Du Pont HLR 150-88

FOR DU PONT USE ONLY

Study Title

IN VITRO BIOCOMPATABILITY EVALUATION OF [redacted] WITH IMR-90 HUMAN DIPLOID FIBROBLASTS IN THE MTT COLORIMETRIC ASSAY

Author

Ralph G. Stahl, Jr.

Issue Date

April 11, 1988

Peforming Laboratory

E. I. du Pont de Nemours and Company, Inc.
Haskell Laboratory for Toxicology and Industrial Medicine
Elkton Road, P. O. Box 50
Newark, Delaware 19714

Medical Research No.

[redacted]

Laboratory Project ID

Haskell Laboratory Report No. 150-88

GOOD LABORATORY PRACTICE STATEMENT

This study was conducted according to FDA Good Laboratory Practice Regulations (21 CFR 58). Any areas of noncompliance are documented in the study records. No deviations existed that significantly affected the validity of the study.

Submitter E.I. du Pont de Nemours & Co., Inc.

Sponsor Chemicals and Pigments Department

Study Director Ralph G. Stahl, Jr.

COMPOUND INFORMATION

Material Tested: [redacted]

Synonyms: [redacted]

Medical Research No. [redacted]

Haskell No.: H-17,157

CAS Registry No.: [redacted]

Other Codes: [redacted]

Purity: [redacted]

Contaminants: [redacted]

Sponsor: Chemical and Pigments Department

Material Submitted by: [redacted]
Chemicals and Pigments Department
Jackson Laboratory

Study Initiated/Completed: 2/15/88-2/19/88

Notebook(s): [redacted]

Distribution: [redacted]

There are 12 pages in this report.

RGS/Ames 18-12

IN VITRO BIOCOMPATABILITY EVALUATION OF [redacted] WITH IMR-90 HUMAN DIPLOID FIBROBLASTS IN THE MTT COLORIMETRIC ASSAY

Summary

[redacted] was tested for *in vitro* biocompatability with IMR-90 human diploid fibroblasts. Under the conditions of this assay, [redacted] is cytotoxic.

Work by: Jeffrey T. Turner, Sr. — 3/22/88 (Mo/Day/Yr)
Technician

Study Director: Ralph G. Stahl, Jr. — 3/22/88 (Mo/Day/Yr)
Ralph G. Stahl, Jr., Ph.D.
Research Genetic Toxicologist

Approved by: Awni Sarrif — 4/11/88 (Mo/Day/Yr)
Awni M. Sarrif Ph.D.
Section Supervisor
Molecular and Genetic Toxicology

QUALITY ASSURANCE DOCUMENTATION

STUDY: [redacted] IN VITRO BIOCOMPATABILITY EVALUATION OF [redacted] WITH IMR-90 HUMAN DIPLOID FIBROBLASTS IN THE MTT COLORIMETRIC ASSAY

AUDITS:

Items Audited	Audit Dates
Protocol, records and final report	3/28,29/88; 4/4/88

SHORT-TERM AUDIT REPORT NUMBER: R-318
DATE FINDINGS REPORTED TO MANAGEMENT AND STUDY DIRECTOR: 4/5/88

In-life critical phases from a representative study of this test type are inspected quarterly. Since short-term studies are numerous and routine in nature, the in-life critical phases from one study exemplify the conduct of other studies from the same test type.

Reported by: Joseph C Hamill 4/6/88
Joseph C. Hamill
Quality Assurance Auditor
Date

INTRODUCTION

The purpose of this study was to evaluate the cytotoxic potential of [redacted] in IMR-90 primary human diploid fibroblasts. Toxicity decreases the growth of cells in culture and cell viability. The basis for this test is the alteration of normal cell morphology and a decrease in the cell's ability to metabolize a chemical substrate. First, cells are exposed to the test material for 48-72 hours. Second, cells are then examined microscopically for morphological abnormalities. Third, a soluble dye, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, or MTT, is dissolved in culture medium and added to the cells. MTT is metabolized by the mitochondria in viable respiring cells to an insoluble purple formazan salt (1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan). Fourth, after 2-3 hours, the insoluble formazan trapped in the mitochondria is leached with dimethylsulfoxide. The optical density of the formazan, measured spectrophotometrically, is proportional to the number of viable, respiring cells. A measure of the test material's cytotoxicity, and by inference its potential biocompatability, is obtained by comparing the optical densities from treated cells to those not exposed to the test material.

MATERIALS/METHODS

A. PROTOCOL

The MTT assay is an adaptation of the method developed by Mossman (1983) and modified by Denizot and Lang (1986).

This study was conducted according to protocol Biocompatability-1 filed with the Quality Assurance Section of Haskell Laboratory on October 3, 1986, and updated February 16, 1988. This protocol includes the Standard Operating Procedures: In Vitro Biocompatability Evaluation of Material with Human and Mammalian Cells: Cell Counting Method; In Vitro Biocompatability Evaluation of Material with Human and Mammalian Cells: MTT Colorimetric Method and Microscopic Examination; Preparation of Biomaterials; and Using the IBM PC with the Titertek Multiskan® MC for the MTT Assay.

B. TEST MATERIALS

1. Test Compound

[redacted] Based on information supplied with the test material, the compound was assumed to be stable under the conditions of this assay. No evidence of instability was observed during the study.

2. Negative and Positive Indicators

Negative Control (noncytotoxic): RPMI-1640 culture medium (Gibco Lot #s 12N9273 and 43K8473).

Positive Indicator (cytotoxic): Mitomycin-C (MMC) (Sigma Lot # 66F-0494) at a final concentration of 10 ug/mL. MMC was prepared in distilled deionized water.

The negative control and positive indicator were assumed to be stable under the conditions of this study; no evidence of instability was observed.

C. CELLS AND CELL CULTURE

IMR-90 diploid human fibroblasts were obtained at passage 9 from the American Type Culture Collection (Cat. No. ATCC CCL 186), Rockville, MD. These cells were established in 1977 from the lungs of a 16-week female fetus (Nichols et al. Science 196: 60-63 1977).

IMR-90 cells are cultured routinely in a 5% CO_2 atmosphere at 37°C without antibiotics using RPMI-1640 culture medium (Gibco Lot #s 12N9273 and 43K8473) supplemented with L-glutamine (Gibco Lot # 10N0666) and 10% heat inactivated fetal bovine serum (Gibco Lot # 28N2372). Cells were free of mycoplasma contamination (GenProbe). Because of senescence cells are not used after passage 30.

Twenty-four hours before the test, cells were seeded into Corning 96-well (half-well area) tissue culture dishes at a density of 60,000 cells/cm^2. Column 1 of the dish was left blank as a control for optical density measurements. Columns 2-12 recei d 100 uL of culture medium/cell suspension.

D. PREPARATION OF TEST MATERIALS

The test material, [redacted] was filter sterilized through a 0.22 u filter (Millipore Millex-GS) before use.

E. DOSE SELECTION

Cells were plated in 100 uL of medium per well. Either 10, 25, 50 or 100 uL of test material was added directly to the medium (9%, 20%, 33%, or 50% final concentration of test material, respectively). Concentrations greater than 50% significantly dilute the culture medium, reduce cell growth, and therefore were not tested. The negative control received no test material. Approximately 10 uL of MMC, the positive indicator, stock solution was added to 100 uL of culture medium (final concentration was 10 ug/mL). Treatment wells were individually labeled with the appropriate concentration of test material. After the test material was added, dishes were placed in a 5% CO_2, 37°C incubator for 72 hours.

G. CYTOTOXICITY DETERMINATIONS

1. Microscopic Examination

All cells were examined under 250X phase contrast magnification for signs of toxicity after the 72 hour exposure. Observations for cell rounding, sloughing and detachment, and lysis were in accordance with guidelines of the American Society for Testing Materials (ASTM, 1983) and the National Heart, Lung and Blood Institute (1979).

2. Measurement of Effects on Growth

After 72 hours of exposure, all 96-well dishes were removed from the incubator. Each dish was inverted to remove culture medium. Fifty microliters of a 1 mg/mL solution of MTT (Sigma Lot # 126F5054) prepared in RPMI-1640 culture medium (without phenol red; Gibco Lot # 14N0066) was added to each well. The dishes were returned to the 37°C incubator for about 3 hours. Afterwards, the dishes were removed and inverted to remove the MTT solution. Approximately 50 uL of dimethlysulfoxide (DMSO; Sigma Lot # 116F34861) was added to each well. The purple formazan color was allowed to develop at room temperature for about 30 minutes. Each dish was then placed in the reading chamber of a Titertek Multiskan® MC connected to an IBM-PC. The optical density was measured at 560 (test wavelength) and 690 (reference wavelength) nanometers. Data were captured on an IBM-PC, stored on a data diskette, and printed.

H. STATISTICAL ANALYSES

Optical densities from treated and control cultures were compared using RS/1 computerized statistical software (BBN Software Products Corporation, Book 2: Graphics and Statistics, Cambridge, MA, 1984). A student t-test, assuming unequal variances, compared each test concentration with the untreated control. The null hypothesis was that the optical density in the treated culture was less than that of the control. All comparisons were at the 95% level of confidence (alpha = 0.05).

I. CLASSIFICATION GUIDELINES

The guidelines below are used to aid in the classification of a test sample along with sound scientific judgement and experience.

A test sample is classified as CYTOTOXIC when:

A. The optical densities in exposed cultures are significantly less than untreated controls or negative control at the 0.05 level of significance as measured during the test period,

AND

B. Cell rounding, sloughing or lysis was observed.

A test sample is classified as NONCYTOTOXIC when:

A. The optical densities in exposed cultures are not significantly less than untreated controls or negative control at the 0.05 level of significance as measured during the test period,

AND

B. Cell rounding, sloughing or lysis was not observed.

A test sample is classified as EQUIVOCAL when:

A. Neither of the criteria for a cytotoxic nor noncytotoxic classifcation is satisfied.

G. ACCEPTABILITY CRITERIA

An acceptable assay is based on a single trial in which the negative and positive indicators respond in the appropriate manner. In addition, contaminated wells are excluded from data analysis. There must be a minimum of 8 noncontaminated wells for each exposure, and the positive and negative indicators. The trial is repeated if these criteria are not met.

H. RETENTION OF RECORDS

All raw data and the final report are stored in the archives of Haskell Laboratory for Toxicology and Industrial Medicine, Newark, Delaware or in the Du Pont Records Management Center, E. I. du Pont de Nemours and Co., Inc., Wilmington, Delaware.

RESULTS/DISCUSSION

Results of [redacted] cytotoxicity to IMR-90 human cells are shown in Table 1. [redacted] was toxic at concentrations of 20%, 33% and 50%, causing widespread cell lysis. Cell morphology was not affected at the 10% concentration of [redacted] There were significant decreases ($p < 0.001$) in optical densities at all concentrations of [redacted] tested. MMC, the positive indicator, significantly reduced optical density and caused widespread cell lysis.

Under the conditions of this assay, [redacted] is cytotoxic.

REFERENCES

American Society for Testing and Materials (1983) Designation F813-83 Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices American Society for Testing and Materials, Philadelphia, PA.

Denizot, F. and R. Lang (1986) Rapid colorimetric assay for cell growth and survival: Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Journal Immunological Methods 89: 271-277.

Green, L.M., J.L. Reade and C.F. Ware (1984) Rapid colorimetric assay for cell viability: Application to the quantitation of cytotoxic and growth inhibitory lymphokines. Journal Immunological Methods 70: 257-268.

Mossman, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal Immunological Methods 65: 55-63.

National Heart, Lung and Blood Institute (1979) Guidelines for Physiochemical Characterization of Biomaterials. Devices and Technology Branch, National Heart, Lung and Blood Institute, NIH Publication No. 80-2186, Washington, D.C.

RS/1® (1984) RS/1® User's Guide, Book 2, BBN Software Products Corp., Cambridge, MA.

Table 1.

In Vitro Biocompatability of H-17,157 With IMR-90 Human Cells.
Results of MTT Colorimetric Assay After 72 Hour Exposure.

Well #	Control	50% H-17157	33% H-17157	20% H-17157	9% H-17157	MMC 10 ug/mL
1	0.221	0.005	0.003	-0.002	0.272	0.001
2	0.256	0.005	0.004	-0.004	0.221	-0.001
3	0.270	0.008	0.010	-0.001	0.202	0.004
4	0.267	0.010	0.005	-0.004	0.160	-0.005
5	0.256	0.007	0.002	-0.003	0.237	-0.002
6	0.273	0.010	0.012	0.003	0.179	0.004
7	0.259	0.013	0.006	0.001	0.170	0.002
8	0.226	0.013	0.005	-0.002	0.235	-0.001
9	0.248	0.013	0.003	-0.003	0.252	-
10	0.267	0.012	0.003	0.000	0.005	-
11	0.274	0.009	0.003	0.001	0.224	-
12	0.254	0.004	0.003	-0.003	0.220	-
13	0.275	0.013	-0.001	-0.002	0.241	-
14	0.251	0.011	0.007	0.006	0.238	-
15	0.290	0.014	0.006	0.001	0.226	-
16	0.226	0.016	0.001	-0.003	0.255	-
Mean	0.257	0.010	0.005	-0.001	0.209	0.001
Stdev	0.019	0.003	0.003	0.003	0.060	0.001
p value		0.000*	0.000*	0.000*	0.004*	0.000*

Data are optical densities corrected for reagent blanks.
Stdev = Standard deviation.
p value = Probability that the mean is less than that of the control.
* = Significant at the 95% level of confidence.
- = Indicates not tested.
([redacted]) = H-17,157