AR226-0161
Pk-6

PROTOCOL TITLE: **Range finding Cytotoxicity Assay of T-6292, T-6293, T-6294, T-6295 in Rat Hepatocytes**

SRI Study No. B010-95

RESEARCH CLIENT

3M Medical Department
Medicine, Health Physics, Industrial Hygiene, Toxicology
3M Center
Building 220-2E-02
St. Paul, MN 55144-1000

Study Monitor: Steven C. Gordon, Ph.D., D.A.B.T.

TESTING LABORATORY

SRI International
Toxicology Laboratory
333 Ravenswood Avenue
Menlo Park, CA 94025

Study Director: Carol E. Green, Ph.D., D.A.B.T.
Telephone: (415) 859-4083
FAX: (415) 859-2889

APPROVALS:

Steven C. Gordon
Research Client's Authorized Representative

6/27/95
Date

Carol E. Green
SRI Study Director

7/3/95
Date

v. May 3, 1995

004030

I. STUDY OBJECTIVE

The objective of this study is to evaluate the cytotoxicity of 4 different test articles as measured by MTT conversion. The EC_{50} and EC_{10} values will be estimated for each test article and these data will be used to select a suitable concentration of each test article for subsequent *in vitro* metabolism studies.

II. APPROACH

Hepatocytes will be isolated from adult male Sprague-Dawley rats. The cells will be allowed to attach in monolayer culture in the wells of a 96 well plate. The cells will then be exposed to either test article, solvent, or positive control in 4 replicate cultures. In this experiment, the compounds will be tested in 6 different concentrations at a single time point (24 hours). The concentrations for each test article are described below in the Experimental Design.

III. TEST ARTICLE

A. Test Article Identification

Name: T-6292

Lot No.: To be provided by Research Client

Molecular weight: 571.2

Name: T-6293

Lot No.: To be provided by Research Client

Molecular weight: 658.28

Name: T-6294

Lot No.: To be provided by Research Client

Molecular weight: 527.2

Name: T-6295

Lot No.: To be provided by Research Client

Molecular weight: 538.1

B. Purity and Stability

Documentation of the identity, strength, purity, and stability of the test articles will be the responsibility of the Research Client. The positive control, acetaminophen, and the solvent control, DMSO, will be obtained from Sigma Chemical Co. (St. Louis) and their documentation of the identity and purity of these chemicals will be used.

C. Handling and Storage

On receipt, the test articles will be placed in a secondary container (lightproof if required) and stored as recommended by the Research Client. A Material Safety Data Sheet (MSDS) or other comparable document specifying any hazards and identifying first aid and clean-up procedures in case of an accidental spill shall be provided by the Research Client and shall accompany the test articles. The test articles will be logged in and stored in Building L, Room LB286.

D. Disposition of the Test Article

Any remaining unused portion of the test articles will be returned to the Research Client after completion of the study.

IV. TEST SYSTEM

A. Background and Justification for Selection of the Test System

The liver is the major site of metabolism of most organic chemicals, both endogenous and foreign. Species-related differences in the metabolism of xenobiotics are well known and have been documented to be correlated to the toxic effects of chemicals on certain species (Caldwell, 1980; Calabrese, 1983). The proposed colorimetric cytotoxicity assay has been used to determine mammalian cell survival and proliferation (Mosmann, 1983; Otoguro et al., 1991). This assay detects living, but not dead cells. Because the signal generated is

dependent on the degree of activaiton of the cells, cytotoxicity, proliferation, and activation can all be measured. This assay depends on the fact that MTT (which is yellow in aqueous solution at neutral pH) is metabolized by mitochondria of living cells to a blue formazan dye. When used as a cytotoxicity assay, spectrophotometric quantitation of the formazan generated by cells after their incubation with the test article will give an indication of the number and metabolic activity of the surviving cells.

B. Test Species

Rat. Adult male Sprague-Dawley rats will be used for the preparation of hepatocytes. They will be purchased from Charles River Laboratories and will weigh approximately 200-250 g at the time of purchase.

The rats to be used for this study will be housed in Building L, where the cell isolation will be performed. Rats will be taken from the shipping containers, weighed, examined for general health, and placed three per cage in 22 x 12½ x 8-inch suspended polycarbonate cages labeled with their quarantine number assignments. Animals will be quarantined in the same room in which they will be housed. Rats will be fed (*ad libitum*) Purina Certified Rodent Chow (#5002) and the feed supplier will provide SRI with an analytical report identifying the diet to be free of contaminants for each lot of feed received. A copy of each analytical report will be available as part of the study record. Rats will receive purified (deionized and UV-treated) drinking water *ad libitum*. Temperature will be maintained at 72 ± 4°F and relative humidity maintained between 35 and 65%. A light cycle of 12 hours light and 12 hours dark will be maintained, with light starting at 06:00.

The laboratory species will be quarantined for at least 3 days before use in an experiment. The Laboratory Animal Medicine Department at SRI will issue documentation to the Study Director on the health of animals during the quarantine period. Before isolation of hepatocytes, the rats will be anesthetized with sodium pentobarbital (65 mg/kg).

V. EXPERIMENTAL DESIGN

A. Hepatocyte Isolation

Rat hepatocytes will be isolated by either whole liver or biopsy perfusion as convenient (Green et al., 1983). In brief, the whole liver or wedges of the organ will be perfused first with a Ca^{+2}-free buffer, followed by a buffer containing collagenase. Hepatocytes will be combed free from the digested tissue and purified by simple differential centrifugation.

Isolated hepatocytes will be plated onto collagen-coated culture dishes (35 mm diameter) in a modified Waymouth's 752/1 culture medium (CMH1a) that contains 11.2 μg/ml alanine, 12.8 μg/ml serine, 24.0 μg/ml asparagine, 84.0 μg/ml gentamicin sulfate, 0.168 μg/ml aminolevulinic acid, 5.0 μg/ml oleic acid, 5.0 μg/ml linoleic acid, 1.0 μg/ml D,L-tocopherol, 288 ng/ml testosterone, 272 ng/ml estradiol, 393 ng/ml dexamethasone, 7.9 μg/ml thyroxin, 30 ng/ml glucagon, 0.02 U/ml insulin, 0.1% ITS (Collaborative Research, Inc.; final concentrations: 5 μg/ml transferrin, 5 μg/ml insulin, and 5 ng/ml selenium), 0.2 mM L-ascorbic acid 2-phosphate, and 0.2% BSA. The initial plating medium will contain 10% fetal bovine serum. After 2 to 3 hr of incubation at 37°C in an atmosphere of 95% air:5% CO_2, the medium will be aspirated to remove nonviable, unattached cells and replaced with CMH1a containing the test article.

B. Range Finding Cytotoxicity Experiment (MTT Assay)

1. Preparation and Administration of Test Articles and Controls. Concurrent solvent controls will be examined in the experiment. Solvent control samples will consist of culture medium and DMSO at the same concentration used to dissolve the test articles. DMSO will be used so that the final concentration does not exceed 10 μl/ml (1%). Acetaminophen (10 mM) will be the positive control and will be prepared as a stock solution in culture medium.

Immediately before the experiment, an aliquot of each test article (solid or pure liquid) will be diluted in DMSO to form a concentrated stock solution. Serial dilutions will be made from these initial stocks.

2. Test Article Concentrations. Six test article concentrations will be studied: 0.01, 0.05, 0.10, 0.50, 1.0, 5.0, 10.0 mM.

3. Incubation of Hepatocytes with Test Article. Four replicate monolayer cultures of isolated rat hepatocytes will be incubated at 37°C for 24 hours with the test article or control. A negative control (no cells) will also be measured for determination of background staining.

4. MTT Assay. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay will be performed as described by Scudiero et al. (1988) as modified by this laboratory (Otoguro et al., 1990). MTT (Sigma) will be dissolved at 5 mg/ml in calcium and magnesium free phosphate buffered saline (CMF-PBS) and sterile-filtered with a 0.2 μm filter unit. The MTT solution will be diluted 1:5 in CMH1a, and 150 μl will be added to each of the 24 wells in the dish. After a 2 hour incubation at 37°C, the incubation medium will be

aspirated and 450 μl of DMSO will be added to solubilize the MTT-formazan product. After mixing, the absorbance at 540 nm will be measured.

5. **Data Collection.** The net change (%) relative to the solvent control will be plotted against the chemical concentration. The EC_{50} values will be determined by a computer statistical program designed for simultaneous fitting of families of sigmoidal dose response curves using the four-parameter logistic equation (De Lean et al., 1988).

VI. REPORTS

A summary report containing the results of this experiment will be issued to the Study Monitor. The test article concentrations for the metabolism study will be selected based on these results.

IX. RECORDS TO BE MAINTAINED

The laboratory notebooks, all raw data, relevant communications, and the original copy of the Final Report will be transferred to the SRI Records Center, Building B, after completion of the study. These materials will be maintained for 10 years; then the Research Client will be contacted concerning future disposition of the records.

X. REFERENCES

Calabrese, E. J. John Wiley & Sons, New York, pp. 203-282, 1983.

Caldwell, J. In *Enzymatic Basis of Detoxication*, Vol. I. William B. Jakoby (Ed.), Academic Press, New York, pp. 85-114, 1980.

DeLean, A., P. J. Munson, and D. Rodbard. 1978. *Am. J. Physiol.* **235**, E97-E102.

Green, C. E., J. E. Dabbs, and C. A. Tyson. Anal. Biochem. **129**, 269-276, 1983.

Mosman, T. J. Immunological. Meth. **65**, 55-63, 1983.

Otoguro, K., K. Komiyama, S. Omura, and C.A. Tyson. ATLA **19**, 356-360 (1991).

FACSIMILE TRANSMISSION

Message to: Dr. Steve Gordon

Company: 3M

FAX Number: 612/733-1773

Date: October 26, 1995

RE: Cytotoxicity assay

Number of Pages: 5

From: Carol Green
Direct Phone: (415) 859-4083
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Toxicology Laboratory
333 Ravenswood Avenue
Menlo Park, CA 94025
FAX (415)859-2889
(415)326-5512(alt)

CC: V. Weizer
C. Tyson

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Enclosed are copies of the data from the MTT assay. You will also find some notes prepared by the technician on the solubility of the compounds in the medium. After reviewing the raw data (absorbance values) for T-6292, I found that the controls for that plate were low. As a result, I think the assay for that chemical may need to be repeated. Based on the appearance of the cells treated with T-6292, severe toxicity occurred between 0.1 and 0.5 mM. Please review the results and call me so we can discuss this further.

Carol

Project 7565 Rat MTT Assay

Project 7565
MTT Assay using Rat hepatocytes

Hepatocytes isolated on 10/16/95.
Cells exposed to test articles for 24 hours.
MTT assay performed on 10/17/95.
At the 24 hour time point the media containing test article was aspirated from the plate and replaced with MTT assay solution.
MTT Assay solution was incubated on the cells for 2 hr
MTT assay solution was removed after 2 hr and the cells were lysed with DMSO releasing into the DMSO the Formazan Blue Dye.
The plates were read on a Molecular Devices Kinetic Plate reader at 540nm.
Data calculations were performed on the Softmax operating program for the plate reader.

T-6295 FC-95

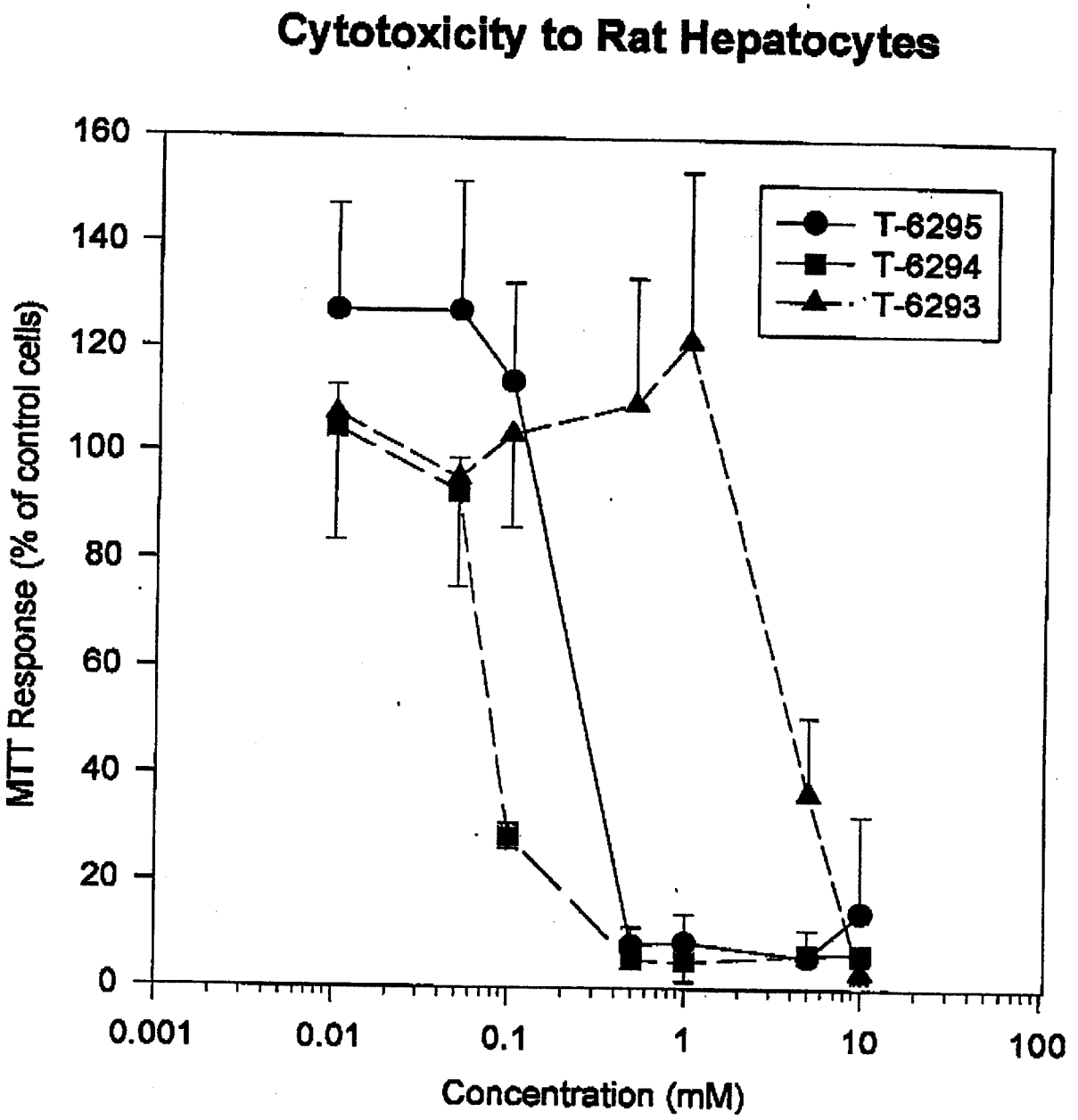
(mM)	% of control cells	SD
0.01	127.3	19.68
0.05	127.3	24.42
0.1	113.8	18.92
0.5	8.318	3.492
1.0	8.796	5.443
5.0	6.13	5.184
10.0	14.58	17.89

T-6294 FX-12

(mM)	% of control cells	SD
0.01	104.7	8.301
0.05	92.73	6.273
0.1	48.43	2.411
0.5	5.366	1.669
1.0	4.983	3.694
5.0	6.54	0.919
10.0	6.807	0.883

Project 7565 Rat MTT Assay

T-6293	FC-807						
(mM)			% of control cells		SD		
0.01			107.4		23.8		
0.05			95.22		19.66		
0.1			103.5		17.53		
0.5			109.6		24.2		
1.0			121.6		32.13		
5.0			36.71		14.49		
10.0			2.969		1.586		
T-6292	FC-10						
(mM)			% of control cells		SD		
0.01			149.8		52.49		
0.05			128.1		38.54		
0.1			404.5		52.7		
0.5			25.99		17.9		
1.0			17.31		4.426		
5.0			227.1		60.39		
10.0			13.59		4.414		
APAP	Positive control						
(mM)			% of control cells		SD		
10			36.45		8.027		


Cytotoxicity to Rat Hepatocytes
MTT Response (% of control cells)
160
140
120
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
Concentration (mM)
T-6295
T-6294
T-6293

Notes on solubility of compound during MTT experiment of Oct.16-17.

T-6292 Visibly precipitated out at 10, 5, and 1 mM concentrations

T-6293 Not all solubilized at 10, 5, and 1 mM concentrations in the DMSO added CMH1a and after agitation appeared to have gone into solution.

T-6294 Visibly precipitated out at 10 and 5 mM concentrations. No visible precipitate at 1 mM concentration.

T-6295 Visibly precipitated out at the 10 mM concentration. No visible precipitate at the 5 and 1 mM concentration.

Notes on appearance of cells after incubation with test articles for approximately 22 hours.

T-6292 Rows 3-5 some cells spread out some cells rounded. 0.01, 0.05, 0.1 mM
Rows 6-9 very few cells spread out most rounded. 0.5, 1.0, 5.0, 10 mM
Row 9 crystals present dark colored media.

T-6293 Rows 3-6 no visible abnormalities 0.01 – 0.5 mM
Rows 7-10 have a lot of debris, the cells are rounded or unattached. 1.0, 5.0, 5.0, 10.0*

T-6294 Rows 3-5 no visible abnormalities. 0.01 - 0.1 mM
Rows 6-9 cells not spread out, rounded. 0.5 - 10 mM

T-6295 Rows 3-5 cells mostly spread out with some rounded. 0.01 - 0.1 mM
Rows 6-7 cells not spread out with some debris. 0.5 - 1.0 mM
Rows 8-9 a lot of debris, the cells were rounded. 5.0 - 10.0 mM

APAP rows 2-3 no visible abnormalities. Controls
Rows 4-6 few to no spread out cells. APAP

Appearance of T6295 after 2 hour incubation with MTT solution
Wells 8-9 had a heavy yellow precipitate.
Well 7 had a medium yellow precipitate.
Well 6 had a light yellow precipitate.
Wells 1-5 no visible precipitate.

The other Plates had no remarkable abnormalities.

* For plate T-6293 one row of wells was repeated. When read by plate reader and by visible observation of color change rows 8 and 9 were 5.0 mM concentration. This corresponds with an interruption during the application of test articles and time I noticed the error.
vlw 10/24/95

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Facsimile Transmittal

From: Carol Green

Direct Phone: 415-859-4083

Message To: Dr. Steve Gordon

Company: 3M

Fax Number: 612 733-1773

Date: December 12, 1995

This Message Transmitted from: Fax No. (415) 859-2889

Number of Pages: 3 including this transmittal sheet

MESSAGE:

Enclosed are the data from the repeat NTT assay. We believe that the reversal in toxicity at 5 & 10 mM was due to the low solubility of the test articles at these concentrations, even in DMSO. Because the compounds were poorly solubilized, we made separate stock solutions for 10, 5, & 1 mM. Subsequent concentrations were serial dilutions of 1 mM. (Same as first experiment).

Sorry for my handwriting but my computer is ill today.

Carol.

Please call the sender directly if you have any problem with the reception of this message.

004041

Project 7565 Rat MTT Assay

Project 7565							
MTT Assay using Rat hepatocytes							
Hepatocytes isolated on 12/6/95.							
Cells exposed to test articles for 24 hours.							
MTT assay performed on 12/7/95.							
At the 24 hour time point the media containing test article was aspirated							
from the plate and replaced with MTT assay solution.							
MTT Assay solution was incubated on the cells for 2 hr							
MTT assay solution was removed after 2 hr and the cells were lysed with DMSO							
releasing into the DMSO the Formazan Blue Dye.							
The plates were read on a Molecular Devices Kinetic Plate reader at 540nm.							
Data calculations were performed on the Softmax operating program for the plate reader.							
T-6293	FC-807						
(mM)			% of control cells		SD		
0.01			84.01		4.495		
0.05			104.7		23.85		
0.1			108.2		14.95		
0.5			45.28		15.69		
1.0			6.739		1.440		
5.0			55.96		18.52		
10.0			97.4		10.36		
T-6292	FC-10						
(mM)			% of control cells		SD		
0.01			106.1		24.85		
0.05			110.0		41.77		
0.1			59.52		33.77		
0.5			7.882		2.914		
1.0			9.208		2.199		
5.0			24.44		9.503		
10.0			93.92		23.96		
APAP	Positive control						
(mM)			% of control cells		SD		
10			32.34		12.80		

EC_{10} = 0.065 mM

EC_{50} = 0.137 mM

Notes on solubility of compound during MTT experiment of Dec.6-7

T-6292 Compound was not all solubilized at 10, 5, and 1 mM concentrations. The 1 mM concentration appeared to be the most solubilized in the DMSO and CMH1a and after agitation appeared to have gone into solution.

T-6293 Compound was not all solubilized at 10, 5, and 1 mM concentrations. The 1 mM concentration appeared to be the most solubilized in the DMSO and CMH1a and after agitation appeared to have gone into solution. Sonicated 10, 5 and 1 mM concentrations for 10 minutes after addition of CMH1a to increase solubility.

Notes on appearance of cells after incubation with test articles for approximately 22 hours.

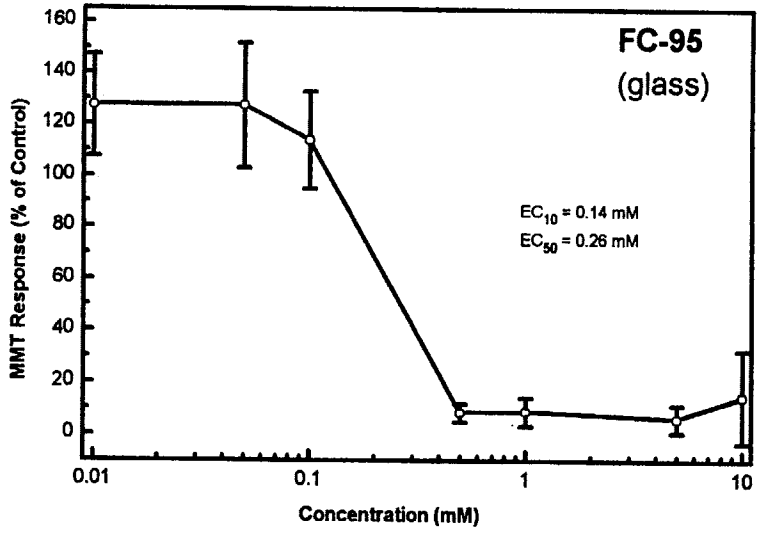
T-6292 Rows 8-9 Some cells spread out some cells rounded. Visible crystals of compound. (5,10 mM)
Rows 6-7 Few to no cell-cell attachments most all cells appear Visible crystals of compound. (1.0, 0.5 mM)
Row 5 Some flattened cells with cell-cell attachment. (0.1 mM)
Row 4 More cell-cell attachment. Few to no crystals. Some rounded cells. (0.05 mM)
Row 3 Cells slightly better than row 4. (0.01 mM)
Row 2 A couple of rounded cells otherwise good. (Normal media)

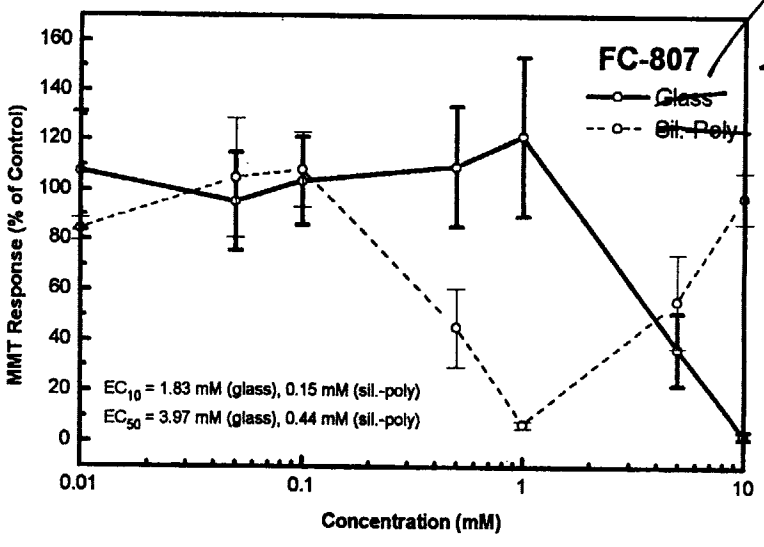
T-6293 Row 9 Cells visible many rounded with some spread having cell-cell attachment. (10.0 mM)
Rows 6-8 Cells not visible due to grainy debris. (5.0, 1.0, 0.5 mM)
Row 5 Very grainy cells rounded. (0.1 mM)
Row 4 Some cells are clumped together growing on top of each other, grainy debris present. (0.05 mM)
Row 3 Good with grainy appearance. (0.01 mM)
Row 2 A couple of rounded cells otherwise good. (Normal media)

APAP rows 2-5 no visible abnormalities. (Normal media)
Rows 6-9 few to no spread out cells.(10mM APAP)

vlw 12/7/95

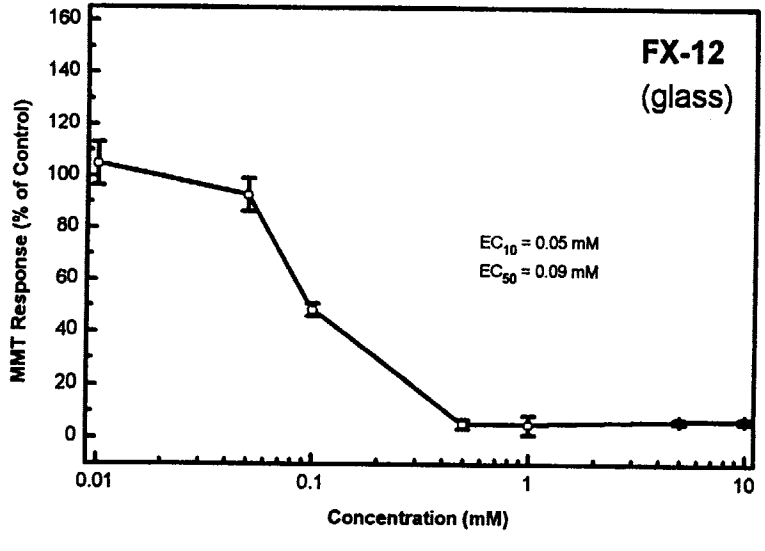
T-6295

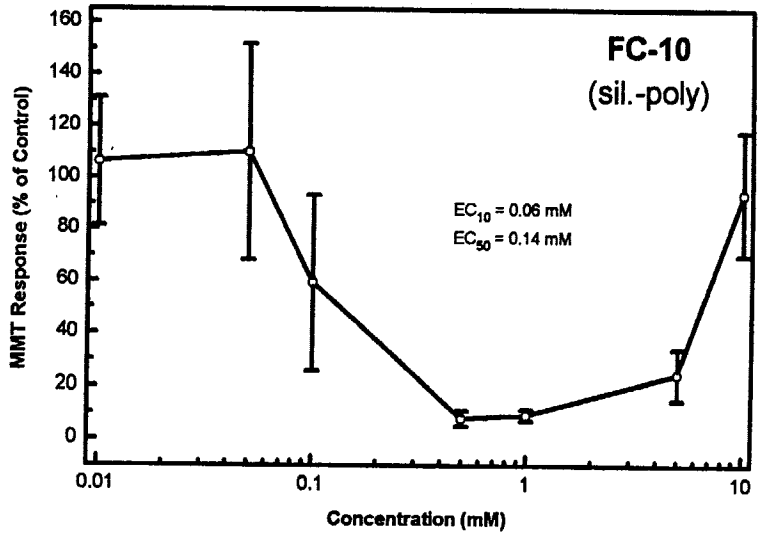




plastic
T-6293
— silanized glass

T-6294





T-6292

FACSIMILE TRANSMISSION

Message to: Dr. Steve Gordon

Company: 3M

FAX Number: 612/733-1773

Date: January 16, 1996

RE: Rat hepatocyte study

Number of Pages: 3

From: Carol Green
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CC: V. Weizer
C. Tyson

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Enclosed are copies of the data from the LDH assay that we performed on rat hepatocytes incubated with the test articles following the protocol for the metabolism study. We used 100 μM of each as discussed. No significant toxicity is evident except with 6294, which did increase LDH release. I suggest that we send the other samples from 6292, 6293, and 6295 to ABS for analysis. Do you agree?

We can discuss these results after you have a chance to review them. I would also like to discuss the project budget because the LDH assay was not included in the original protocol and we now may need to repeat the incubation for 6294 metabolism. I will talk to you soon about these issues.

C04046

1/16/96 VLW

LDH assay for 7565 / B011-R1

incubation performed on 1/11/96

LDH ~~analized~~ analyzed on Gemeni on 1/12/96

Samples stored 0-5°C untill analysis

Cells in 35 mm dish were scraped into 1.0 ml of PBS after washing with PBS

Cells were diluted/lysed with 1.0 % triton X-100 in PBS 1.0 ml

Cell samples were further diluted as necessary

2X = 100 µl cell mix + 100 µl PBS — 6294 c + d, 6293 c,

3X = 100 µl cell mix + 200 µl PBS — normal, 6292 c + d, 6293 d, 6295 c + d

Media samples were aspirated into microfuge tubes and stored untill analysis at 0-5°C

Normal cells were incubated with 2 ml of medium

Test articles were incubated with 1 ml of medium

Media samples were further diluted as necessary

2X = 100 µl medium + 100 µl PBS — 6294 c+d

remaining samples were not diluted normal cells were in 2 ml of media so 2X

Caluculations:

Final LDH C = dilution C X LDH/Cell

Final LDH M = dilution M X LDH/media

Total = Final LDH C + Final LDH M

% released into media = Final LDH M / Total (expressed as %)

* Spiking correction
Cg 111456

SAMPLE		LDH/CELL	dilution C	final LDH C	LDH/MEDIA	Dilution M	final LDH M	Total	% released into media	
normal a		384	6	2304	207	2	414	2718		15.2%
normal a		360	6	2160	211	2	422	2582		16.3%
normal b		438	6	2628	242	2	484	3112		15.6%
normal b		456	6	2736	248	2	496	3232		15.3%
									Average	15.6% ± 0.5 **

SAMPLE		LDH/CELL	dilution C	final LDH C	LDH/MEDIA	Dilution M	final LDH M	Total	% released into media	
6292 c		779	6	4674	808	1	808	5656		14.3%
6292 c		745	6	4470	815	1	815	5285		15.4%
6292 d		749	6	4494	717	1	717	5211		13.8%
6292 d		750	6	4500	732	1	732	5232		14.0%
									Average	14.4% ± 0.7 ***
6293 c		909	4	3636	484	1	484	4120		11.7%
6293 c		969	4	3876	509	1	509	4385		11.6%
6293 d		627	6	3762	552	1	552	4314		12.8%
6293 d		649	6	3894	560	1	560	4454		12.6%
									Average	12.2% ± 0.6 ***
6294 c		712	4	2848	722	2	1444	4292		33.6%
6294 c		722	4	2888	704	2	1408	4296		32.8%
6294 d		760	4	3040	532	2	1064	4104		25.9%
6294 d		763	4	3052	544	2	1088	4140		26.3%
									Average	29.7% ± 4.1 ***
6295 c		451	6	2706	547	1	547	3253		16.8%
6295 c		459	6	2754	574	1	574	3328		17.2%
6295 d		709	6	4254	286	1	286	4540		6.3%
6295 d		653	6	3918	284	1	284	4202		6.8%
									Average	11.8% ± 6.0 ***

*** Calculated standard deviations by IJK 1/96