UNSCHEDULED DNA SYNTHESIS IN
RAT PRIMARY HEPATOCYTES

TEST ARTICLE B34
Lot No. 2nd Subm.

FINAL REPORT

MICROBIOLOGICAL ASSOCIATES INC.

5221 RIVER ROAD, BETHESDA, MARYLAND 20816

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Author

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Study Completed On

June 3, 1987

Performing Laboratory

MICROBIOLOGICAL ASSOCIATES, INC.
5221 RIVER ROAD
BETHESDA, MARYLAND 20816

Laboratory Study Number

T5227.380

QUALITY ASSURANCE STATEMENT

Study Title: UNSCHEDULED DNA SYNTHESIS IN RAT PRIMARY HEPATOCYTES

Study Number: T5227.380

Study Director: Rodger D. Curren, PH.D.

Initiation Date: 86/10/23

Review Completed Date: 87/06/03

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice regulations (21CFR58), the U.S. EPA GLPs (40CFR792 and 40CFR160), and the OECD guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of the study.

INSPECT ON 86/10/06 - 86/10/06,TO STUDY DIR 86/10/06,TO MGMT 86/10/06

PHASES: PROTOCOL REVIEW

INSPECT ON 86/12/19 - 86/12/19,TO STUDY DIR 86/12/19,TO MGMT 86/12/29

PHASES: DETERMINATION OF LDH LEVELS

INSPECT ON 87/05/19 - 87/05/19,TO STUDY DIR 87/05/19,TO MGMT 87/06/03

PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Mona S Kanter 6/3/87

Quality Assurance
RA/QA Department

Date

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UNSCHEDULED DNA SYNTHESIS IN RAT PRIMARY HEPATOCYTES

FINAL REPORT

Test Article : B34

Lot : 2nd Subm.

MA Study No.: T5227.380

Test Article Description: White Powder

Storage Conditions: Refrigerated in an amber bottle

Date Sample Received: August 23, 1986

Initiation Date: October 23, 1986

Completion Date: June 3, 1987

Sponsor: Lorillard Research Center
P.O. Box 21688 420 English Street
Greensboro, NC 27420-1688

Sponsor's Investigator: J. Daniel Heck, Ph.D.

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC.
5221 River Road
Bethesda, Maryland 20816

Study Director: Rodger D. Curren, Ph.D. 6/3/87 Date

Laboratory Technician: Katherine T. Ault, B.A. (for K.T.A.) 6/3/87 Date

Laboratory Technician: Nagasundari Durvasula, M.S. (for N.D.) 6/3/87 Date

Laboratory Technician: Barbara J. Head, A.S. 6/3/87 Date

Laboratory Technician: Kathleen Wallace, B.S. 6/3/87 Date

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SUMMARY

Lorillard Research Center's test article, B34, was tested in the Unscheduled DNA Synthesis Test using rat primary hepatocytes. The test article was tested at eight dose levels ranging from 2.5 ug/ml to 2500 ug/ml and was fully evaluated at five dose levels from 7.5 to 1250 ug/ml .

The results of the UDS assay indicate that under the test conditions, the test article did not cause a significant increase in the mean number of net nuclear grain counts (i.e., an increase of at least 5 counts over the control), at any dose level. Therefore, the test article is considered negative in this study.

INTRODUCTION

This study was conducted from October 23, 1986 to May 8, 1987 at Microbiological Associates, Inc. The experimental procedure employed was essentially that of Williams, G.M. (Cancer Research 37:1845-1851, 1977) and is described in detail in the specific protocol for this study (see Appendix).

The purpose of the study was to evaluate the test article, B34, for its ability to induce unscheduled DNA synthesis in rat primary hepatocytes as measured by autoradiographic methods.

MATERIALS AND METHODS

Indicator Cells

Primary rat liver cell cultures derived from the livers of normal adult male Sprague-Dawley rats were used in this study. The animals were obtained from Frederick Cancer Research Facility and Charles River Laboratories, Inc. and were quarantined for at least one week prior to the initiation of the study. The animals were maintained on standard laboratory diet throughout the quarantine period.

The procedure used for obtaining rat hepatocyte cultures (HPC) was essentially that of Williams, et al., (In Vitro 13:809-817, 1977). Each rat used was sacrificed by inhalation of metofane. The animal was dissected and perfused first with 0.5mM EGTA solution and then with a collagenase solution. The liver was removed from the animal and the cells were dissociated, counted, and seeded into 35 mm dishes containing coverslips (5×10^5 viable cells/dish). The cells were seeded in Williams Medium E (WME) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units of penicillin and 100 ug of streptomycin/ml or 50 ug/ml gentamicin. The cultures were incubated at 37 ± 1°C in a humidified 5 ± 1% CO_2 incubator for 90-120 minutes, washed and refed with serum-free medium and used in the test.

Test and Control Articles

The test article, B34, was received on August 23, 1986, and stored refrigerated in an amber bottle. The test article was dissolved and diluted in DMSO (Aldrich, Lot 63309LM) to make up the stock solutions. 7,12-Dimethylbenzanthracene (DMBA) (Kodak, Lot C13) was dissolved in DMSO (Aldrich, Lot 63309LM) and used as a positive control in this study.

The test article was diluted to appropriate concentrations immediately prior to use. Approximately 20 to 30 minutes elapsed between the time the test article was dissolved and the final treatment of cells. All test article and control treatments were done under subdued yellow lights to avoid possible problems of photoinactivation.

Documentation of the stability, purity and the method of synthesis, fabrication or derivation of the test substance is the responsibility of the sponsor.

Identification of Test System

All culture plates were labeled with pen with a code system which clearly identifies the test article or control, test phase, and the experiment number. Slides were similarly labeled with pencil or pen.

Initial Cytotoxicity Test

A preliminary cytotoxicity test was performed to establish an appropriate dose range for the test article. Ten doses ranging from 0.075 to 2500 ug/ml were tested. The test article was tested by treating replicate cultures of HPC 90-120 minutes after seeding. Eighteen to twenty hours later, an aliquot of culture fluid was removed, centrifuged, and the level of lactic acid dehydrogenase (LDH) activity determined. Two replicate plates were used for LDH measurement at each dose level. The relative toxicities were obtained by comparing the treated to untreated control cultures. The LDH values for the single condition of lysed cells plus test article may not be reliable due to substrate exhaustion. Inadvertently the samples for this condition were not further diluted and reassayed.

Unscheduled DNA Synthesis Test

Based on the results of the initial cytotoxicity test, the test article, B34, was tested at eight dose levels. Three replicate plates seeded with 5 x 10^5 HPC/plate were treated with 2.5 ug/ml to 2500 ug/ml of test article. DMSO, which was used to dissolve the test article was also used as the solvent control for the test article. DMBA, at 3 ug/ml and 10 ug/ml, was used as the positive control. DMSO was used as the solvent control for the positive control. Each test article and control dish received ^{3}H-thymidine at a final concentration of 10 uCi/ml. In parallel with the test plates, three cultures per dilution were treated with the same compound for a parallel toxicity test.

The cells were treated for 18-20 hours as described earlier. The parallel toxicity plates were harvested by removal of a portion of the medium for LDH determinations as described in the initial cytotoxicity test to obtain the relative survivals and relative toxicities.

After eighteen to twenty hours of exposure, the cells in the Unscheduled DNA Synthesis assay plates were washed in serum-free WME, swelled in 1% sodium citrate and fixed in ethanol-acetic acid fixative. The coverslips were air-dried, mounted cell side up on glass slides, and allowed to dry. The slides were coated with Kodak NTB emulsion and stored for ten days at 4°C in light tight boxes with desiccant. The slides were then developed in Kodak D-19 developer, fixed in Kodak fixer and stained in hematoxylin-sodium acetate-eosin stain.

Scoring

The slides were read "blind" on an Artek Colony Counter. Nuclear grains were counted in 25 cells in random areas on each of three coverslips per treatment where possible. The net nuclear counts were determined by counting three nucleus-sized areas adjacent to each nucleus and subtracting the average cytoplasmic count from the nuclear count. Replicative synthesis was identified by nuclei completely blackened with grains and such cells were not counted. Nuclei exhibiting toxic effects of treatment, such as dark staining, disrupted membranes or irregular shape, were not counted.

Presentation of Data

For each treatment slide, the net nuclear counts were averaged and the standard deviation (S.D.) determined and recorded on a summary form. Also reported are the grand mean and S.D. for each dose level as well as the percent of cells in repair (cells with $\geq$ 5 net nuclear grains). Means, standard deviations and percent survivals were computed using a LOTUS 1-2-3 program on an IBM PC or compatible computer.

Criteria for Evaluation of Test Results

The results of this study were evaluated according to the criteria described below.

If the mean net nuclear count was increased by at least five counts over the control, the results for a particular dose level were considered significant. A test article was judged positive if it induced a dose-related response and at least one dose produced a significant increase in the average net nuclear grains when compared to that of the control. In the absence of the dose response, a test article which showed a significant increase in the mean net nuclear grain count in at least two successive doses was considered positive. If a test article showed a significant increase in the net nuclear grain count at one dose level without any dose response, the test article was considered to have a marginal positive activity. The test article was considered negative if no significant increase in the net nuclear grain counts at any dose level was observed.

Records

All raw data, final report and stained slides of this study are maintained in the archives of Microbiological Associates, Inc. located at 5221 River Road, Bethesda, Maryland 20816.

RESULTS AND DISCUSSION

The results of the preliminary cytotoxicity assay are recorded in Table 1. Toxicity was present at the highest concentration of test article, 2500 ug/ml. The remaining dose levels were non-toxic. The highest concentration of test article selected for Unscheduled DNA Synthesis assay was 2500 ug/ml.

The results of the parallel cytotoxicity assay are recorded in Table 2. Microscopic examination of the hepatocyte cultures indicated high toxicity through 250 ug/ml and a slight toxicity at 75 and 25 ug/ml. LDH determinations showed 12% toxicity at the highest concentration, 2,500 ug/ml, and 5% toxicity at 1,250 ug/ml. However, when the test article was added directly to lysed cells, the measured LDH levels were much lower than when lysed cells alone were measured (corrected LDH of 150.0 and 383.8, respectively; Table 2). This indicates that the test article interferes with the LDH assay. Microscopic inspection of the slides showed at 2,500 ug/ml the nuclei could not be evaluated due to toxicity. The dose of 25 ug/ml could not be evaluated since cytoplasmic definition was poor. Dose levels of 1,250, 750, 250, 75 and 7.5 ug/ml were fully evaluated for UDS.

The results of the UDS assay are summarized in Table 3. Slides treated with B34 or DMBA were compared to the appropriate negative control. According to the criteria set for evaluating the test results, both doses of the positive control compound, DMBA, induced a significant increase in the average net nuclear count of silver grains. None of the test article doses caused a significant increase (5 counts above the solvent control) in the mean net nuclear counts. The highest concentration of test article, 1250 ug/ml, gave a grand mean of 2.3 net nuclear counts with 24% in repair. While these values are somewhat elevated, they do not meet our criteria for a positive response. All criteria for a valid test were met.

TABLE 1

PRELIMINARY CYTOTOXICITY ASSAY
LDH RELEASE
UNSCHEDULED DNA SYNTHESIS

TREATMENT	DISHES COUNTED	LDH	AVERAGE LDH	CORRECTED LDH	RELATIVE TOXICITY
B34					
2,500 ug/ml	2	275			
		263	269.0	108.0	30%
750 ug/ml	2	174			
		160	167.0	6.0	2%
250 ug/ml	2	131			
		113	122.0	-39.0	-11%
75 ug/ml	2	117			
		114	115.5	-45.5	-13%
25 ug/ml	2	116			
		115	115.5	-45.5	-13%
7.5 ug/ml	2	134			
		136	135.0	-26.0	-7%
2.5 ug/ml	2	138			
		138	138.0	-23.0	-6%
0.75 ug/ml	2	141			
		157	149.0	-12.0	-3%
0.25 ug/ml	2	125			
		123	124.0	-37.0	-10%
0.075 ug/ml	2	139			
		152	145.5	-15.5	-4%

TREATMENT	DISHES COUNTED	LDH	AVERAGE LDH	CORRECTED LDH	RELATIVE TOXICITY
DMSO (Solvent Control for Test Article)					
	2	178			
		144	161.0	0.0	0%
WME (Medium Control)					
	2	162			
		143	152.5	-8.5	-2%
WME +1% Triton	2	566			
		484	525.0	364.0	100%
2500 ug/ml T5227 + 1% Triton	2	409*			
		413*	411.0	250.0	69%

CORRECTED LDH = AVERAGE LDH - SOLVENT CONTROL LDH
RELATIVE TOXICITY = CORRECTED LDH / 100% CORRECTED LDH CONTROL
100% LDH CONTROL = THE AMOUNT OF CORRECTED LDH ACTIVITY RELEASED BY EXPOSURE OF CONTROL CELLS TO 1% TRITON (100% LYSIS).
* VALUES MAY NOT BE RELIABLE DUE TO EXHAUSTION OF SUBSTRATE

TABLE 2

PARALLEL CYTOTOXICITY ASSAY
LDH RELEASE
UNSCHEDULED DNA SYNTHESIS

TREATMENT	DISHES COUNTED	LDH	AVERAGE LDH	CORRECTED LDH	RELATIVE TOXICITY
B34					
2,500 ug/ml	3	110 154 112	125.3	47.3	12%
1,250 ug/ml	3	119 89 87	98.3	20.3	5%
750 ug/ml	3	54 72 60	62.0	-16.0	-4%
250 ug/ml	3	56 43 62	53.7	-24.3	-6%
75 ug/ml	3	42 55 51	49.3	-28.7	-7%
25 ug/ml	3	45 54 54	51.0	-27.0	-7%
7.5 ug/ml	3	54 58 59	57.0	-21.0	-5%
2.5 ug/ml	3	53 71 87	70.3	-7.7	-2%
DMBA					
10 ug/ml	3	162 144 142	149.3	71.3	19%
3.0 ug/ml	3	130 139 147	138.7	60.7	16%
DMSO (Solvent Control for Test Article and DMBA)					
10 ul/ml	3	84 78 72	78.0	0.0	0%
WME (Medium Control)	3	124 103 107	111.3	33.3	9%
DMSO + 1% Triton	3	570 410 404	461.3	383.3	100%
2500 ug/ml T5227 + 1% Triton	3	202 228 254	228.0	150.0	39%

CORRECTED LDH = AVERAGE LDH - SOLVENT CONTROL LDH
RELATIVE TOXICITY = CORRECTED LDH / 100% CORRECTED LDH CONTROL
100% LDH CONTROL = THE AMOUNT OF CORRECTED LDH ACTIVITY RELEASED BY EXPOSURE OF CONTROL CELLS TO 1% TRITON (100% LYSIS).

TABLE 3

SUMMARY OF UDS ASSAY
WITH B34

TREATMENT	RELATIVE SURVIVAL	SLIDE DESIGNATION	NO. OF NUCLEI COUNTED	AVERAGE NET GRAINS PER NUCLEUS	S.D.	GRAND MEAN	S.D.	PERCENT CELLS WITH 5 OR MORE NET NUCLEAR GRAINS
B34								
1250 ug/ml	95%	33A	25	2.9 +/-	3.5	2.3 +/-	3.7	24%
		33C	25	1.1 +/-	3.7			
		33C	25	2.9 +/-	3.9			
750 ug/ml	104%	32A	25	0.2 +/-	3.5	0.4 +/-	3.2	8%
		32B	25	0.7 +/-	3.4			
		32C	25	0.2 +/-	2.7			
250 ug/ml	106%	34B	25	0.1 +/-	3.8	0.0 +/-	3.6	7%
		34B	25	-0.3 +/-	3.5			
		34C	25	0.2 +/-	3.5			
75 ug/ml	107%	36A	25	1.6 +/-	3.3	1.1 +/-	3.7	13%
		36B	25	0.8 +/-	4.5			
		36C	25	1.0 +/-	3.3			
7.5 ug/ml	105%	37A	25	0.5 +/-	2.3	0.5 +/-	2.9	4%
		37B	25	0.9 +/-	1.9			
		37C	25	0.0 +/-	4.1			
DMBA								
10 ug/ml	81%	48A	25	20.4 +/-	7.1	*17.7 +/-	6.3	100%
		48B	25	17.3 +/-	5.9			
		48C	25	15.3 +/-	4.9			
3 ug/ml	84%	50A	25	20.2 +/-	3.9	*23.0 +/-	6.7	100%
		50B	25	22.3 +/-	6.1			
		50C	25	26.4 +/-	8.0			
DMSO (Solvent Control For Test Article and DMBA)								
10 ul/ml	100%	52A	25	1.1 +/-	2.5	0.3 +/-	2.6	4%
		52B	25	0.6 +/-	2.0			
		52C	25	-0.8 +/-	3.1			
WME (Medium Control)								
	91%	47B	25	0.3 +/-	2.6	0.0 +/-	2.4	1%
		47B	25	-0.5 +/-	2.4			
		47C	25	0.1 +/-	2.2			

S.D. Standard Deviation
* Statistically Significant (See Protocol: Section 8.0, Evaluation of Test Results)

CONCLUSION

Lorillard Research Center's test article B34, was tested in the Rat Hepatocyte Unscheduled DNA Synthesis Assay. The test article was tested at eight dose levels ranging from 2.5 ug/ml to 2500 ug/ml and was fully evaluated at five dose levels, 1250, 750, 250, 75 and 7.5 ug/ml.

The results of the UDS assay indicate that under the test conditions, the test article did not cause a significant increase in the Unscheduled DNA Synthesis as measured by the mean number of net nuclear grain counts (i.e., an increase of at least 5 counts over the control), at any dose level. In this study the positive control, 7,12-Dimethylbenzanthracene (DMBA), induced significant increases in the mean number of net nuclear grain counts over that in the solvent control.

APPENDIX

Received by RA/QA 10/3/84

APPROVED

UNSCHEDULED DNA SYNTHESIS IN RAT PRIMARY HEPATOCYTES

1.0 PURPOSE

The purpose of this study is to evaluate the potential of the test article to induce unscheduled DNA synthesis in primary cultures of rat hepatocytes.

2.0 TEST ARTICLE

2.1 Identification: B34

2.2 Analysis:
The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

2.3 MA Study Number: T5227.380

3.0 SPONSOR

3.1 Name: Lorillard Research

3.2 Address: P. O. Box 21688
420 English St.
Greensboro, NC 27420

3.3 Authorized Representative: Connie J. Stone, Ph.D.
J. Daniel Heck, Ph.D.

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20816

4.3 Study Director: Rodger D. Curren, Ph.D.

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MICROBIOLOGICAL ASSOCIATES INC.
A Subsidiary of Darvl Laboratories Inc.

5.0 TEST SYSTEM

Primary hepatocytes obtained from Sprague-Dawley or Fischer rats will be used in this study. Monitoring unscheduled DNA synthesis (UDS) in rat hepatocyte primary cultures (HPC) presents several advantages over other cell types used to monitor possible interactions between the test article and DNA. First, the target cells possess the ability to metabolize many promutagens/procarcinogens to their active form, thus eliminating the need for an exogenous source of metabolic activation. Secondly, HPC's are nearly 100% non-dividing, so no metabolic blocks are needed to inhibit replicative DNA synthesis. Thirdly, the target cells, HPC, are epithelial in origin. Since most human cancers are carcinomas, an assay using epithelial cells to monitor genetic damage may be more relevant to the in vivo situation than a similar assay using fibroblasts. Finally, the test is relatively short-term, requiring less than 20 days to evaluate a chemical.

6.0 EXPERIMENTAL DESIGN

The experimental design of this study consists of a solubility or miscibility test to select a suitable solvent for the test article, a preliminary toxicity test, and then the UDS assay which includes a simultaneous toxicity test. The UDS assay is evaluated on the basis of incorporation of tritiated-thymidine (^{3}H-TdR) into the HPC DNA, presumably as a consequence of DNA repair. This incorporation is evidenced by the presence of silver grains over the nuclei of cells that were coated with a photographic emulsion 8-12 days preceding development. The cells are stained, and the number of grains over the nucleus and adjacent nuclear-sized cytoplasmic areas are counted on an automated colony counter. UDS data are presented as net nuclear grains (the number of nuclear grains minus the number of cytoplasmic grains) per cell. The toxicity data are presented as relative survival, based on viable cell counts after approximately 18-20 hours exposure to test articles.

7.0 METHODS

The methods used are modifications of the procedures used by G. M. Williams (1,2) and J. Bradlaw (3).

7.1 Media and Reagents

7.1.1 Plating Medium: Williams Medium E (WME) buffered with 0.01M HEPES, adjusted to pH 7.3 and supplemented with 10% fetal bovine serum

(FBS), 2mM L-glutamine and 50 ug gentamycin/ml or 100 units of penicillin and 100 ug of streptomycin/ml.

7.1.2 Perfusion (collagenase) solution: Serum-free WME with HEPES, L-glutamine and gentamycin or penicillin and streptomycin at the same concentrations as in the plating medium, pH adjusted to 7.3 and containing 100 units of collagenase (Type I)/ml.

7.1.3 Treatment medium: Serum-free WME plus HEPES, L-glutamine and gentamycin or penicillin and streptomycin at the same concentrations as in the plating medium, adjusted to pH 7.3 and containing 10 uCi ^{3}H-TdR/ml.

7.1.4 EGTA solution: 0.5mM EGTA in Ca^{++}, Mg^{++}, free Hanks' Balanced Salt Solution, buffered with 0.01M HEPES, pH adjusted to 7.3.

7.1.5 Metofane (Methoxyflurane).

7.2 Preparation and Delivery of Test Article

The test article will be dissolved in deionized distilled water, WME, ethanol (CAS #64-17-5), dimethylsulfoxide (DMSO) (CAS #67-68-5), acetone (CAS #67-64-1), or other appropriate solvent. A 100X concentrate of test article in appropriate solvent will be prepared. If WME is the solvent, the 100X concentrate is not required.

An aliquot of 100X test article will be diluted in the serum-free WME treatment medium, vortexed and delivered 2 ml/35mm culture plate. The treatment medium for UDS assay will contain 10 uCi/ml of ^{3}H-thymidine whereas the treatment medium for the toxicity test plates will be without ^{3}H- thymidine. Alternatively, serum-free WME with or without 10 uCi/ml ^{3}H-thymidine will be prepared and 2 ml will be delivered directly into each of the test plates. The test article or its stock dilutions will then be added directly into the test plates using micropipettes. In any case, the final concentration of the solvent in the culture plate will not exceed 1% unless otherwise specified.

The stability of the test article under the actual experimental conditions will not be determined by Microbiological Associates.

7.3 Route of Administration

Test article will be administered by direct addition to the culture medium with a vehicle compatible with the test system. Cells will be exposed to the test article for approximately 18-20 hours. This method of exposure has been shown to be the most effective method with known carcinogens.

7.4 Controls

Each assay will include an untreated control, a solvent control and one untreated control in which no ^{3}H-TdR is added (to check for background not related to the ^{3}H-TdR). Two doses of 2-acetyl- aminofluorene (2-AAF) or 7,12-dimethylbenz(a)- anthracene will be used as a positive control. Both compounds require metabolic activation and historically give a strong positive response in the UDS assay.

7.5 Preparation of Hepatocytes

7.5.1 Animals: Male Sprague-Dawley or Fischer rats, will be used in all tests. The animals used in the study will be received, quarantined for at least one week and housed in the animal room according to the Standard Operating Procedures of Microbiological Associates (OPGT0403).

7.5.2 One rat will be anesthetized by inhalation of metofane and a midventral incision will be made from the pubic bone to the sternum.

7.5.3 A 21 gauge needle will be inserted into the hepatic portal vein and 0.5mM EGTA in Hanks' Balanced Salt Solution at pH 7.3 will then be perfused at approximately 8 ml/minute for 1-2 minutes.

7.5.4 The inferior vena cava will be clamped off at the kidney and the thoracic vena cava will be cannulated through the heart or the heart will be punctured and the perfusion rate increased to approximately 20 ml/minute.

7.5.5 The inferior vena cava will then be cut below the clamp after the return cannula is in place or after puncturing the heart.

7.5.6 After the perfusion of about 120 ml of EGTA solution, 250 ml of collagenase solution will be perfused through the liver at 20-30 ml/ minute.

7.5.7 The liver will be removed from the animal, trimmed of excess fat and the capsule opened at numerous points.

7.5.8 Cells will be removed by shaking the liver in collagenase solution, followed by gentle combing of the liver lobes with a stainless steel comb or by passing the cells through a stainless steel sieve.

7.5.9 The cells will be pooled, counted and approximately 5×10^5 cells will be seeded into preconditioned 35 mm tissue culture plates containing the coverslips. Cells will be seeded into 35 mm plates without the coverslips for the toxicity test. Cells will be seeded in 3 ml of plating medium.

7.5.10 Cells will be seeded into 6 replicate plates per dose level, 3 will be used for evaluating cytotoxicity and the remaining 3 will be used for the UDS assay.

7.6 Dose Selection

A preliminary toxicity test will be performed to establish an appropriate test article dose range for the UDS assay. Based on the information available for the test article, ten decreasing doses will be selected and used in the initial toxicity test. The highest dose of test article will be based on its solubility in water, WME, ethanol, DMSO, acetone, or in another appropriate solvent. The maximum solvent concentration (other than water or WME medium) will be 1% unless otherwise specified by the Study Coordinator. The test article will be tested by treating duplicate cultures of HPC plated 90 to 120 minutes earlier in 35 mm culture plates. Approximately 18-20 hours later, the cells will be washed with phosphate buffered saline (PBS), dissociated with 0.25% Trypsin, and the viable cells will be counted using the trypan blue dye exclusion method. Relative survival will be obtained by comparing treated to control groups. The test article concentrations causing 50-90% and 0-20% relative toxicity will be maximum and minimum doses, respectively, to be used in the UDS assay. Three other intermediate doses will also be tested. Doses will generally be selected to cover a two log range. If a test article fails to cause 50-90% toxicity, the maximum dose will be based on its solubility in the preferred solvent. If the test article is sufficiently soluble, a maximum dose of 10 mg/ml will

be used unless otherwise specified by the Sponsor. If the test article is a liquid, 10 ul/ml will be the highest dose tested in the absence of cytotoxicity.

7.7 UDS Assay

7.7.1 Ninety to 120 minutes after plating, the cells will be washed once in serum-free WME and treated by addition of test article and controls in serum-free WME.

7.7.2 Cells will be exposed to chemicals for 18-20 hours, which allows adequate time for activation, metabolite-DNA interaction, and short-patch and long-patch DNA repair.

7.7.3 Eighteen to twenty hours after exposure, the cells will be washed three times in serum-free WME, swelled in 1% sodium citrate solution and fixed in three fifteen minute changes of ethanol-glacial acetic acid fixative. Toxicity plates will be washed with PBS, dissociated with 0.25% trypsin and the viable cells counted by trypan blue dye exclusion method.

7.7.4 The UDS coverslips will be allowed to dry for at least 1 hour before mounting cell side up on glass slides. The slides will be labelled with a code to identify the dose.

7.7.5 The slides will be dipped in NTB or NTB-2 emulsion at 43-45°C, allowed to drain and dry for at least 1.5 hours at room temperature and stored for 8-12 days (NTB) or 3 days (NTB-2) at 0-4°C in light tight boxes with a desiccant.

7.7.6 Slides will be developed in D-19, fixed in Kodak fixer, stained and permanently mounted with a coverglass.

7.7.7 The net increase in grains/nucleus will be determined by subtracting the cytoplasmic area count from the nuclear area count. Three nuclear-size cytoplasmic area counts will be taken per nucleus and the average value subtracted from the nuclear count. Counts will be made under oil immersion (1000X) using an automatic colony counter.

7.8 Collection of Data

All coded slides will be read blind. Seventy-five randomly selected nuclei (with appropriate background counts) will be counted per dose level. If possible, 25 nuclei will be scored from each of three replicate cultures. Replicative DNA synthesis is evidenced by nuclei completely blackened with grains. Such nuclei will not be counted. Cells exhibiting toxic effects of treatments, such as irregularly shaped or very darkly stained nuclei, or nuclei with a projected image of less than 4 mm^2 will not be counted. For each treatment group, the standardized counts for all slides will be averaged and a standard deviation (S.D.) determined and recorded on a summary form.

8.0 EVALUATION OF TEST RESULTS

If the mean net nuclear count is increased by at least five counts over the control (1,2), the results for a particular dose level will be considered significant. A test article will be judged positive if it induces a dose-related response and at least one dose produces a significant increase in the average net nuclear grains when compared to that of the control. In the absence of the dose response, the test article should show a signficant increase in the mean net nuclear grain count in at least two successive doses. If a test article showed a significant increase in the net nuclear grain count at one dose level without any dose response, the test article will be considered to have a marginal positive activity. The test article will be considered negative if no significant increase in the net nuclear grain counts at any dose level is observed. At the request of the Sponsor, the data will also be analyzed based on the proportion of cells with $\geq$ 5 net nuclear counts (cells in repair). The result will be considered significant if more than 20% of the cells are in repair.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

A test will be accepted if: (a) the positive control compound induces a positive response, significantly greater than the solvent control (b) the difference in the mean net counts per nucleus between the solvent and negative control does not exceed those of the negative control by two standard deviations of the control value (c) if the data is analyzed based on the proportion of cells with $\geq$5 net nuclear counts (cells in repair), the number of cells in repair in the negative control is less than 20%.

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10.0 IDENTIFICATION OF TEST SYSTEM

All plate cultures will be labelled to identify the test article or control, test phase, and the experiment number. Slides will be labelled with a code system which clearly identifies the test article or control, test phase and experiment number.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records
A separate working notebook will be used to record the materials and procedures used to perform this study. Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Specimens
All specimens, such as slides, will be held in storage for ten years or as long as the quality of the preparation affords evaluation and in accordance with the Terms and Conditions.

12.0 FINAL REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. A summary will be presented for each treatment group, indicating the number of slides examined, number of nuclei counted, relative survival and average net counts per nucleus. The report will also include a discussion of results and the Study Director's interpretation of results. A copy of the protocol used for the study and any significant deviation(s) from the protocol will appear as a part of the final report.

13.0 GOOD LABORATORY PRACTICES

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? No
If so, to which agency or agencies? _____

Does the Sponsor request that samples of the Test Article dosing solutions be returned? No
Sponsor requests return of all "A" coded test articles -

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14.0 SCHEDULE OF EVENTS

14.1 Proposed Initiation Date: October 16, 1985

14.2 Scheduled Completion Date: December 15, 1985

15.0 REFERENCES

1. Williams, G.M., The detection of chemical mutagens/ carcinogens by DNA repair and mutagenesis in liver cultures. In: Chemical Mutagens, Vol. VI, F.J. DeSerres and A. Hollaender, eds. Plenum Press, New York pp 71-79, 1979.

2. Williams, G.M., Carcinogen-induced DNA repair in primary rat liver cell cultures, a possible screen for chemical-carcinogens. Canc. Lett., 1:231-237, 1977.

3. Bradlaw, J. FDA, personal communication

J. Daniel Heck
SPONSOR'S AUTHORIZED REPRESENTATIVE

10/5/85
DATE PROTOCOL APPROVED BY SPONSOR

Rodger D. Curtis 10/2/85
STUDY DIRECTOR DATE

88208767

PROTOCOL AMENDMENT

DATE: April 10, 1986

SPONSOR: Blanket for Many Sponsors

SPONSOR'S TEST ARTICLE DESIGNATION: Blanket for Many Test Articles

MA STUDY NO: Blanket for Many Sponsors PROTOCOL NO.: SPGT380

PROTOCOL TITLE: Unscheduled DNA Synthesis in Rat Primary Hepatocytes

AMENDMENT(S): (INCLUDE LOCATION IN PROTOCOL, AMENDMENT, AND REASON)

Protocol SPGT380
Section 7.6 Dose Selection Line 18 and
Section 7.7.3 UDS Assay Line 8

Alternatively, the supernatant will be removed from the cell cultures and tested for its level of Lactic Acid Dehydrogenase (LDH). Leakage of LDH from the cytoplasm into the culture medium is also a function of cell plasma membrane integrity.

Measurement of LDH leakage, although of comparative sensitivity with the trypan blue vital dye staining, offers a less subjective alternative to cell counting procedures.

APPROVAL:

A. Daniel Hack — 4/21/86
SPONSOR REPRESENTATIVE/ INVESTIGATOR — DATE

Rodney T. Tolman — 4/11-/86
STUDY DIRECTOR — DATE

88208768

PROTOCOL AMENDMENT

DATE: December 1, 1986

SPONSOR: Lorillard Research Center

SPONSOR'S TEST ARTICLE
DESIGNATION: B34

MA STUDY NO: T5227.380 PROTOCOL NO: SPGT380

PROTOCOL TITLE: Unscheduled DNA Synthesis in Rat Primary Hepatocytes

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AMENDMENT(S): (INCLUDE LOCATION IN PROTOCOL, AMENDMENT, AND REASON)

Protocol SPGT380

Section 7.5.4 Preparation of Hepatocytes Line 4 and
Section 7.5.6 Preparation of Hepatocytes Line 3

Change "...approximately 20 ml/minute" to "... 20 to 40 ml/minute"

Reason for Change: To increase the number of usable hepatocyte preparations

APPROVAL:

J. David Heck — 12/5/86
SPONSOR REPRESENTATIVE/ INVESTIGATOR — DATE

Rodger D. Curren — 12/1/86
STUDY DIRECTOR — DATE

11/86
KAW

88208769

DATE: April 23, 1987

SPONSOR: Lorillard Research Center

SPONSOR'S TEST ARTICLE
DESIGNATION: B34

MA STUDY NO: T5227.380 PROTOCOL NO: SPGI380

PROTOCOL TITLE: Unscheduled DNA Synthesis in Rat Primary Hepatocytes

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AMENDMENT(S): (INCLUDE LOCATION IN PROTOCOL, AMENDMENT, AND REASON)

Protocol SPGI380

1) Location: Section 7.1.2 Media and Reagents Line 5
Amendment: Change "...containing 100 units" to "... containing 80 to 100 units"
Reason: To increase the number of usable hepatocyte preparations

APPROVAL:

J. Daniel Heck — SPONSOR REPRESENTATIVE/ INVESTIGATOR — 5/15/87 DATE

Richard D. Owen — STUDY DIRECTOR — 4/30/87 DATE

4/87
KAW