells will be examined microscopically at approximately 1500x magnification under oil immersion and the field displayed on the video screen of an automatic counter. UDS will be measured by counting nuclear grains and subtracting the average number of grains in three nuclear-sized areas adjacent to each nucleus (cytoplasmic count). This value is referred to as the net nuclear grain count. The coverslips will be coded to prevent bias in grain counting.

The net nuclear grain count will be routinely determined for 50 randomly selected cells on each coverslip analyzed for UDS. Only normally appearing nuclei will be scored, and any occasional nuclei blackened by grains too numerous to count will be excluded as cells in which replicative DNA synthesis occurred rather than repair synthesis. The mean net nuclear grain count will be determined from triplicate coverslips (150 total nuclei) for each treated animal. Occasionally, a coverslip may be recounted at a later date or by a different technician. Since a different cell population will generally be scored, the average of these repeated counts for 50 cells will be used in the calculation of the mean for each animal.

V. EXPERIMENTAL METHODS: CELL PROLIFERATION ANALYSIS

A. Treatment and Dose Levels

All animals will be dosed as described in the UDS section. Five animals from each dose level and control group will be used to analyze cell proliferation at 72-hours.

B. Implantation of Osmotic Pumps

ALZET® Model 2ML1 osmotic pumps will be preloaded with 2000 µl of BrdU at a concentration of 20 mg/ml. 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.

C. 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, 5 μ paraffin embedded sections will be taken from the left lateral, right median, and right anterior lobes. Similarly prepared sections of the duodenum will also be made. The sections will be mounted on slides and a sample from the duodenum will be included on each slide. Slides will also be prepared for analysis by a pathologist to determine if any abnormalities are observed.

D. Immunohistochemical Staining

The slides will be deparaffinized and rehydrated prior to staining using 1) the BIOGENIX Supersensitive Kit using DAB stain and hematoxylin counterstain, and 2) hematoxylin and eosin.

E. 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 will be examined to determine if differences in labeling are observed. If a significant difference in labeling index (LI) among the liver lobes is observed, all the lobes will be counted. If no differences are observed, labeled hepatocytes in the left 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.

VI. 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:

For UDS:

- The mean net nuclear grain count for triplicate cultures (usually a total of 150 cells) ± standard deviation for each animal analyzed for UDS.
- The mean percent of cells containing five or more net nuclear grains for triplicate cultures for each animal analyzed for UDS.
- The average cytoplasmic count for triplicate cultures for each animal analyzed for UDS.
- The level of scheduled DNA synthesis (percent of heavily labeled cells) for each animal.
- Perfusion and culture data for individual animals.

For Cell Proliferation:

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

VII. ASSAY ACCEPTANCE AND EVALUATION CRITERIA: UDS

An assay normally will be considered acceptable for evaluation of the test results only if all of the criteria listed below are satisfied. This listing may not encompass all test situations, so the study director must exercise scientific judgment in modifying the criteria or considering other causes that might affect reliability and acceptance.

1. The viability of the hepatocytes collected from the perfusion process normally exceeds 70%. A variety of factors can affect cell yield and viability, so values below 70% are not uncommon nor necessarily detrimental. The toxicity of the treatment with the test article may be reflected in perfusion viability, therefore no lower limit will be set.

2. The viability of the monolayer cell cultures used for the assay treatments must be 70% or greater. Normally, the viability of attached cells is about 85%.

3. The positive control is used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS and S-phase. For test materials causing weak or no UDS activity, the average response to the positive control treatments must exceed either criteria used to indicate UDS. The positive control for S-phase must have greater than 1% of the cells in scheduled DNA synthesis. For test materials clearly causing a dose-

related UDS or S-phase activity, an assay will be acceptable in the absence of a positive control lost for technical reasons.

4. Data obtained per animal will be acceptable as part of the evaluation if obtained from two replicate cultures and at least 50 nuclei per culture for UDS. Data should be available from 2 of the 3 animals treated.

5. A minimum of 3 dose levels will be analyzed for both UDS timepoints. Repeat trials need only augment the number of analyzed dose levels in the first trial to achieve a total of 3 concentrations, but must include at least one dose previously assayed as acceptable.

Several criteria have been established which, if met, will provide a basis for evaluation of a test article as active in the UDS assay. The criteria for a positive response are based on a statistical analysis of the historical data as described by Casciano and Gaylor (11).

1. The test article will be considered active in the UDS assay at applied concentrations that cause:

- An increase in the mean net nuclear grain count to at least five grains per nucleus above the concurrent negative control average leading to a positive number, and/or;

- An increase in the number of nuclei with five or more net grains such that the average percentage of these nuclei in test cultures is 10% above the percentage in negative control cultures.

Generally, if the first condition is satisfied, the second condition will also be met. However, satisfaction of only one condition can also indicate UDS activity. Different DNA-damaging agents can give a variety of nuclear labeling patterns, and weak agents may strongly affect only a small minority of the cells. Therefore, both of the above conditions will be considered in an evaluation. In cases where increases are not observed in all three animals, the test material will be considered active for that condition if cells from two of the three animals show increases. If the negative control cultures show an average less than -5 or more than one grain per nucleus, the assay will normally be considered invalid.

2. The test article is considered negative if none of the above criteria are met.

When results are neither clearly positive nor clearly negative, the presence of a dose response, the frequency distribution of cellular responses, and the reproducibility of data among animals is considered

in the evaluation. Groups in which one out of three animals shows increases in labeling will be decided on a case by case basis depending on the level of activity in cells from the active animal, the level of activity in cells from the inactive animals and the presence or absence of activity in surrounding groups.

The positive control nuclear labeling will not be used as a reference point to estimate mutagenic or carcinogenic risk associated with the UDS activity of the test article. UDS elicited by test agents in this assay is probably more dependent on the type of DNA damage inflicted and the available repair mechanisms than on the potency of the test agent as a mutagen or carcinogen.

Some forms of DNA damage are repaired without the incorporation of new nucleic acids. Thus, the positive control will be used to demonstrate that the rats were responsive and the methodology was adequate for the detection of UDS.

VIII. ASSAY EVALUATION CRITERIA : CELL PROLIFERATION

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 (12). Control versus treatment group comparisons were done with Dunnet's t-test (13,14). In the case of variance heterogeneity, rank transformation of the data will be 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 $p \leq 0.05$ will be considered significantly different than the control group.

IX. REFERENCES

1. Mirsalis, J.C., Tyson, C.K., and B.E. Butterworth: Detection of genotoxic carcinogens in the in vivo - in vitro hepatocyte DNA repair assay. Environ. Mutagenesis, 4:553-562, 1982.

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

3. 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.

4. 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.

5. Williams, G.M.: The detection of chemical mutagens-carcinogens by DNA repair and mutagenesis in liver cultures. In, Chemical Mutagens, Vol. 6. F. De Serres and A. Hollaender, (Eds.), Plenum Press, NY, 1980, pp. 61-79.

6. 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.

7. 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.

8. 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.

9. 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.

10. 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.

11. Casciano, D.A. and D.W. Gaylor: Statistical criteria for evaluating chemicals as positive or negative in the hepatocyte DNA repair assay. Mutation Research, 122:81-86, 1983.

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

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

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

X. 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.

XI. 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.

XII. 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.