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GENOTOXICITY TEST ON

T-5710.1

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

FINAL REPORT

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

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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) and the Organization of Economic Cooperation and Development Principles of Good Laboratory Practice C(81)30 (Final) Annex 2, issued 1979-1980 (effective 1981). 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.

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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 was administered to rats at doses of approximately 810, 405, 203, and 101 mg/kg for the measurement of both UDS and cell proliferation.

The test material, T-5710.1 did not induce significant changes in UDS as measured by the nuclear labeling of rat hepatocytes. Primary hepatocyte cultures were prepared at two sacrifice times, approximately 2-3 hours and 15-16 hours after administration of a single oral dose. For each sacrifice time, three male rats were treated with test material suspended in corn oil.

The hepatocyte cultures were incubated with 10 $\mu\text{Ci/ml}$ $^3\text{HTdr}$ for about 4 hours and autoradiography was performed the $^3\text{HTdr}$ -treated cultures. After autoradiography, three treatment groups from each sacrifice time 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-5710.1 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, no evidence for an increase in cell proliferation was 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, right median and anterior 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. No statistically significant increases in cell proliferation were observed.

T-5710.1 was therefore considered negative for the induction of both UDS and cell proliferation in rat liver cells.

Genotoxicity Test on T-5710.1 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.: 15516
- B. Identification: T-5710.1
- 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 4, 1993
- C. Experimental Termination Date: May 6, 1993

VI. SUPERVISORY PERSONNEL:

- A. Study Director: Maria A. Cifone, Ph. D.
- B. Scientist: Marie 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 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 ³H-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 measures 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 ³HTdr 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. (CrI: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 324.5-439.1 grams for the early timepoint and 343.2-437.2 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% CO₂.

For the cell proliferation assay, 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 CO₂ and exsanguinated prior to removal of the livers and duodenum (control organ). The organs were fixed in 10% Neutral Buffered Formalin.

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 ³HTdr, 47 Ci/mMole (WME-treat).

C. Osmotic Pumps and Label for Cell Proliferation Analysis

ALZET® osmotic pumps (ALZA Corporation, Palo Alto, 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 control consisting of three rats for the UDS assay and five rats for cell proliferation were treated by oral gavage with the vehicle, corn oil (10 ml/kg, Duke, 2B2517:59) 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 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, the test article was suspended in corn oil at a concentration of 81.1 mg/ml and serial dilutions made prior to dosing for each timepoint and used fresh. 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 solutions 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 810 mg/kg was selected for both timepoints based on Ld_{50} 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; 2-3 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 tattoo number.

An attachment period of 1.8-2.1 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 17.8-18.9 hours (2-3 hours timepoint) or 18.3-18.8 hours (15-16 hours timepoint). Nuclei were then swollen by addition of 1% sodium citrate to the coverslips (containing the cell monolayers) for 7-10 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 8 days at 4°C. The emulsions were then developed in D19, fixed, and stained with hematoxylin and eosin.

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 ($\pm$ 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 from each dose level and control group were used to analyze cell proliferation at 72-hours after a single oral dose.

B. Implantation of Osmotic Pumps

ALZET® Model 2004 osmotic pumps (Lot# 042205) were preloaded with 2000 μ l 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). 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 the 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 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 at approximately 400 to 600X magnification. 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 8 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, terminal body weights and terminal liver weights were calculated for each treatment group using the individual animal mean values. Statistical analysis 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, terminal body weight or terminal liver weight percentage in a dose group that deviates from the 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-5710.1 was suspended in corn oil at a concentration of 81.1 mg/ml. The test material appeared to form a cream colored, opaque viscous suspension in the vehicle. Individual dosing stocks of 81.1, 40.6, 20.3 and 10.1 mg/ml

were prepared just prior to dosing. Three rats per dose level were treated with 810, 405, 203, and 101 mg/kg for both the 2-3 hours and 15-16 hours groups, in volumes which did not exceed 10 ml/kg.

For the early timepoint, perfusions were initiated 2.1-2.6 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 78.2%-98.6% of the total cells collected in the perfusate (Table 1). The attachment efficiency varied from 61.5%-104.1% and the viability of the attached cells was very good, ranging from 88.9%-97.5%.

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 vehicle control animals was -0.32 and the average percent of cells containing five or more net nuclear grains was 3.89%. A positive response consisted of average mean net nuclear grain counts exceeding 4.68 (5 net grains above the control value) or at least 13.89% 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 both 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 2-3 hours timepoint.

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 2-3 hours study was low and did not interfere with the assay.

For the later timepoint, perfusions were initiated 15.2-15.6 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 79.5%-92.2% of the total cells collected in the perfusate (Table 3). The attachment efficiency varied from 60.9%-91.1% and the viability of the attached cells was very good, ranging from 83.5%-97.9%.

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 = -0.54, average percent of cells containing five or more net nuclear grains = 2.50%). The criteria were mean net nuclear grain counts exceeding 4.43 or at least 12.5% 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 large increases in nuclear labeling exceeding both 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.

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 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 1 (vehicle control) was eliminated from the evaluation because of extremely high labeling indices. This animal was treated at a different time than the other animals and may have been dosed improperly. One animal from Group 6 (positive control) was also 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 liver weights of the dosed animals were not significantly different from control animals and no obvious changes were observed at autopsy. The mean liver weight of the positive control was slightly elevated ($p \leq 0.05$) as expected considering the large increase in DNA synthesis (and subsequent cell proliferation) that was occurring. The high dose animals (Group 5) had a mean terminal body weight that was less than control value ($p \leq 0.05$) indicating some toxicity.

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, except where necrosis was observed. The necrotic areas were noted but not included in the count.

The mean background labeling index (Group 1) was 0.61 which indicates that less than 1% of the nuclei had undergone DNA synthesis during the 72-hour labeling period. No significant increases were observed in the dosed groups (Groups 2 through 5) and all mean labeling indices were below 1% which is expected for 12-week old male rats. 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-5710.1 did not induce significant increases in the LI in the liver in male rats after a single oral dose at concentrations from 101 mg/kg to 810 mg/kg. Large increases in the labeling index were observed in the positive control animals. The mean labeling index of the positive control animals was 31.51.

XVI. CONCLUSIONS

The test material, T-5710.1 did not induce significant changes in the nuclear labeling of rat primary hepatocytes at either the 2-3 hours timepoint or the 15-16 hours timepoint when doses of 810, 405 and 203 mg/kg were delivered by oral gavage. T-5710.1 was therefore evaluated as inactive in the induction of UDS in rat liver cells.

The test material did not induce significant changes in the number of S-phase cells following a single oral dose of T-5710.1. The animals were labeled for 72 hours following dosing and all mean labeling indices of the treated groups were below 1%. The mean labeling index of the positive control animals was 31.51. T-5710.1 was therefore evaluated as inactive 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 ³H-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.

XIII. EXPERIMENTAL DATA TABLES

TABLE 1

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

Client: 3M Corporation

HWA Assay No.: 15516-0-494

Client Code: T-5710.1

Trial Initiation Date: 23-Mar-93

Test Condition	Animal Number	Target Dose Level(1)	Perfusion Viability	Attachment Efficiency(2)	Attachment Viability(2)
Vehicle Control - Corn Oil (ml/kg)					
	34449	10.0	78.2	92.3	93.5
	34448	10.0	82.8	83.9	94.5
	34447	10.0	86.1	67.6	90.4
Positive Control 1 - 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)					
	34437	810	83.3	89.7	95.2
	34436	810	85.2	84.6	92.9
	34435	810	88.3	74.3	92.8
	34425	405	79.4	75.1	92.0
	34424	405	87.2	75.9	88.9
	34423	405	90.9	79.6	93.1
	34413	203	91.3	86.0	95.2
	34412	203	85.7	62.8	95.5
	34411	203	98.6	63.3	95.3
	34401	101	98.0	70.2	97.5
	34400	101	83.5	61.5	92.3
	34399	101	87.9	104.1	94.6

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

TABLE 2

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

Client: 3M Corporation

HWA Assay No.: 15516-0-494

Client Code: T-5710.1

Trial Initiation Date: 23-Mar-93

Test Cond.	Animal Number	Target Dose ¹	Mean Net Nuclear Grains (MNNG) $\pm$ SD ²	% Cells w/ $\geq$ 5 NNG ³	Mean Cyto. Grains ⁴	Percent S-Phase Cells ⁵
Vehicle Control - Corn Oil (ml/kg)						
	34449	10.0	-1.17 $\pm$ 0.48	2.00	6.00	0.20
	34448	10.0	0.47 $\pm$ 0.51	6.67	4.61	0.47
	34447*	10.0	-0.27 $\pm$ 0.24	3.00	4.63	0.30
Positive Control 1 - DMN (mg/kg)						
	34443	10.0	16.10 $\pm$ 0.76	96.00	8.95	0.07
	34442	10.0	15.66 $\pm$ 4.30	95.33	6.41	0.87
	34441	10.0	13.13 $\pm$ 1.77	92.67	7.71	0.13
Test Material - (mg/kg)						
	34437	810	-0.17 $\pm$ 0.41	2.00	4.86	0.13
	34436	810	-0.03 $\pm$ 0.70	6.67	5.68	0.13
	34435*	810	0.24 $\pm$ 0.00	4.00	4.89	0.20
	34425*	405	-0.07 $\pm$ 0.64	4.00	4.39	0.40
	34424*	405	-0.51 $\pm$ 1.37	4.00	4.04	0.50
	34423*	405	-0.89 $\pm$ 0.81	2.00	6.58	0.30
	34413*	203	-0.23 $\pm$ 2.11	7.00	7.72	0.50
	34412*	203	0.62 $\pm$ 0.03	9.00	5.07	0.10
	34411	203	-0.52 $\pm$ 0.87	6.67	7.15	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.

* 1 slide(s) not analyzed; UDS = average of mean NNG counts on 2 coverslips (100 total cells).

DMN = Dimethylnitrosamine

TABLE 3

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

Client: 3M Corporation

HWA Assay No.: 15516-0-494

Client Code: T-5710.1

Trial Initiation Date: 25-Mar-93

Test Condition	Animal Number	Target Dose Level(1)	Perfusion Viability	Attachment Efficiency(2)	Attachment Viability(2)
Vehicle Control - CORN OIL (ML/KG)					
	34452	10.0	81.6	75.1	83.5
	34451	10.0	86.9	89.7	97.9
	34450	10.0	88.1	66.3	92.0
Positive Control 1 - DMN (MG/KG)					
	34446	15.0	80.3	79.4	88.4
	34445	15.0	86.2	60.5	92.5
	34444	15.0	89.6	68.2	93.1
Test Material - (MG/KG)					
	34440	810	87.4	91.1	93.9
	34439	810	82.9	76.6	94.4
	34438	810	80.2	66.9	86.8
	34428	405	79.7	76.6	93.7
	34427	405	88.1	72.4	91.0
	34426	405	92.2	79.5	97.1
	34416	203	79.5	73.5	89.7
	34415	203	89.7	82.6	95.6
	34414	203	85.1	60.9	89.1
	34404	101	92.1	75.0	93.9
	34403	101	85.9	83.3	96.5
	34402	101	87.3	85.5	95.7

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

TABLE 4

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

Client: 3M Corporation

HWA Assay No.: 15516-0-494

Client Code: T-5710.1

Trial Initiation Date: 25-Mar-93

Test Cond.	Animal Number	Target Dose ¹	Mean Net Nuclear Grains (MNNG) $\pm$ SD ²	% Cells w/ $\geq$ 5 NNG ³	Mean Cyto. Grains ⁴	Percent S-Phase Cells ⁵
Vehicle Control - CORN OIL (ML/KG)						
	34452 ^a	10.0	0.04 $\pm$ 0.37	4.00	3.61	0.00
	34451	10.0	-0.73 $\pm$ 0.68	2.67	6.69	0.27
	34450	10.0	-0.73 $\pm$ 0.40	1.33	4.55	0.00
Positive Control 1 - DMN (MG/KG)						
	34446	15.0	3.95 $\pm$ 2.86	38.67	2.59	0.00
	34445	15.0	4.05 $\pm$ 3.38	38.00	4.58	0.07
	34444	15.0	5.01 $\pm$ 2.62	48.00	3.32	0.07
Test Material - (MG/KG)						
	34440	810	-0.29 $\pm$ 1.40	9.33	8.54	0.07
	34439	810	-0.40 $\pm$ 0.48	1.33	5.26	0.00
	34438	810	-0.24 $\pm$ 1.07	6.67	5.79	0.00
	34428	405	-0.10 $\pm$ 0.23	3.33	5.47	0.00
	34427	405	0.75 $\pm$ 0.20	7.33	4.54	0.27
	34426	405	-0.40 $\pm$ 0.12	2.67	6.67	0.13
	34416	203	0.43 $\pm$ 0.40	7.33	5.21	0.27
	34415	203	0.63 $\pm$ 0.41	7.33	5.46	0.13
	34414	203	0.05 $\pm$ 0.54	7.33	7.37	0.07

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(s) not analyzed; UDS = average of mean NNG counts on 2 coverslips (100 total cells).

DMN = Dimethylnitrosamine

Table 5
Cell Proliferation Summary

Client: 3M Corporation

HWA Assay No.: 15516-0-494

Client Code: T-5710.1

Trial Initiation Date: 5-Mar-93

Group/Sex ^a	Dose Level (mg/kg)	Labeling Index ^b (%)	Liver Weight (g)	Terminal Body Weight (g)
1M ^c	0 ^d	0.61 ± 0.18	16.42 ± 1.21	421.6 ± 13.6
2M	101	0.49 ± 0.46	17.16 ± 1.46	420.0 ± 23.7
3M	203	0.26 ± 0.26	17.24 ± 1.17	407.5 ± 20.6
4M	405	0.50 ± 0.38	18.48 ± 3.06	389.1 ± 40.8
5M	810	0.34 ± 0.25	18.32 ± 1.10	365.1 ± 24.6*↓
6M ^c	15 ^e	31.51 ± 15.86**↑	19.48 ± 2.06*↑	459.8 ± 30.1

*Five animals per group unless indicated

^bPercentage of labeled hepatocyte nuclei per total number of hepatocytes counted (at least 2000)

^cFour animals per group

^dVehicle control, 10 ml/kg of corn oil

^ePositive control, 15 mg/kg of DMN

*Significant at $p \leq 0.05$

**Significant at $p \leq 0.01$

↑ Increase in the mean

↓ Decrease in the mean

Table 6
Cell Proliferation Assay
Individual Animal Data

Animal Number	Group /Sex	Mean Labeling Index (%)	Terminal Body Weight (g)	Terminal Liver Weight (g)
33459	1M	0.62	432.0	16.02
34460	1M	0.81	402.2	14.89
34461	1M	0.62	429.9	17.24
34463	1M	0.38	422.2	17.53
Group Mean		0.61	421.6	16.42
Group SD		0.18	13.6	1.21
N		4	4	4
34464	2M	0.19	430.9	17.66
34465	2M	1.24	429.7	16.34
34466	2M	0.62	425.2	17.85
34467	2M	0.19	435.8	18.85
34468	2M	0.19	378.2	15.10
Group Mean		0.49	420.0	17.16
Group SD		0.46	23.7	1.46
N		5	5	5
34469	3M	0.43	393.0	16.21
34470	3M	0.05	391.0	18.16
34471	3M	0.10	442.1	18.51
34472	3M	0.67	408.5	15.86
34473	3M	0.10	402.9	17.45
Group Mean		0.27	407.5	17.24
Group SD		0.27	20.6	1.17
N		5	5	5

Table 6 (Continued)
 Cell Proliferation Assay
 Individual Animal Data

Animal Number	Group /Sex	Mean Labeling Index (%)	Terminal Body Weight (g)	Terminal Liver Weight (g)
34474	4M	0.95	401.6	20.22
34475	4M	0.19	369.9	14.87
34476	4M	0.29	379.8	18.83
34477	4M	0.19	342.6	16.06
34478	4M	0.86	451.4	22.42
Group Mean		0.50	389.1	18.48
Group SD		0.38	40.8	3.06
N		5	5	5
34479	5M	0.29	346.3	17.80
34480	5M	0.24	375.2	19.22
34481	5M	0.29	369.1	17.57
34482	5M	0.76	398.6	19.75
34483	5M	0.10	336.2	17.26
Group Mean		0.34	365.1	18.32
Group SD		0.25	24.6	1.10
N		5	5	5
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

XIV. APPENDIX A: HISTORICAL CONTROLS

HISTORICAL NEGATIVE CONTROLS

IN VIVO/IN VITRO UNSCHEDULED DNA SYNTHESIS ASSAY

Number of data points is 20

Animal #	UDS Grains/ Nucleus $\pm$ SD *	% of Nuclei with ≥ 5 Net Nuclear Grains	Average Cyto **Grains **
1	-0.23 $\pm$ 0.33	2.7	6.17
2	-1.39 $\pm$ 0.49	0.0	7.76
3	-0.99 $\pm$ 0.47	1.3	8.19
4	0.12 $\pm$ 0.89	4.7	7.69
5	-1.43 $\pm$ 0.76	0.7	10.61
6	-0.55 $\pm$ 0.45	0.0	3.55
7	-0.67 $\pm$ 0.32	0.0	3.93
8	0.11 $\pm$ 0.63	0.0	2.66
9	-0.11 $\pm$ 0.25	0.7	2.66
10	-0.05 $\pm$ 0.39	0.0	4.25
11	-1.05 $\pm$ 0.31	0.0	2.79
12	-0.01 $\pm$ 0.89	6.7	7.55
13	-0.38 $\pm$ 0.30	1.3	8.55
14	-0.27 $\pm$ 0.22	2.7	6.58
15	-1.38 $\pm$ 0.31	0.7	9.89
16	-0.18 $\pm$ 0.33	4.0	8.18
17	-0.57 $\pm$ 0.34	3.3	8.97
18	-0.21 $\pm$ 0.47	2.0	7.94
19	-0.59 $\pm$ 0.38	0.0	7.75
20	-0.62 $\pm$ 0.52	0.0	7.20
Average:	-0.52	1.5	6.56
SD ^a	$\pm$ 0.50	$\pm$ 1.0	$\pm$ 2.44
Range:			
Low	-1.74	0.0	2.66
High	0.12	6.7	10.61

- * UDS = Average of net nuclear grain counts $\pm$ 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

HISTORICAL POSITIVE CONTROLS

IN VIVO/IN VITRO UNSCHEDULED DNA SYNTHESIS ASSAY

4-Hour Timepoint

Number of data points is 9

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

* UDS = Average of net nuclear grain counts $\pm$ 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

HISTORICAL POSITIVE CONTROLS

IN VIVO/IN VITRO UNSCHEDULED DNA SYNTHESIS ASSAY

15-Hour Timepoint

Number of data points is 12

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

- * UDS = Average of net nuclear grain counts $\pm$ 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