INDUCTION OF HEPATIC MICROSOMAL ENZYMES
IN RATS BY B111

MICROBIOLOGICAL ASSOCIATES INC.

5221 RIVER ROAD, BETHESDA, MARYLAND 20816

853974583

Final Report
I-5049.401

INDUCTION OF HEPATIC MICROSOMAL ENZYMES IN RATS BY B111

Final Report for
Lorillard Research Center
420 English St.
Greensboro, N.C. 27420

February 27, 1986

By
Microbiological Associates Inc.
5221 River Rd.
Bethesda, MD. 20816

89397459

Table of Contents

	Page
I. Data Page	1
II. Introduction	2
III. Purpose	3
IV. Test Article Identification and Properties	4
V. Test Description	5
VI. Methods	6
A. Animals	
B. Treatment	
C. Enzyme Preparation	
D. Assays Used to Assess Enzyme Activity	
VII. Results	9
VIII. Conclusions	10
IX. References	11
X. Quality Assurance Statement	12
XI. Tables	13
XII. Protocol	14

89397460

ABBREVIATIONS

7EC - 7-Ethoxycoumarin O-deethylase

ETR - 7-Ethoxyresorufin O-deethylase

MTD - Maximum tolerated dose

PNAS - para-Nitroanisole O-demethylase

S9 - Supernatant from 9000xg centrifugation

89397461

Final Report
I-5049.401

I. DATA PAGE

Test Article Identity: B111

Initiation Date: January 20, 1986

Completion of Dosing: January 23, 1986

Completion of *In Vitro* Assays: February 5, 1986

Review Date: See Review Completed Date, Page 12

MA Study Number: I-5049.401

MA Notebook Number: 5049.401

Archives Location: 5221 River Road, Bethesda, Maryland 20816

Sponsor: Lorillard Research Center
420 English St.
Greensboro, NC 27420

Authorized Representative: J. Daniel Heck, Ph.D.

Testing Facility: Microbiological Associates Inc.
5221 River Road
Bethesda, Maryland 20816

Technical Staff: David R. Dansie, Lloyd C. Campbell, Guillermo Martinez, Raymond W. Nims, Calvin V. Dove, Lana L. Braithwaite

Study Director: Raymond M. David, Ph.D., D.A.B.T. 2/27/86 Date

89397462

II. INTRODUCTION

The Cytochromes P-450 are a group of hemoproteins which are associated with the microsomal or lipid portion of a cell. Their designation as P-450 resulted from the observed maximum of the reduced hemoprotein-carbon monoxide complex at 450 nm in a difference spectrum (Omura & Sato, 1964). These hemoproteins also have enzymic activity, and can metabolize relatively lipophilic substrates to forms which are more water soluble. Such reactions are a normal function of the body and result in the formation of many important hormones. A great variety of non-physiologic compounds (xenobiotics) can also be metabolized by these enzymes, e.g. alkyl halides, aromatic hydrocarbons, aliphatic amines, etc. Such broad substrate specificity is enhanced by the fact that the relative population of isozymes can be altered by the presence of a potential substrate. Such alterations may increase the amount of only one specific isozyme that metabolizes that substrate, or all P-450 activity can be increased uniformly. In either case, the phenomenon of induction can have important consequences not only to the xenobiotic substrate that induced the activity, but also to physiologic substrates.

Of the number of compounds that have been shown to induce P-450, most have tended to fall into one of two categories based on their similarity to two classic inducers: phenobarbital and methylcholanthrene (Snyder and Remmer, 1979). Phenobarbital (PB) induces general P-450 activity, while methylcholanthrene (MC) induces a different type of P-450 called P_1-450 or P-448, named because of the shift in spectral maximum from 450 to 448 nm. Both MC and PB induced forms of P-450 can be induced by certain chlorinated biphenyls such as Aroclor 1254 (Parkinson et al., 1983).

A number of reactions have been used to assay P-450 activity. Three primary assays can be employed to examine P-450 and P-448 activity.

1) p-Nitroanisole O-demethylation - The demethylation of p-nitroanisole has been used as a marker for P-450 activity in the liver (Thurman et al., 1977).

2) 7-Ethoxycoumarin O-deethylation - The O-deethylation of 7EC has been shown to be a sensitive marker for P-448 and P-450 induction (Greenlee and Poland, 1978).

3) Ethoxyresorufin O-deethylation - Ethoxyresorufin deethylation is a sensitive marker for P-448 induction in rat liver, kidneys, and lungs (Nims et al., 1984).

89397463

III. PURPOSE

The purpose of this study is to determine if orally administered test material induces cytochrome P-450 and/or P-448 activity in rat liver.

89397464

IV. TEST ARTICLE IDENTIFICATION AND PROPERTIES

Test article Identification:	B111
Test Article Number:	T05049A
Quantity received:	50g
Date received:	January 16, 1986
Expiration date:	June 11, 1986
Date sample returned:	Stored at Microbiological Associates Inc.
Description:	Clear pale yellow liquid
Storage conditions:	Refrigerated (amber bottle)
Purity:	100%
Solubility:	Corn oil[a]
Handling Conditions:	Routine safety precautions

Protocol says C.O. so why put this in here

[a] Sponsor had suggested propylene glycol, but this was changed after consultation.

89397465

V. TEST DESCRIPTION

Groups of rats (8 rats per group) were treated daily by gavage with one of 2 concentrations of test material for 4 consecutive days. A control group (8 rats per group) was treated with vehicle only. Dose volumes were based on individual body weights on Day 1. Oral administration of test material was the route of administration specified by the Sponsor. Dosing volumes were 10 ml/kg body weight.

On day 5, all surviving animals were weighed and sacrificed by carbon dioxide asphyxiation. Their livers were excised, washed in cold 1.15% KCl, and weighed. The livers were homogenized in 2 volumes (by weight) of 1.15% KCl, centrifuged at 9,000 x g for 20 minutes, and the supernatants frozen at -70°C until assay. Tissues from the first six animals in each group that exhibited the greatest weight increase during the 4 day dosing period were used for assay. On the day assays were performed, the supernatants were thawed at room temperature and centrifuged at 100,000 x g for 60 min. Pellets were resuspended in 1.15% KCl and assayed for enzyme activity using specific substrates. A positive control of S9 from animals treated with Aroclor 1254 was also assayed at the same time to validate the assay procedure.

89397466

VI. METHODS

A. Animals

Female Sprague-Dawley rats were obtained from Charles River Breeding Laboratories, Kingston, New York, at 6 weeks of age. Animals were vaccinated against Sendai virus and quarantined for at least 14 days. Stringent disease control procedures were followed during quarantine to assure the use of healthy animals. Rats were observed for signs of illness and cultures from the respiratory tract were examined for the presence of pathogens. In addition, sera from sentinel animals were examined for antibody titers to common rodent viruses and bacteria (Reovirus type 3, Pneumonia virus of mice, Sendai virus, Encephalomyelitis virus (GD VII), Mouse adenovirus, Toolan H-1 virus, *Mycoplasma pulmonis*, Kilham rat virus, Lymphocytic choriomeningitis virus, Rat coronavirus, Sialodacryoadenitis virus). The animals were judged to be healthy prior to utilization in this study and were 9 weeks old at initiation of dosing.

Animals were housed 4 per cage in an AAALAC-accredited facility under a controlled environment of 71 ± 3° F, 44 ± 20% relative humidity, and a 12 hour light/dark cycle. Rats were housed in polycarbonate autoclavable cages with filter top cage lids. Corn-cob bedding was used and animals had free access to certified laboratory rodent chow which had been analyzed for environmental contaminants. Water and food were provided *ad libitum*.

B. Treatment with Test Materials

Test material was administered at two dose levels to give information regarding the dose-response. The doses recommended by the Sponsor for test material B111 were 1250 mg/kg for the High Dose and 625 mg/kg for the Low Dose. Eight female rats per group were given one of two doses by gavage for 4 consecutive days prior to sacrifice. A vehicle control group was also included and treated concurrently. The vehicle employed was corn oil since the solvent suggested by the Sponsor, propylene glycol, is inappropriate for use in gavage studies. By agreement with the Sponsor, lipid-soluble test materials are delivered using corn oil as the vehicle in gavage studies. Animals were divided into groups and the final doses given are as follows:

7 1/2 MTD

Remove

Dose	Treatment	Number of Animals
High	1250 mg/kg	8 per group
Low	625 mg/kg	8 per group
Control	vehicle (corn oil)	8 per group

Dose Preparation:

Test material was stored according to Sponsor instructions and routine safety precautions were observed in handling. Test material was prepared in corn oil according to Sponsor instructions. Because of the stability of the

89397467

material as specified by the Sponsor, dosing solutions were prepared in advance of initiation of dosing but not more than 4 days before. Dosing solutions were stored at 4° C between treatments.

Volume:

The volume of liquid administered to the test animals was 10 ml/kg body weight. Daily volumes over the 4 day period were based on individual body weights taken on Day 1. Concentrations were adjusted so that a constant dosing volume was given at both dose levels. The test material was administered in a single dose by gavage, using a 16 gauge gavage needle.

Body Weights:

Animals were weighed prior to treatment on Day 1 and on Day 5 prior to sacrifice.

C. Enzyme Preparation

On day 5, animals were sacrificed by carbon dioxide asphyxiation. At sacrifice, the liver of each animal was weighed, washed in ice cold 1.15% KCl, and homogenized in 2 volumes (by weight) of 1.15% KCl using a Polytron homogenizer. All tissues and homogenates were kept at 4°C (on ice) during preparation. Homogenates were centrifuged at 9000 x g for 20 minutes using a Sorvall RC2-B or Beckman J2-21 refrigerated centrifuge. Aliquots of the supernatant (S9) were frozen at -70°C until assay. On the day assays were performed, the S9 fractions were thawed at room temperature and centrifuged at 100,000 x g for one hour to prepare microsomal pellets. The pellets were resuspended in 10 ml of 1.15% KCl using 6-8 strokes of a glass-to-glass homogenizer.

Prior to incubation with substrate, an aliquot of each enzyme preparation was assayed for protein content using the Biuret method as described by Layne (1957) and each was diluted with 1.15% KCl to an appropriate concentration for the assay. All assays were performed in duplicate, and the average absorbance or fluorescence used for the final calculation.

D. Assays Used to Assess Enzyme Activity

1) p-Nitroanisole O-demethylase (PNAS)

PNAS activity was determined according to the method of Thurman _et al_. (1977) with modification. Microsomal protein (1.5 mg) was incubated with 1.2 mM p-nitroanisole in the presence of 0.7 mM NADPH, 10 mM $MgCl_2$, and 0.2 M phosphate buffer at pH 7.4. After 15 minutes of incubation, the reaction was stopped with trichloroacetic acid and the amount of p-nitrophenol was determined spectrophotometrically at 436 nm.

8S397468

2) 7-Ethoxycoumarin O-deethylase (7EC)

7EC activity was determined using the method of Greenlee and Poland (1978) with modification. Approximately 0.3 mg of microsomal protein was incubated with 0.5 umole of 7-ethoxycoumarin in the presence of 0.5 umole NADPH, 0.5 umole NADH, 5 umole $MgCl_2$, and 65 umole of phosphate buffer at pH 7.4 for 20 minutes. At the termination of the reaction, the product, 7-hydroxycoumarin, was extracted into chloroform and back-extracted into an alkaline aqueous solution. The amount of product was determined fluorometrically at an excitation wavelength of 330 nm and emission wavelength of 455 nm.

3) 7-Ethoxyresorufin O-deethylase (ETR)

ETR activity was determined according to the method of Nims *et al*. (1984) with modification. Approximately 0.3 mg of microsomal protein was incubated with 1.7 mM 7-ethoxyresorufin in the presence of 125 mM NADPH, 25 mM $MgCl_2$, and 0.05 M Tris buffer at pH 7.5. The amount of resorufin produced was determined fluorometrically at an excitation wavelength of 522 nm and emission wavelength of 586 nm after a 15 minute incubation (positive controls were incubated for 3 minutes).

Enzyme activity was determined *in vitro* using microsomal suspensions from individual rat livers. Activity is expressed as product formed/mg microsomal protein/hour incubation.

A positive control was assayed with each group of samples. This positive control consisted of a microsomal suspension prepared on the day of assay from frozen hepatic S9 from Sprague-Dawley rats treated with Aroclor 1254 (MAI Catalog #82-150A).

CRITERIA FOR A VALID TEST

At least 6 animals from each group must survive dosing before enzyme assays are performed for that group.

The positive control should demonstrate enzyme activity that is $\geq$ 150% of control levels. Assays were repeated in the event that the positive control failed to demonstrate this degree of induction.

EVALUATION OF TEST RESULTS

Enzyme activity is presented for individual animals and groups as the quantity of product formed per mg microsomal protein per hour incubation time. Group means for each assay were compared by Analysis of Variance and Studentized Range Test (Armitage, 1971). A probability of 0.05 was used to determine significance.

89397469

VII. RESULTS

Female Sprague Dawley rats were quarantined for at least 14 days prior to utilization, during which time their health status was evaluated by observation. In addition, sentinel animals were sacrificed and cultures from the respiratory tract were examined for pathogens. Sera from sentinel rats were examined for titers to common rodent viruses (see Section VI, Methods). Animals were free of titers to rodent viruses and all animals were vaccinated against Sendai virus. No pathogens were found in the cultures of the respiratory tract, and the animals were judged to be healthy prior to the initiation of the study.

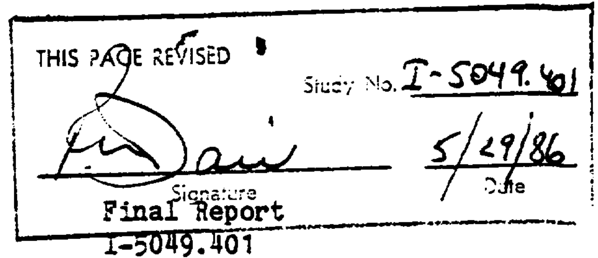
Animals were treated with 1250 mg/kg, 625 mg/kg B111 in corn oil or vehicle alone (corn oil) for 4 consecutive days. Survival to dosing was 100% in all groups.

Body weights on days 1 and 5 are presented in Table 1. These data were used to determine which samples were used for subsequent assays. Tissues from the first 6 animals in each group to gain the most weight were used for subsequent in vitro assays.

Mean liver weights and liver-to-body weight ratios of all animals used for in vitro assays are presented in Table 2. No significant differences were observed in liver weights or liver-to-body weight ratios between the treated and control groups.

Enzyme activities from individual animals are presented in Table 3 with mean activities presented in Table 4. The activity of 7EC in animals treated with the high dose of B111 was significantly greater than in the low dose group and in the controls (Studentized Range Test, $p \leq 0.05$). 7EC activity was 1.2 - 1.7 fold higher in treated animals, respectively, than in controls. A dose-related trend was observed in 7EC activity but no significant difference was seen between the low dose and control groups. No significant difference in PNAS or ETR activity was observed between treated and control groups.

89397470



VIII. CONCLUSIONS

The criteria for a valid test were satisfied in that 6 animals per group survived dosing and the activity of the positive control was at least 1.5-fold above the activity in control samples. Three assays for cytochrome P-450 and P-448 activity in rat liver were used to compare the effect of two doses of test material B111 on hepatic enzyme activity. 7EC activity in the high dose group was significantly greater than that observed in the low dose group or in the controls. No significant difference in PNAS or ETR activity between B111-treated and control groups was observed, however. Based on these results, B111 may be a weak inducer of cytochrome(s) P-450 activity, although the type of induction is unclear.

853974771

IX. REFERENCES

Armitage, P. Statistical Methods in Medical Research, New York: John Wiley and Sons, pp. 99-104, 1971.

Greenlee, W.F., and Poland, A. (1978). An Improved Assay of 7-Ethoxycoumarin O-Deethylase Activity: Induction of Hepatic Enzyme Activity in C57BL/6J and DBA/2J Mice by Phenobarbital, 3-Methylcholanthrene and 2,3,7,8-Tetrachlorodibenzo-p-dioxin. J. Pharmacol. Exptl. Therap., 205:596-605

Layne, E. (1957). Spectrophotometric and Turbidimetric Methods for Measuring Proteins. In Methods in Enzymology III, S. P. Colowick and N.O. Kaplan, eds. New York: Academic Press Inc., pp. 450-451.

Nims, R.W., Prough, R.A., and Lubet, R.A. (1984). Cytosol-Mediated Reduction of Resorufin: A Method for Measuring Quinone Oxidoreductase. Arch. Biochem. Biophys., 229: 459-465.

Omura, T., and Sato, R. (1964). The Carbon Monoxide-binding Pigment of Liver Microsomes. I. Evidence for Hemoprotein Nature. J. Biol. Chem., 239: 2370-2378.

Parkinson, A., Thomas, P.E., Ryan, D.E., Reik, L.M., Safe, S.H., Robertson, L.W., Levin, W. (1983). Differential Time Course of Induction of Rat Liver Microsomal Cytochrome P-450 Isozymes and Epoxide Hydrolase by Aroclor-1254. Arch. Biochem. Biophys., 225: 203-215.

Snyder, R., and Remmer, H. (1979). Classes of Hepatic Microsomal Mixed Function Oxidase Inducers. Pharmac. Ther., 7: 203-244.

Thurman, R.G., Marazzo, D.P., Jones, L.S., and Kauffman, F.C. (1977). Mixed Function Oxidation in Hemoglobin-free Perfused Rat Liver. A Simple Method for the Continuous Kinetic Determination of p-Nitroanisole O-demethylation. J. Pharmacol. Exptl. Therap., 201: 498-506.

89397472

X. QUALITY ASSURANCE STATEMENT

85397473

QUALITY ASSURANCE STATEMENT

Study Title: INDUCTION OF HEPATIC MICROSOMAL
ENZYMES IN RATS

Study Number: 15049.401

Study Director: Raymond M. David, Ph.D.

Initiation Date: 86/01/20

Review Completed Date: 86/04/01

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 Good Laboratory Practice regulations 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 86/01/20 - 86/01/20,TO STUDY DIR 86/01/20,TO MGMT 86/01/20

PHASES: PROTOCOL REVIEW

INSPECT ON 86/01/24 - 86/01/24,TO STUDY DIR 86/01/24,TO MGMT 86/02/04

PHASES: LIVER TISSUE COLLECTION
LIVER WEIGHTS

INSPECT ON 86/03/17 - 86/03/27,TO STUDY DIR 86/03/27,TO MGMT 86/04/01

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.

Joseph Appelbaum 4/1/86

Quality Assurance Date
RA/QA Department

83397474

XI. TABLES

89397475

Table 1

Individual Body Weights, Liver Weights, and Liver-to-Body Weight Ratios

Dose	Animal Number	Body Weights (g) Day 1	Body Weights (g) Day 5	Liver Weight(g)	Liver Ratio[a]
1250 mg/kg:	6307	220	235	12.3	5.23
	6308	203	217	10.0	4.61
	6309	203	221	11.7	5.29
	6310	224	235	-[b]	-
	6311	222	245	9.9	4.04
	6312	221	244	13.0	5.33
	6313	219	231	-[b]	-
	6314	211	228	11.0	4.82
625 mg/kg:	6315	212	231	10.4	4.50
	6316	204	218	10.0	4.59
	6317	217	231	9.4	4.07
	6318	211	229	11.0	4.80
	6319	206	219	10.3	4.70
	6320	208	228	10.0	4.39
	6321	221	233	-[b]	-
	6322	201	211	-[b]	-
Control:	6323	214	224	-[b]	-
	6324	203	224	10.8	4.82
	6325	221	241	10.7	4.44
	6326	211	223	9.4	4.22
	6327	213	235	10.0	4.26
	6328	204	225	8.9	3.96
	6329	221	241	11.3	4.69
	6330	209	216	-[b]	-

[a] Grams per 100 grams body weight.

[b] Tissue from this animal not used for assays because of low weight gain.

Table 2

Mean[a] Liver Weights and Liver-to-Body Weight Ratios

Dose	Liver Weight (g)	Liver-to-Body Weight Ratio[b]
1250 mg/kg:	11.3 ± 1.2	4.89 ± 0.51
625 mg/kg:	10.2 ± 0.5	4.51 ± 0.26
Control:	10.2 ± 0.9	4.40 ± 0.32

[a] Data presented as mean ± standard deviation. Means include only those animals used for subsequent enzyme assays.

[b] Grams per 100 grams body weight.

Table 3

Enzyme Activities of Individual Rats Treated with B111

Dose	Animal Number	Enzyme Activity (nmole/mg protein/hr)		
		PNAS	7EC	ETR
1250 mg/kg:	6307	44.1	67.8	2.56
	6308	32.5	50.5	1.60
	6309	47.7	75.3	2.56
	6311	37.6	69.3	1.28
	6312	32.5	57.3	1.28
	6314	35.4	57.3	0.64
625 mg/kg:	6315	25.3	30.1	1.12
	6316	32.5	37.7	1.28
	6317	39.0	52.7	3.04
	6318	43.4	69.3	2.88
	6319	36.1	52.7	2.56
	6320	17.3	30.1	0.64
Control:	6324	28.2	45.2	1.60
	6325	28.2	33.9	1.12
	6326	26.0	31.6	0.64
	6327	32.5	37.7	0.96
	6328	28.9	33.1	0.96
	6329	39.0	46.7	1.28
Positive Control	C_1	201.7	464.8	99.84
	C_2	208.2	534.9	94.08

89397478

Table 4

Mean[a] Enzyme Activities in Rats Treated with B111

Dose	Enzyme Activity (nmole/mg protein/hr)		
	PNAS	7EC	ETR
1250 mg/kg:	38.3 ± 6.3	62.9 ± 9.3[bc]	1.65 ± 0.77
625 mg/kg:	32.3 ± 9.6	45.4 ± 15.5	1.92 ± 1.03
Control:	30.5 ± 4.7	38.0 ± 6.5	1.09 ± 0.33

[a] Data presented as mean ± standard deviation.

[b] Significantly different from control, Studentized Range Test, $p \leq 0.05$.

[c] Significantly different from low dose group, Studentized Range Test, $p \leq 0.05$.

XII. PROTOCOL

85397480

PROTOCOL AMENDMENT

Date: February 12, 1986

Study Number: I-5049.401

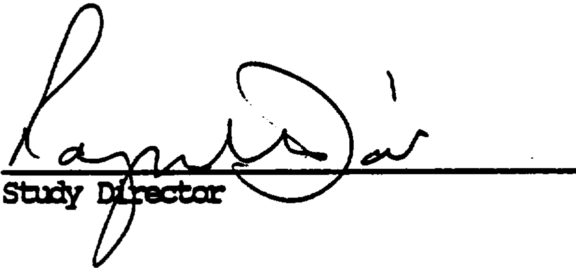
Protocol Title: Induction of Hepatic Microsomal Enzymes in Rats (B111)

Amendment:

1. Page 1, Proposed Completion Date: Change " 1/24/85" to 1/24/86".

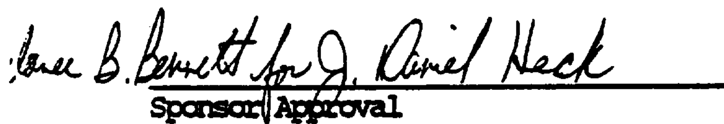
Reason:

1. To correct typographical error.



Study Director

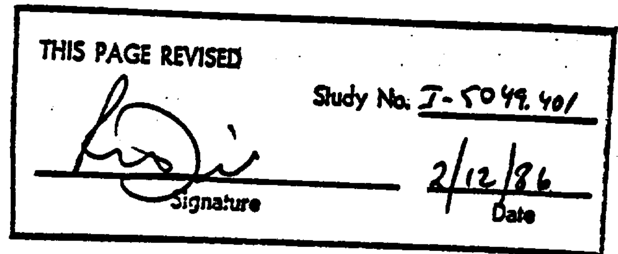
Date 2/14/86



Sponsor Approval

Date 3/4/86

89397481



PROTOCOL 1601.401
(I-5049.401)

Study Number: I-5049.401 Scheduled Initiation Date: 1/20/86

Proposed Completion Date: 1/24/86

INDUCTION OF HEPATIC MICROSOMAL ENZYMES IN RATS

1.0 PURPOSE

The purpose of this study is to determine if test materials induce cytochrome P-450 and/or P-448 activity in rat liver.

2.0 SPONSOR

2.1 Name: Lorillard Research Center

2.2 Address: 420 English St., Greensboro, N.C., 27420

2.3 Authorized Representative: D. Heck

3.0 TESTING FACILITY

3.1 Name: Microbiological Associates Inc.

3.2 Address: 5221 River Road, Bethesda, Maryland 20816

3.3 Study Director: R. M. David

4.0 TEST ARTICLE

4.1 Name (or Code): B111

4.2 Test Article Number: T05049A

4.3 Date sample received: see Test Article Receipt Form

4.4 Amount of sample received: see Test Article Receipt Form

4.5 Vehicle: Unless specified by the Sponsor, the following vehicles will be used, in order of preference, depending on the solubility determinations performed by the Study

85397482

7.2 Enzyme Preparation

On day 5, all surviving animals will be weighed and sacrificed by carbon dioxide asphyxiation. Their livers will be excised, washed in cold 1.15% KCl, and weighed. The livers will be homogenized in 2 volumes (by weight) of 1.15% KCl, centrifuged at 9,000 x g for 20 minutes, and the supernatants frozen at -70°C until assay. The first six animals in each group that exhibit the greatest weight increase during the 4 day dosing period will be used for assay. On the day assays are to be performed, the supernatants will be thawed at room temperature and centrifuged at 100,000 x g for 60 min. Pellets will be resuspended in 1.15% KCl and assayed for enzyme activity using specific substrates.

7.3 Disposition of Animals:

Test Groups:

8 rats per group; 2 dose groups; 16 rats total.

Control Groups:

8 rats per control group; 8 rats total.

7.4 Dose Preparation:

Two doses, either based on the MTD or as designated by the Sponsor, will be administered: a high dose at approximately 1/2 the Maximum Tolerated Dose (MTD), and a low dose at approximately 1/4 the MTD. Test material will be prepared in an appropriate vehicle depending on the solubility as determined by the Study Director or unless specified by the Sponsor. The appropriate vehicles are, in order of preference, water, 1-2% carboxymethylcellulose, or corn oil or other vehicle as specified by the Sponsor. Based on the stability of the material as listed in the Test Article Characterization Form, prepared solutions will be kept refrigerated between dosings. Dosing volumes will be no more than 5.0 ml/500 g body weight, unless otherwise specified by the sponsor.

8.0 METHODS

8.1 Receipt, Quarantine, Vaccination, Monitoring and Randomization:

Animals are obtained at 6-7 weeks of age from a source monitored and known to be free of adventitious agents, vaccinated against Sendai virus, and quarantined for 14 days. Stringent disease

89397483

control procedures are followed during quarantine to assure the use of healthy animals. Animals are examined each working day during the quarantine period for deaths or signs of illness. Dead or ill animals are separated from healthy ones and any unusual observations are reported to the Study Director and recorded in the raw data book. Animals are randomized according to standard operating procedures during or at the end of quarantine and individually identified by a unique 4 digit numbered ear tag.

8.2 Animal Care:

All animals have free access to certified laboratory diet and water *ad libitum*. Certified diet is lot numbered and dated. The nature and level of contaminants in the feed will not interfere with this study. The water source is Washington Suburban Sanitary Commission (WSSC), Potomac Plant, no additional treatment; water meets USEPA drinking water standards.

Animals are group housed in an AAALAC accredited facility in "shoe box" type cages equipped with filter covers. The light cycle is regulated at 12 hours light/12 hours dark and the temperature and humidity are controlled and monitored. Cages are changed biweekly and feeders are changed weekly.

8.3 Gavage Treatment:

The test material will be administered daily for 4 consecutive days in a single dose by gavage using a stomach tube or intubation cannula. If a single dose is not possible, the dose may be given in smaller fractions over several hours (less than 24 hours). It may be necessary to provide animals with food and water depending on the length of the dosing schedule.

8.4 Doses:

Maximum Tolerated Dose (provided by Sponsor): ______

2500 mg/kg

High Dose: 1250 mg/kg

Low Dose: 625 mg/kg

Vehicle (if specified by Sponsor): corn oil

89397484

8.5 Enzyme Assays:

The following reactions will be used to assess enzyme activity:

8.5.1 p-Nitroanisole O-demethylase (PNAS)

PNAS activity will be determined according to the method of Thurman et al. (1977) with modification. Microsomal protein (1.5 mg) will be incubated with 1.2 mM p-nitroanisole in the presence of 0.7 mM NADPH, 10 mM $MgCl_2$, and 0.2 M phosphate buffer at pH 7.4. After 15 minutes of incubation, the reaction is stopped with trichloroacetic acid and the amount of p-nitrophenol is determined spectrophotometrically at 436 nm.

8.5.2 7-Ethoxycoumarin O-deethylase (7EC)

7EC activity will be determined using the method of Greenlee and Poland (1978) with modification. Approximately 0.3 mg of microsomal protein will be incubated with 0.5 umole of 7-ethoxycoumarin in the presence of 0.5 umole NADPH, 0.5 umole NADH, 5 umole $MgCl_2$, and 65 umole of phosphate buffer at pH 7.4 for 20 minutes. At the termination of the reaction, the product, 7-hydroxycoumarin, is extracted into chloroform and back-extracted into an alkaline aqueous solution. The amount of product is determined fluorometrically at an excitation wavelength of 330 nm and emission wavelength of 455 nm.

8.5.3 7-Ethoxyresorufin O-deethylase (ETR)

ETR activity will be determined according to the method of Nims et al. (1984) with modification. Approximately 0.3 mg of microsomal protein will be incubated with 1.7 mM 7-ethoxyresorufin in the presence of 125 mM NADPH, 25 mM $MgCl_2$, and 0.05 M Tris buffer at pH 7.5. The amount of resorufin produced is determined fluorometrically at an excitation wavelength of 522 nm and emission wavelength of 586 nm after a 3 minute incubation.

Enzyme activity will be determined in vitro using microsomal suspensions from individual rat livers. Activity will be expressed as product formed/mg microsomal protein/hour incubation.

A positive control will be assayed with each group of samples. This positive control will consist of a microsomal suspension

89397485

prepared on the day of assay from frozen hepatic S9 from Sprague-Dawley rats treated with Aroclor 1254.

9.0 CRITERIA FOR A VALID TEST

At least 6 animals from each group must survive dosing before enzyme assays will be performed for that group.

The positive control should demonstrate enzyme activity that is $\geq$150% of control levels in at least two assays. Assays will be repeated in the event that the positive control and the samples show no induction.

10.0 EVALUATION OF TEST RESULTS

10.1 Enzyme activity will be presented for individual animals and groups as the quantity of product formed per mg microsomal protein per hour incubation time. Group means for each assay will be compared by Analysis of Variance and Studentized Range Test (Armitage, 1971).

10.2 Liver weights and liver-to-body weight ratios will be presented for individual animals. Group means will be compared by Analysis of Variance and Studentized Range Test (Armitage, 1971).

11.0 RECORDS AND SAMPLE ARCHIVES

11.1 Records:

11.1.1 Upon completion of the final report, all raw data and reports will be retired to the archives located at 5221 River Road, Bethesda, MD. 20816

11.1.2 The archives will be maintained by the Regulatory Affairs/Quality Assurance Unit.

11.2 Test Article:

Test article will be stored under appropriate conditions with access restricted to authorized personnel. Sponsor will be consulted regarding final disposal.

12.0 REFERENCES

Armitage, P. Statistical Methods in Medical Research, New York: John Wiley and Sons, pp. 99-104, 1971.

89397486

PROTOCOL 1601.401
(I-5049.401)

Greenlee, W. F., and Poland, A. An Improved Assay of 7-Ethoxycoumarin O-Deethylase Activity: Induction of the Hepatic Enzyme Activity in C57BL/6J and DBA/2J Mice by Phenobarbital, 3-Methylcholanthrene and 2,3,7,8 - Tetrachlorodibenzo-p-dioxin. J. Pharmacol. Exp. Ther. 205: 596-605, 1978.

Nims, R. W., Prough, R. A., and Lubet, R. A. Cytosol-Mediated Reduction of Resorufin: A Method for Measuring Quinone Oxidoreductase. Arch. Biochem. Biophys. 229: 459-465, 1984.

Thurman, R. G., Marazzo, D. P., Jones, L. S., Kauffman, F. C. The Continuous Kinetic Determination of p-Nitroanisole O-Demethylation in Hemoglobin-Free Perfused Rat Liver. J. Pharmacol. Exp. Ther. 201: 498-504, 1977.

Telephone approval 1/16/86

Bruce B. Bennett for J. Daniel Heck 3/4/86
Sponsor Approval Date

[signature] 1/17/86
Study Director Date

[signature] 1/20/86
RA/QA Date

89397487

89397488