150 pp

AR 226 - 0947

Telomer Research Program Update and Status Report

Presented to U.S. EPA, OPPT

23 October, 2000

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TRP Member Companies

- Asahi Glass
- Atofina
- Clariant
- Daikin
- DuPont

Telomer Research Program (TRP)

- Industry-sponsored Research Program
- Focus on data gathering common to Telomer manufacturers
- Key Events Timeline
  - *June 20, 2000* : Formal Organization Proposal
  - *July 7, 2000* : Met with U.S. EPA
  - *July 11, 2000*: Met with MITI
  - *August 14-15, 2000* : First Meeting as Formal Consortium
  - *October 23, 2000*: Present Update to U.S. EPA
  - *October 30, 2000*: Present Update to MITI

TRP Guiding Principles

- Participation by all Telomer Manufacturers
- Expeditious and Effective Research of Publishable Quality
- Early and Full Disclosure of Results
- Equal Sharing of Costs
- Structure modeled on an earlier and very successful cooperative research program
- Structure: a Management Committee which acts on recommendations from a Research Committee
- Use independent third party to administer program

TRP Management Committee

- Oversee the affairs of the TRP
- One member designated by each participant company
- Appoint the Chair and other members of the Research Panel
- Approve projects for funding by TRP
- Have custody of the funds; determine financial needs of TRP and assess parties such amounts
- Formulate a policy for proper reporting of project results to the parties, to government agencies, and others

TRP Research Panel

- Comprised of representatives from each participant company
- Compile relevant existing studies
- Recommend research testing and develop study protocols
- Recommend how above can be executed and insure it gets accomplished
- Identify laboratory facilities that have the capabilities to conduct the recommended tests.
- Obtain bids or quotes from laboratories and present for approval (via Administrator)

TRP Research Quality

- Standards such as Good Laboratory Practices as established by the OECD and applicable country laws
- Encourage peer review and publication in the open literature

TRP Information Sharing

- Relevant telomer toxicology research information that has already been developed by a participant shall be shared with the TRP
- Testing results by or for the TRP shall be promptly disseminated to each participant
- A policy to govern disclosure and publication of research results will be developed
- Early and full disclosure to government and regulatory agencies

TRP Initial Meeting : 14-15 August, 2000

- Information Handling and Sharing
- Chemicals to be Tested
- *Toxicology Studies*: acute, genetic toxicity, developmental toxicity; initial 45-day range-finder followed by 90-day subchronic with recovery, followed by multi-generation reproduction study
- *Pharmacokinetics*: *In Vitro* Metabolism, Mass Balance and ADME (absorption, distribution, metabolism, elimination)
- *Environmental Fate*: atmospheric fate, acute aquatic toxicity, bioconcentration, and biodegradation
- Communications including meetings with regulatory authorities

TRP Information Handling & Sharing

- Condition of membership in TRP to share toxicology data on representative compounds from each participant
- Proposal to establish single repository for either final reports or summary data; all submissions due Oct 2000

  -Summary data in standard format

  -Text in English

  -Identify key contact in each company
- Results from TRP work will be available to all members
- Initial repository will be Haskell Laboratory but this could transition to RAND at a later time

TRP Chemical to be Tested

- Telomer 8-2 Alcohol:
  $n\text{-}C_8F_{17}CH_2CH_2OH$

- Purity >99%, and characterized
- Sample made, 20 kg, now in analysis

Acute Oral Toxicity - Fixed Dose Method

Objective: To assess the acute oral toxicity (lethality, clinical signs of response) by administration of gavage doses to rats

Method:

- Rats, male and female
- Single oral doses
- Sighting Study: - 5-50-500-2000 mg/kg, 1 rat/dose
  - clinical signs; mortality to 14 days
  - select first dose on available information, analogous chemicals
- Main Study: - 5M/5F/group
  - initial dose - toxicity response, no mortality in sighting study
  - test up/down dependent on results

Results: - Lethal level(s)
- Principal clinical signs
- Recovery/time to recovery

Repeated-Dose Oral Toxicity Gavage Range-Finding Study in Rats

Objective: To determine dose levels for use in 90-day toxicity study which will enhance ability to achieve NOAEL, LOAEL

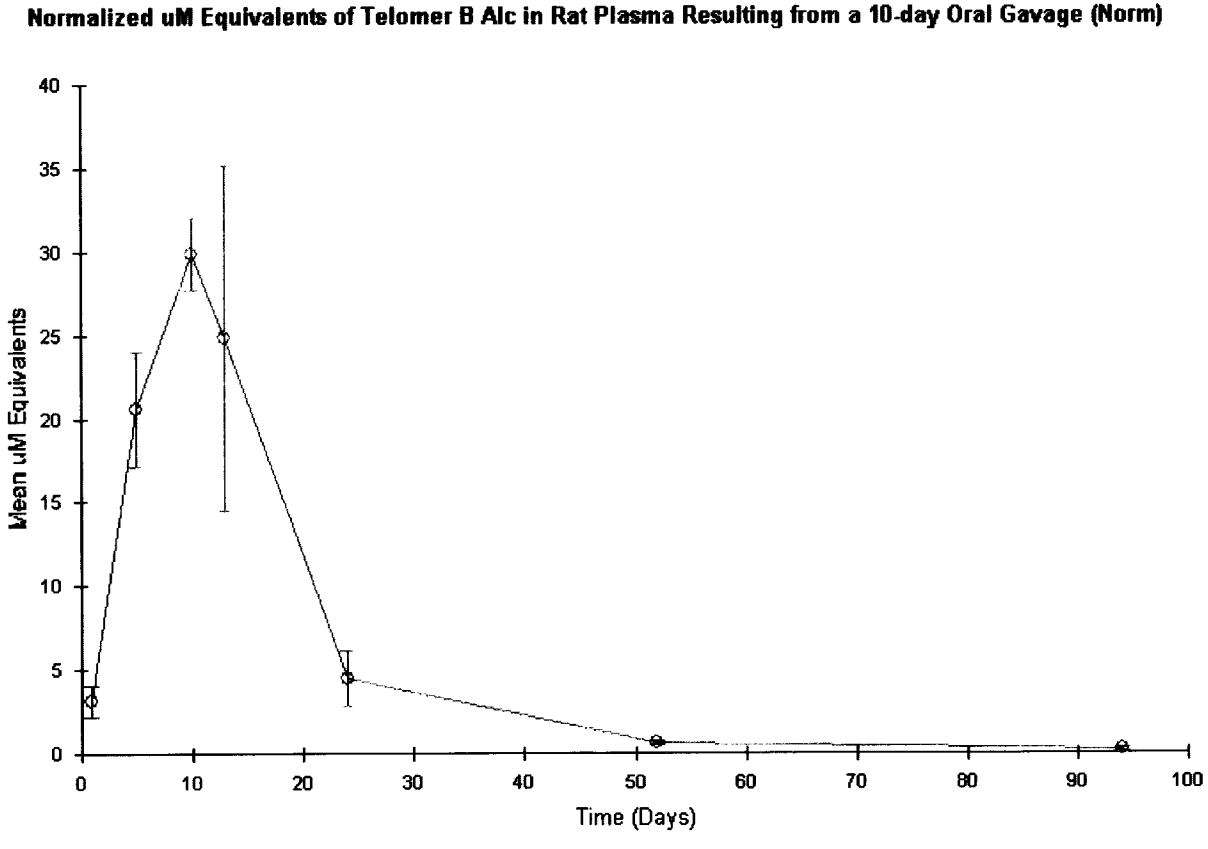
Method:

- Rats, 5M/5F/group, 1 control, 3 graded treatment levels, expect 3-8 week duration
- Endpoints Measured:
  - *In vivo* (body weight, clinical signs)
  - Blood organofluoride- pretest (day-4)
    day 1, weekly
    Post-dosing day 3, weekly
  - Collect urine and feces (to expand pharmacokinetics model if indicated)

Results:

- *In vivo* response
- Blood organofluoride level to steady-state, define clearance profile
- Evaluate bio-response, bio-uptake and clearance versus dose

Previous Study with Telomer B Alcohol (C8-2)



Telomer Research Program Oct 2000

Subchronic Toxicity - 90-Day Gavage Study in Rats with Recovery

Ojective: To evaluate the subchronic toxicity and the kinetics of uptake and clearance of this chemical in the blood.

Methods:

- Rats, 10M/10F/group, oral gavage, 90 days, control and 3 test levels; 10M/10F/group control and high 4 wk reversibility
- Parameters: *In vivo*, clinical pathology, neurobehavior, pathology
- 5M/5F/group Blood Kinetics:

•Results:

- Determine target organ(s) and dose required to produce effect
- Determine reversibility of change(s) produced
- Determine uptake and clearance of organofluoride in blood

Goal: Establish target site, potency, (NOAEL….LOAEL….)

Developmental Toxicity Study in Rats

Ojective: To evaluate the potential effect on the fetal rat following administration of the chemical to the maternal rat from implantation to the end of gestation.

Methods:

- Rats, oral gavage doses gestation day 6 through 21;
  control plus 3 graded dose levels
- Parameters - maternal - body weights, clinical signs, resorptions, implantations, gestation time
  - fetal - weight, structural integrity (external, internal, skeletal)

Results:

- To evaluate the ability of chemical to effect either the maternal or fetal rat (or both) and the types and severity of the induced changes
- The potency of the chemical
  - maternal NOAEL....LOAEL
  - fetal NOAEL.....LOAEL

Multi-Generation Reproduction Study in Rats

Objective: To evaluate the effect of the chemical on the gonadal function, conception, parturition, and the growth and development of offspring of rats over 2 generations (involving the production of at least 1 set of litters in each generation).

Methods:

- Rats, 30M/30F/group, graded dietary levels, control + 3 test levels
- Feed chemical 70 days pre-mating (P1 and F1) until sacrifice
- Parameters - body weight, clinical signs
  - reproductive function (mating, fertility, fecundity, gestation, implantations, pup survival and lactation, developmental landmarks, sperm assessments, estrous cycling)
  - pregnancy - growth, clinical signs,
  - selected histopathology (focus on reproductive organs)

Results:

- Reproductive system as a target (yes or no)
  - functional (1st, 2nd generation)
  - histologic (1st, 2nd generation)
  - effects in offspring
- Potency of effects in offspring
- NOAEL.....LOAEL....

Genetic Toxicity - Bacterial Reverse Mutation Test

Ojective: To evaluate the mutagenic potential by measuring the ability to induce reverse mutations at selected loci of several strains of *S. typhimurium* and at the tryptophane locus of *E. coli* (with and without S-9 activation)

Methods:

- Tester strains - *Salmonella typhimurium*
  TA98; TA 100
  TA1535; TA 1537
  E. Coli WP2uvrA
- 5 graded test concentrations
  - selected based on evidence of toxicity and precipitate
- Incubate for 48/72 hours with and without S-9 mix
- Score revertant colonies/plate (all in triplicate)
- Score against negative + positive controls run concurrently

Results:

- Control revertant rates must be within historical data
- Positive control rates at least 3x control
- Positive results - 3x control rate for TA1535/TA1537
  - 2x control rate for TA98, TA100, WP2uvrA

Genetic Toxicity - In Vitro Mammalian Chromosome Aberration Test

Objective: To evaluate the clastogenic potential based on its ability to induce chromosome aberrations in human peripheral blood lymphocytes

Method:

- Expose human lymphocytes to at least 4 graded concentrations of the test agent (with positive and negative controls)
- Non-activated system treatment times - 4 and 20 hours
- S-9 activated system treatment time - 4 hours
- Arrest dividing cells in metaphase; harvest and evaluate at 20 hours
- Score a minimum of 200 spreads/dose level (100/duplicate treatment tube)

Results:

- Chromatid-type aberrations - chromatid/isochromatid breaks
  - exchange figures
- Chromosome-type aberrations - breaks
  - dicentrics/rings
- Fragments (can be chromatid or chromosome)
- Analyze % aberrant cells statistically (versus negative control)

Genetic Toxicity - Rat Bone Marrow Erythrocyte Micronucleus Test

Objective: To evaluate the clastogenic potential as measured by its ability to induce micronucleated polychromatic erythrocytes in rat bone marrow

Method:

- Single IP injection of rats at 3 graded doses (from pilot study but not to exceed 2000 mg/kg)
- 5M/5F/group
- Negative and positive (cyclophosphamide) controls
- Sacrifice and collect bone marrow from 5/sex/group at 24 hours; 5/5 from high dose only at 48 hours.
- Examine bone marrow cells - 2000 micronucleated normocytes per rat

Results:

- Proportion of micronucleated polychromatic erythrocytes/ polychromatic erythrocytes
- Apply appropriate statistics versus controls
- Valid test only if control incidence of micronucleated cells <0.3%; positive control statistically elevated

OECD 474 uses mice; consider oral gavage vs. IP

TRP Pharmacokinetics Study

Metabolism of Telomer 8-2 Alcohol by Mammalian Microsomes

Objective: **Determine the rate of test material metabolism and metabolic product identification in liver microsomes prepared from various mammalian species.**

Method:

- **Microsomes prepared for rat, rabbit, dog, monkey and human**
- **Samples analyzed by HPLC-MS**
- **Determine linearity of reaction rate and protein concentration**
  - ** **Reaction media contains an NADP regeneration system, cofactors, microsomes, test compound and phosphate buffer at pH 7.4, 37C**
  - * **Determine kinetics of test compound**
  - ** **Reactions carried out at constant initial test material concentration and at a constant protein concentration**
  - ** **Time course of metabolism by microsomes evaluated**

Results:

- **Rate of microsomal metabolism of test compound by each species tested**
- **Identity of metabolic products**
- **Estimates of test material half-life *in-vitro* and intrinsic clearance**

Note: Protocols still in draft form

TRP Pharmacokinetics Study

Metabolism of Telomer 8-2 Alcohol by Mammalian Hepatocytes

Objective: To evaluate the rate of metabolism and identification of metabolic products in cultured liver cells from several mammalian species.

Method:

* Prepare hepatocytes from rat, mouse, rabbit, monkey, dog and human
* Negative (vehicle) and positive (ethoxycoumarin) controls included
* Hepatocytes and compound incubated at 37C for 2 hrs
* Samples analyzed by HPLC-MS for test compound and metabolites

Results:

* Qualitative and quantitative description of metabolic activity toward test material in several species
* Description of metabolism in a metabolically "complete" system
* Identity of test material metabolites in a whole cell system

Note: Protocols still in draft form

TRP Pharmacokinetics Study

Toxicokinetics of Telomer 8-2 Alcohol (TBA) in the Rat

Objective: **Determine the absorption, distribution, metabolism and excretion of Telomer 8-2 alcohol in male and female rats following oral and dermal dosing.**

Method:

- * **Conduct pilot experiments**
  - ** **Radiolabeled and cold TBA used as test material**
  - ** **2 male and 2 female rats**
  - ** **Determine plasma kinetics following a single high oral dose of TBA**
  - ** **Determine excretion and metabolism following a single high oral dose of radiolabeled TBA**
- * **Conduct absorption experiments**
  - ** **TBA test material**
  - ** **4 male and 4 female rats**
  - ** **Single low and single high oral dose**
  - ** **Single low and single high dermal exposure (8 hrs)**
  - ** **Single low intravenous dose**
  - ** **Follow plasma concentrations of test material**

Note: Protocols still in draft form

TRP Pharmacokinetics Study

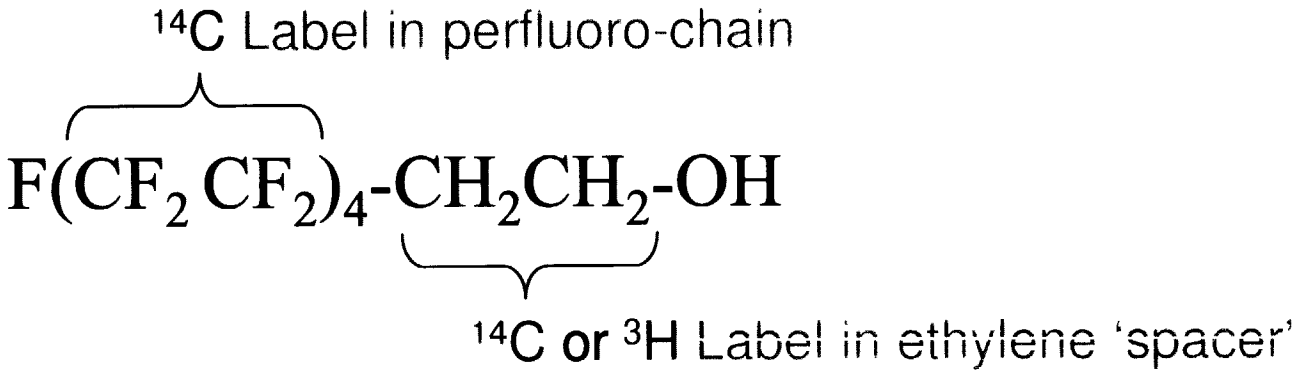
Toxicokinetics of Telomer 8-2 Alcohol (TBA) in the Rat (Continued)

* **Distribution experiments**
    - ** **Radiolabelled TBA test material**
    - ** **4 male and 4 female rats**
    - ** **Conduct oral and dermal exposures**
    - ** **Evaluate tissue distribution of test material at Tmax and Tmax/2**
* **Excretion and metabolism experiments**
    - ** **Radiolabelled TBA test material**
    - ** **4 male and 4 female rats**
    - ** **Single low and high oral doses**
    - ** **Single low and high dermal doses**
    - ** **Daily dose of TBA with last dose being radiolabelled TBA**
    - ** **Identify excretion rates and metabolic profiles**

Results:

* **Metabolic profiles of TBA**
* **Qualitative and quantitative description of biodistribution of TBA**
* **Description of absorption characteristics of TBA**
* **Sex linked differences in TBA handling in rats**
* **Description of TBA metabolism at varying concentrations and between sexes**

Radiolabeled Fluorotelomer Alcohol



- Need two-labeled compounds: perfluoro-chain and ethylene 'spacer'
- Expect Fluorinated chain label to be difficult
- Expect Ethylene 'Spacer" Label to be Straightforward

TRP Environmental Fate & Effect Study

- Initially focused on four areas:

-Atmospheric Degradation

-Biodegradation

-Acute Aquatic Toxicity (Daphnia, Algae, Fish)

-Chronic Aquatic Toxicity (Daphnia)

-Bioconcentration and chronic fish (simultaneous study based on result from fish acute)

- Protocols still in draft form; Completion expected end of October

TRP Environmental Fate Study

- **Objective**: Laboratory Study of Atmospheric Degradation of n-$C_8F_{17}CH_2CH_2OH$

  -Determine persistence via reaction with OH radical

  -Determine major oxidation products via reaction with O_2 and NO_x(NO, NO_2)

  -Determine reactivity of the oxidation products.

TRP Environmental Effect Study

- **Objective**: Conduct Acute and Chronic Studies on Daphnia and Fish*

  -Acute: conduct simple limit test on Daphnia and fish in preparation for the chronic studies

  -Chronic studies:

  *Standard 21-day Daphnia reproduction test (OECD 211) carried out as a semi-static study

  *Modified 14-day fish sub-acute test (OECD 204) increased to maximum 56 day exposure with depuration step and whole body and tissue concentration measurements providing insights into bioconcentration

*Algae to be addressed in later work

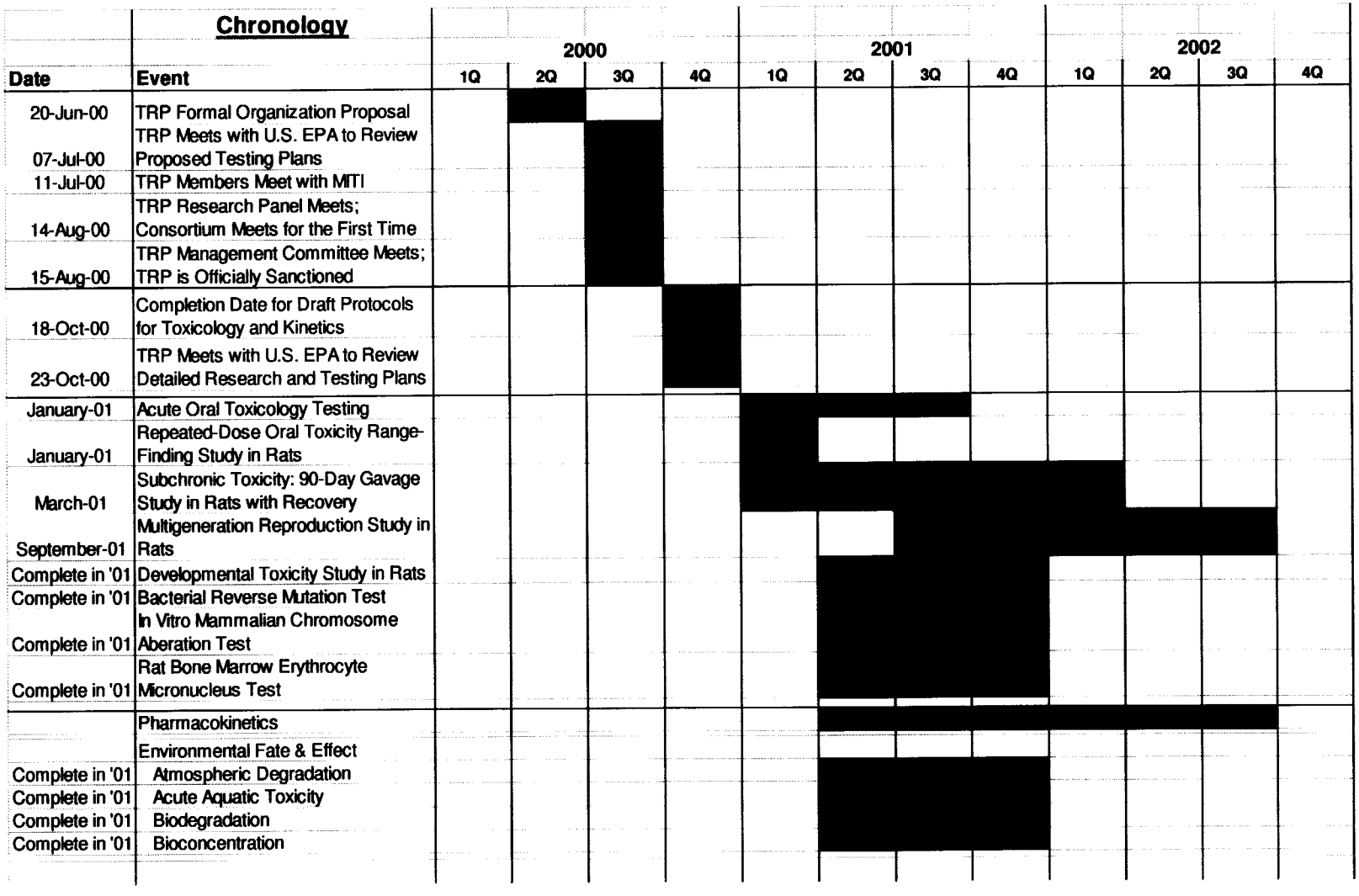
TRP Environmental Fate Study

- **Objective**: determine the rate and/or amount of biodegradation and potential mineralization of the subject compound

  -Use Draft ISO standard test (ISO/DIS 14592 pt1), potentially with ^{14}C label on ethylene to assess biodegradation of alkyl portion

  -Zahn-Wellens test with activated sludge to assess whether the subject compound is degradable or mineralizable; part of this test will also assess whether there is an inhibition of activity of bacteria under the test conditions and attempt to determine the fugacity of the substance or its degradation products by measurement of trapped volatiles

TRP Toxicology, Pharmacokinetics, and Environmental Fate & Effect Timelines*



Date	Chronology Event	2000 1Q	2000 2Q	2000 3Q	2000 4Q	2001 1Q	2001 2Q	2001 3Q	2001 4Q	2002 1Q	2002 2Q	2002 3Q	2002 4Q
20-Jun-00	TRP Formal Organization Proposal		■										
07-Jul-00	TRP Meets with U.S. EPA to Review Proposed Testing Plans			■									
11-Jul-00	TRP Members Meet with MITI			■									
14-Aug-00	TRP Research Panel Meets; Consortium Meets for the First Time			■									
15-Aug-00	TRP Management Committee Meets; TRP is Officially Sanctioned			■									
18-Oct-00	Completion Date for Draft Protocols for Toxicology and Kinetics				■								
23-Oct-00	TRP Meets with U.S. EPA to Review Detailed Research and Testing Plans				■								
January-01	Acute Oral Toxicology Testing					■	■	■					
January-01	Repeated-Dose Oral Toxicity Range-Finding Study in Rats					■							
March-01	Subchronic Toxicity: 90-Day Gavage Study in Rats with Recovery					■	■	■	■	■			
September-01	Multigeneration Reproduction Study in Rats							■	■	■	■	■	
Complete in '01	Developmental Toxicity Study in Rats						■	■	■				
Complete in '01	Bacterial Reverse Mutation Test						■	■	■				
Complete in '01	In Vitro Mammalian Chromosome Aberation Test						■	■	■				
Complete in '01	Rat Bone Marrow Erythrocyte Micronucleus Test						■	■	■				
	Pharmacokinetics						■	■	■	■	■	■	
	Environmental Fate & Effect												
Complete in '01	Atmospheric Degradation						■	■	■				
Complete in '01	Acute Aquatic Toxicity						■	■	■				
Complete in '01	Biodegradation						■	■	■				
Complete in '01	Bioconcentration						■	■	■				

*TRP will present results as available on '01

Telomer Research Program Oct 2000

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TRP Path Forward Items

- Complete Respective Protocols
- Complete Information Database
- Submit and Obtain Competitive Bids on the Key Work Streams
- Complete the Telomer 8-2 Alcohol Characterization and Decision on Labeled Compounds Preparation
- Update MITI and other respective regulatory groups
- Meet as a Consortium to Finalize Remaining Details in Nov/Dec 2000
- Begin Workstreams per Timeline Jan 2001

«Title»: Acute Oral Toxicity - Fixed Dose Method

Protocol

Contain NO CBI

INTRODUCTION

This test is designed to assess the acute oral toxicity produced when a test substance is administered by the oral route (gavage) to rats.

SPONSOR AND TEST FACILITY

This study is sponsored by <facility, location>. The sponsor's approval was effective the date the sponsor authorized the work. The study will be conducted at <facility, location>, in accordance with all applicable Good Laboratory Practice standards.[1,2] The study design is based on European Economic Communities (EEC)[3] and Organization for Economic Cooperation and Development (OECD),[4] test guidelines. Areas of noncompliance will be documented in the final report.

MATERIALS AND METHODS

A. Test Substance

The test substance will be supplied by the sponsor. The test substance will be assigned a unique number.

B. Test Species

Male and female Crl:CD®(SD)IGS BR rats will be obtained from Charles River Laboratories, Inc. The address of the supplier (city/state) will be documented in the study records and final report. The Crl:CD®(SD)IGS BR rat has been selected on the bases of extensive experience with this strain and its suitability with respect to longevity, hardiness, sensitivity, and low incidence of spontaneous diseases.

C. Animal Husbandry

All animals will be housed in stainless steel, wire-mesh cages suspended above cage boards. Animal rooms will be maintained at a temperature of 22 ± 3°C and a relative humidity of 50% ± 20%. Animal rooms will be artificially illuminated (fluorescent light) on an approximate 12-hour light/dark cycle.

All animals will be provided tap water *ad libitum*. All animals will be fed PMI Nutrition International, Inc. Certified Rodent LabDiet® 5002 *ad libitum* except during fasting.

As specified in the <> Laboratory animal health and environmental monitoring program, the following procedures are performed periodically to assure that contaminant levels are below those that would be expected to impact the scientific integrity of the study:

- Water samples are analyzed for total bacterial counts, and the presence of coliforms, lead, and other contaminants.
- Feed samples are analyzed for total bacterial, spore and fungal counts.
- Samples from freshly washed cages and cage racks are analyzed to ensure adequate sanitation by the cagewashers.

Certified animal feed is used, guaranteed by the manufacturer to meet specified nutritional requirements and not to exceed stated maximum concentrations of key contaminants, including specified heavy metals, aflatoxin, chlorinated hydrocarbons, and organophosphates. The presence of these contaminants below the maximum concentration stated by the manufacturer would not be expected to impact the integrity of the study.

The animal health and environmental monitoring program is administered by the attending laboratory animal veterinarian. Data are maintained separately from study records and may be included in the final report at the discretion of the study director.

D. Pretest Period

Upon arrival at <> Laboratory, all animals will be housed 1 per cage, sexes separate in quarantine. The rats will be:

- quarantined for at least 5 days.
- identified by cage identification.
- weighed at least 3 times during quarantine.
- observed with respect to weight gain and any gross signs of disease or injury.

The animals will be released from quarantine by the laboratory animal veterinarian or designee on the basis of acceptable body weights and clinical signs.

E. Assignment to Groups

Animals of each sex, selected on the bases of adequate body weight gain and freedom from any clinical signs of disease or injury, will be assigned to the study. After assignment to the study, each animal will be housed individually. Information on the cage labels will include the unique animal number and an individual identification number assigned to each animal. The last 3 or 4 digits of the animal number will be marked on the tail of each animal. At study start (test day 0)

the animals will be between 8 and 12 weeks of age. On test day 0, the body weights will be within ± 20% of the mean within a sex.

F. Dosing and Evaluations

1. Fasting

For both the sighting and main studies, the animals will be fasted prior to dosing by withholding food overnight (approximately 16-18 hours). Food will be returned to the animals approximately 3-4 hours after dosing.

2. Sighting Study

The effects of various doses will be investigated in single animals. Normally female animals will be used in the absence of information derived from structure-activity relationships or other information indicating that males will be the more sensitive sex. Dosing will be sequential, allowing at least 24 hours before dosing the next animal. All animals will be carefully observed for signs of toxicity for at least 7 days; if signs of moderate toxicity persists at 7 days, the animal will be observed for up to an additional 7 days. The following initial dose levels will be considered: 5, 50, 500, and 2000 mg/kg. If the initial dose chosen does not produce severe toxicity, and the next higher level produces mortality, then it will be necessary to investigate one or more intermediate dose levels as appropriate. In this way it should be possible to build up information on the dose level(s) that produce(s) some signs of toxicity and the minimum dose level that produces mortality.

An effort will be made to select the initial dose using evidence from related chemicals. In the absence of such information, it is suggested that the 500 mg/kg dose is used in the first instance. If no signs of toxicity are seen at the initial dose, then the next higher dose level will be investigated. If no mortality occurs at 2000 mg/kg, the sighting test is complete and the main study should be conducted at this dose level. If severe effects, necessitating humane killing are seen at the initial dose (e.g. 500 mg/kg), the next lower dose (e.g. 50 mg/kg) will be given to another animal. If this animal survives, further animals will then be dosed with the appropriate intermediate dose levels between the fixed doses. Normally, one would not expect to use more than 5 animals in this procedure.

3. Main Study

At least 10 animals (5 male and 5 female) will be used for each dose level investigated. The dose level to be used in the main study is selected from one of four levels, 5, 50, 500, and 2000 mg/kg body weight and will be that which is judged on the basis of the results from the sighting study likely to produce evident toxicity but no mortality. If the data from the sighting study suggest that mortality will occur at 5 mg/kg, the substance can be investigated at a lower dose level.

In most cases it is likely that the data obtained from the sighting study will be adequate to allow the appropriate dose level to be selected in the main study (i.e. a level that produces evident toxicity but no mortality). However if evident toxicity is not seen at the initial dose level, the substance will be retested at the next higher level. If animals die at the initial dose chosen, or a severe reaction requires removal of animals from the study for animal welfare reasons, the substance will be retested at the next lower dose level.

4. Dose Volume

When practical, liquid test substances will be administered neat. Solid test substances will be suspended or dissolved in a vehicle. Variability in volume will be minimized by adjusting the concentration to ensure a constant volume at all dose levels. The dose volume will not exceed 1 mL/100 g of body weight, except in the cases of aqueous solutions where 2 mL/100 g may be used. The volume of dosing material administered to each animal will be based on the animal's fasted body weight determined on the day of dosing.

5. Frequency

The test substance will be administered in a single dose. If a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours. Where a dose is administered in fractions, it may be necessary to provide the animals with food, depending on the length of the dosing period.

6. Vehicle

The use of an aqueous mixture should be considered first, followed by consideration of a mixture in corn oil, and then by consideration of a possible mixture in other vehicles. Commonly used vehicles in the order of preference are water, methylcellulose in water, Mazola® corn oil or equivalent, mixtures of acetone and water, or mixtures of acetone and corn oil. Corn oil, methylcellulose, and acetone will be used as supplied; any contaminants that can reasonably be expected to be present are not expected to affect the integrity of the study. The vehicle used will be documented in the study records and final report.

7. Observations and Body Weights for Sighting Study and Main Study

Except where animals need to be removed from the study and humanely killed for animal welfare reasons, animals will be observed for at least 7 days (sighting study) or 14 days (main study) after dosing. However, the duration of observation will not be fixed rigidly. It will be determined by the toxic reactions, rate to onset and length of recovery period, and may thus be extended when considered necessary. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed. All

observations are systematically recorded with individual records being maintained for each animal.

A careful clinical examination will be made at least twice on the day of dosing and once each day thereafter (weekends and holidays excluded except when warranted by the condition of the animals). Animals obviously in pain or showing severe signs of distress will be humanely killed. Additional observations may be necessary during the first few days after dosing so that the test may be terminated if it becomes apparent that the initial dose level chosen was too high. When animals are killed for humane reasons or are found dead, the time of death will be recorded as precisely as possible. The rats will be checked twice daily for mortality and signs of illness, injury, and abnormal behavior.

Individual weights of animals will be determined at the time of fasting, shortly before the test substance is administered, daily for the next 3 days, and weekly thereafter. If significant weight loss ($\geq 2\%$ of the previously determined body weight) occurs, all animals in the group will be weighed daily until there is no significant weight loss. At the end of the test surviving animals are weighed and then sacrificed.

STATISTICAL METHODS

Descriptive statistics (e.g. mean, standard deviation, etc.) may be used.

SAFETY AND HOUSEKEEPING

Good housekeeping procedures will be practiced to avoid potential health hazards. To avoid skin contact, gloves will be worn when handling the test substance. Animal carcasses and feces will be incinerated.

RECORDS AND SAMPLE RETENTION

All original records will be retained at <facility, location>. Laboratory-specific or site-specific records such as personnel files and equipment records will be retained at the facility where the work was done.

REFERENCES

1. EPA/FIFRA Good Laboratory Practice Standards (40 CFR 160). (1989).

2. OECD Principles of Good Laboratory Practice (as revised in 1997, published in ENV/MC/CHEM(98)17 (OCDE/GD(92)32).

3. Official Journal of the European Communities. Part B1bis: Methods for the Determination of Toxicity. (1992).

4. OECD Guidelines for the Testing of Chemicals, No. 420. (1992).

SIGNATURE

Approved by: _____ _____

«sd», «SDDegree»
Study Director

Date

cc: <>

<Cmpd> Repeated-Dose Oral Toxicity
Gavage Range-Finding Study in Rats

Protocol

Contain NO CBI

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TABLE OF CONTENTS

INTRODUCTION

The test substance, <Cmpd>, is a <compound> used as an < >.

Dose levels for this study were selected based on <>.

OBJECTIVE

The objective of this oral gavage range-finding study is to determine the dose levels for use in a 90-day oral subchronic study. The oral route of administration was selected because <>.

SPONSOR AND TEST FACILITY

This study is sponsored by <sponsor name>. The sponsor's approval was effective the date the sponsor submitted the work. The study will be conducted at <laboratory, location>.

STUDY DESIGN

The study design is as follows:

Group[a]		No./Group		Main Study	Metabolism		Dosage[b] mg/kg/day	Treatment
Male	Female	Male	Female		Serial bleed	Tissue/sample collection		
I	II	5	5	5/sex	All rats	All rats	0	Control
III	IV	5	5	5/sex	All rats		<>	<Cmpd>
V	VI	5	5	5/sex	All rats		<>	<Cmpd>
VII	VIII	5	5	5/sex	All rats	All rats	<>	<Cmpd>

a Each rat will be assigned its own animal number. Rats will have the last 3 digits of the unique animal number marked on their tails.
b Weight/weight concentration of test substance.

Study Parameters	Frequency
Dose Administration	Test Day 0 – to attainment of steady state. Vehicle animals will be dosed until last dose group attains steady state for total fluorine in blood.
Body Weight	Twice per week at 3-4 day intervals
Clinical Observations	At every weighing
Mortality/Moribundity Checks	At least twice daily
Serial bleeding for fluorine analysis	Predosing – Day –4 During Dosing – Day 1 and weekly thereafter Postdosing – Day 3, 1 week, 2 weeks, 1 month, 45 days, 60 days, and 75 days
Collection of urine and feces	Once steady state is obtained for 3 consecutive days
Necropsy (Anatomical Pathology)	
• Select Tissue/Sample Collection	All rats in the 0 mg/kg/day (Control) and <> mg/kg/day <Cmpd>-group). Date of sacrifice will depend on the rate of elimination of fluorine from the blood after dosing is stopped.

MATERIALS AND METHODS

A. Test Substance

The test substances will be supplied by the sponsor. The test substance(s) were assigned <> Laboratory Number(s) <>.

B. Test Species

Male and female Crl:CD®(SD)IGS BR rats will be obtained from Charles River Laboratories, Inc. The address of the supplier (city/state) will be documented in the study records and final report. The Crl:CD®(SD)IGS BR rat has been selected on the bases of extensive experience with this strain and its suitability with respect to hardiness, longevity, sensitivity, and low incidence of spontaneous diseases.

C. Animal Husbandry

All rats will be housed in stainless steel, wire-mesh cages suspended above cage boards. Animal rooms will be maintained at a temperature of 22 ± 3°C and a relative humidity of 50 ± 20%. Animal rooms will be artificially illuminated (fluorescent light) on an approximate 12-hour light/dark cycle.

All rats will be provided tap water *ad libitum*. All rats will be fed PMI Nutrition International, Inc. Certified Rodent LabDiet® 5002 *ad libitum*.

As specified in the <> Laboratory animal health and environmental monitoring program, the following procedures are performed periodically to assure that contaminant levels are below those that would be expected to impact the scientific integrity of the study:

- Water samples are analyzed for total bacterial counts, and the presence of coliforms, lead, and other contaminants.
- Feed samples are analyzed for total bacterial, spore and fungal counts.
- Samples from freshly washed cages and cage racks are analyzed to ensure adequate sanitation by the cagewashers.

Certified animal feed is used, guaranteed by the manufacturer to meet specified nutritional requirements and not to exceed stated maximum concentrations of key contaminants, including specified heavy metals, aflatoxin, chlorinated hydrocarbons, and organophosphates. The presence of these contaminants below the maximum concentration stated by the manufacturer would not be expected to impact the integrity of the study.

The animal health and environmental monitoring program is administered by the attending laboratory animal veterinarian. Data are maintained separately from study records and may be included in the final report at the discretion of the study director.

D. Pretest Period

Upon arrival at <> Laboratory, all rats will be housed 1 per cage, sexes separate, in quarantine. The rats will be:

- quarantined for a minimum of 5 days.
- identified temporarily by cage identification.
- weighed at least 3 times during quarantine.
- observed with respect to weight gain and any gross signs of disease or injury.

The rats will be released from quarantine by the laboratory animal veterinarian or designee on the bases of body weights and clinical signs of all rats.

Rats that are accidentally killed or removed from study during the pretest period will be discarded without necropsy. Rats that are found dead or sacrificed *in extremis* during the pretest period will be sent to Pathology and given a gross examination to check for the presence of disease. Dependent upon these findings, further diagnostic procedures may be employed at the discretion of the study director, a pathologist, or the laboratory animal veterinarian. The results will not be reported in the final report unless considered significant to the evaluation of the study.

E. Assignment to Groups

Rats of each sex, selected on the bases of adequate body weight gain and freedom from any clinical signs of disease or injury will be distributed by computerized, stratified randomization into study groups as designated in the Study Design, so that there are no statistically significant differences among group body weight means within a sex. The weight variation of selected rats will not exceed ± 20% of the mean weight for each sex.

After assignment to groups, each rat will be housed individually. Information on the cage labels will include the unique animal number and the unique individual identification number assigned to each rat. The last 3 or 4 digits of the unique animal number will be tattooed on the tail of each rat.

At study start (test day 0) the rats will be 7 weeks old. On test day 0, when possible, rats with body weights that are not within ± 20% of the mean within a sex, will be removed from study and replaced with rats having body weights within that range (subject to the same selection criteria as the original rats).

Rats that have not been assigned to a test group or which have been removed from study on test day 0, for out-of-range body weight, will be released for other laboratory purposes, or be sacrificed by carbon dioxide asphyxiation and discarded without pathological evaluation, at the discretion of the study director.

F. Dosing Solution Preparation and Sampling

The test substance will be <suspended> in <>. Dosing solutions of the test substance will be prepared <daily>.

G. Administration of Dosing Suspensions

The test substance will be suspended in <>. The rats will be dosed by intragastric intubation until steady state is obtained (estimated to be <> days) at a volume of 1 mL/100 grams of body weight. Animals will be dosed 7 days a week. The amount of test substance each rat receives will be based on the body weights collected twice a week and the solutions concentration. Control rats will be dosed with <> at a volume of 1 mL/100 grams of body weight.

The following suspension concentrations will be used for each dose: <> mg/ml for the <> mg/kg/day dose; <> mg/ml for the <> mg/kg/day dose; and <> mg/ml for the <> mg/kg/day dose. Control rats will be dosed with the same vehicle used to administer the test compounds at a volume of 1 ml/100 grams of body weight.

H. Body Weights

The rats will be weighed twice per week at 3 to 4 day intervals during the study.

I. Clinical Observations and Mortality

During the test period, cage-site examinations to detect moribund or dead rats and abnormal behavior and/or appearance among rats will be conducted at least twice daily throughout the study. Moribund rats will be sacrificed and discarded without necropsy. At every weighing, each rat will be individually handled and examined for abnormal behavior and appearance. Clinical signs noted will be recorded. Animals that do not exhibit clinical signs of toxicity will be recorded as "no abnormality detected" (NAD) in the study records.

Prior to sacrifice of moribund rats, if possible and at the discretion of the clinical pathologist and/or study director, blood samples for hematology and clinical chemistry measurements may be collected for clinical pathology evaluations.

J. Total Fluorine Level Evaluation

Prior to dosing (test day –4), 1, and 7 days after study start, and weekly thereafter until steady state for total fluorine in blood is attained, blood (approximately 0.5 – 1 mL) will be collected from the orbital sinus of each animal while under light carbon dioxide anesthesia for total fluorine analysis. On the day of blood collection, blood will be collected from the animals prior to dosing. The blood will be collected in glass tubes containing EDTA while on ice and stored in the refrigerator until they are sent to <> within 24 hours for total fluorine analysis. For each bleeding, blood should be collected at approximately the same time of day. Once steady state is reached for a particular dose group, administration of that dose of test article will end. (Note – the control group will continue to be dosed with vehicle until the last test article group achieves steady state).

Blood will also be collected for total fluorine analysis at 3, 7, 14, 30, 45, 60, and 75 days postdosing for each dose group. The blood samples collected postdosing may be analyzed as needed to answer questions regarding distribution and metabolism of the test substances.

Additionally, once a dose group attains steady state, the animals will then be placed in metabolism cages for collection of feces and urine for 3 days. Urine and feces will be collected in 24 hour increments. The exact time period of collection of urine and feces will need to be documented along with the total volume of urine obtained. The urine and feces obtained from a particular dose group will be pooled (sexes separate). Blood, urine, and feces will be sent to <> for total fluorine analysis. The samples will be sent to <> in coolers containing ice. Additionally, urine samples may be provided to the Clinical Pathology Group for method development studies.

K. Anatomical Pathology

1. Pretest

See Materials and Methods, Section D. Pretest Period.

2. Dosing Phase

All rats in the control and high dose groups will be sent to Pathology for gross evaluation and collection of tissues. Rats will be euthanatized by carbon dioxide anesthesia and exsanguination.

The following tissues will be collected and weighed from rats in the control and high-dose groups sacrificed by design. The tissues will be placed in plastic freezer bags and stored in the freezer until analysis of total fluorine levels.

Digestive System
liver

Reproductive System
Male
testes

Miscellaneous
fat

The liver, testes, and fat will be sent to Jackson Laboratory for total fluorine evaluation in coolers containing ice.

SAFETY AND HOUSEKEEPING

Good housekeeping procedures will be practiced to avoid contamination of dosing solution preparation facilities and potential health hazards. To avoid skin contact, gloves will be worn when handling the test substance or dosing solutions. In addition, the test substance will be handled in a chemical hood. Dosing solutions will be prepared in properly ventilated areas. Animal carcasses and feces will be incinerated.

RECORDS AND SAMPLE RETENTION

All original records will be retained at <>. Laboratory-specific or site-specific records such as personnel files and equipment records will be retained at the facility where the work was done.

PROTOCOL APPENDIX

Study Function	Study Personnel
Study Director:	<> <Senior Research Scientist>
Study Dates	
Prebleed	<>
Initiation of Test Substance Administration	<>
Scheduled Sacrifice	Dependent upon the elimination rate of fluorine from the blood of the various dose groups

SIGNATURES

Approved by: _____ _____

<>
Primary Technician

_____ _____

<>
Management

_____ _____

<>
Study Director

cc:

<CMPD>: Subchronic Toxicity: 90-Day Gavage Study in Rats with Recovery

Protocol

Contain NO CBI

TABLE OF CONTENTS

INTRODUCTION

The test substance, <CMPD>, is under consideration for use as <use>.

In a <duration> range finding study conducted with <CMPD> in rats, there was <results>

Dose levels for this 90-day gavage study were selected based on <results> observed in the range-finding study.

OBJECTIVE

The objective of this study is to evaluate the potential subchronic toxicity and reversibility of toxicity of <CMPD> when administered by gavage to male and female rats. A supplemental group will also be used to determine <> in the blood during the course of the study. The oral route of administration was selected because it provides an effective means of compound delivery..

SPONSOR AND TEST FACILITY

This study is sponsored by <name, address of sponsor>. The sponsor's approval was effective the date the sponsor authorized the work. The study will be conducted at <facility, location>. This study will be conducted in compliance with U.S. EPA TSCA (40 CFR part 792) Good Laboratory Practice Standards, which are consistent with the OECD Principles of Good Laboratory Practice (as revised in 1997) published in ENV/MC/CHEM(98)17 and MAFF Japan Good Laboratory Practice Standards (59 NohSan No. 3850) [1,2,3] Areas of noncompliance will be documented in the final report.

The study design is based on European Economic Communities (EEC),[4] U.S. Environmental Protection Agency (EPA) Office of Prevention, Pesticides, and Toxic Substances (OPPTS),[5] Organization for Economic Cooperation and Development (OECD),[6] and the Ministry of Agriculture, Forestry and Fisheries (MAFF) Japan[7] test guidelines.

STUDY DESIGN

The study design is as follows:

Group		Number/Group		
Male	Female	Male	Female	Daily Dosage[a]
I	II	25	25	0 (Control)
III	IV	15	15	<> mg/kg
V	VI	15	15	<> mg /kg
VII	VIII	25	25	<> mg/kg

a Weight/weight of test substance (adjusted for sponsor-supplied purity of active ingredient).

Study Parameters	Frequency
Body Weight	Day 0 and weekly thereafter
Food Consumption	Day 0 and weekly thereafter
Clinical Observations	Day 0 and weekly thereafter
Mortality/Moribundity Checks	Twice daily
Clinical Pathology	Week 7 and 13; optional at end of recovery phase (week 17)
Neurobehavioral Evaluations	Pretest and week 13; optional at end of recovery phase (week 17)
Necropsy	
Gross Observations	Week 13 and 17
Organ Weights	

MATERIALS AND METHODS

A. Test Substance

The test substance of known purity will be supplied by the sponsor. The test substance was assigned <> Laboratory Number <>. The stability of <CMPD> will be confirmed by analyses near the beginning and end of the study.

B. Test Species

SPF male and female Crl:CD®(SD)IGS BR rats will be obtained from Charles River Laboratories, Inc. The address of the supplier (city/state) will be documented in the study records and final report. The Crl:CD®(SD)IGS BR rat has been selected on the bases of extensive experience with this strain and its suitability with respect to longevity, hardiness, sensitivity, and low incidence of spontaneous diseases.

C. Animal Husbandry

1. Housing

All rats will be housed in stainless steel, wire-mesh cages suspended above cage boards.

2. Cage Rack Positioning

Cage racks will be relocated within the animal room at least every other week and cages on the racks will be repositioned every 2 weeks.

3. Environmental Conditions

Animal rooms will be maintained at a temperature of 22 ± 3°C and a relative humidity of 50% ± 20%. Animal rooms will be artificially illuminated (fluorescent light) on an approximate 12-hour light/dark cycle.

4. Water and Food

All rats will be provided tap water *ad libitum*. All rats will be fed PMI Nutrition International, Inc. Certified Rodent LabDiet® 5002 *ad libitum*.

5. Animal Health Monitoring

As specified in the <> Laboratory animal health and environmental monitoring program, the following procedures are performed periodically to assure that contaminant levels are below those that would be expected to impact the scientific integrity of the study:

- Water samples are analyzed for total bacterial counts, and the presence of coliforms, lead, and other contaminants.
- Feed samples are analyzed for total bacterial, spore and fungal counts.
- Samples from freshly washed cages and cage racks are analyzed to ensure adequate sanitation by the cagewashers.

Certified animal feed is used, guaranteed by the manufacturer to meet specified nutritional requirements and not to exceed stated maximum concentrations of key contaminants, including specified heavy metals, aflatoxin, chlorinated hydrocarbons, and organophosphates. The presence of these contaminants below the maximum concentration stated by the manufacturer would not be expected to impact the integrity of the study.

The animal health and environmental monitoring program is administered by the attending laboratory animal veterinarian. Data are maintained separately from study records and may be included in the final report at the discretion of the study director.

D. Pretest Period

Upon arrival at <> Laboratory, all rats will be housed 1 per cage, sexes separate, in quarantine. The rats will be:

- quarantined for at least 5 days.
- identified temporarily by cage identification.
- weighed at least 3 times during quarantine.
- observed with respect to weight gain and any gross signs of disease or injury.
- given an ophthalmology examination.

The rats will be released from quarantine by the laboratory animal veterinarian or designee on the basis of acceptable body weights and clinical signs of all rats.

Rats that are accidentally killed or removed from study during the pretest period will be discarded without necropsy. Rats that are found dead or sacrificed *in extremis* during the pretest period will be sent to Pathology and given a gross examination to check for the presence of disease. Dependent upon these findings, further diagnostic procedures may be employed at the discretion of the study director, a pathologist, or the laboratory animal veterinarian. The results will not be reported in the final report unless considered significant to the evaluation of the study.

E. Assignment to Groups

Rats of each sex, selected on the basis of adequate body weight gain and freedom from any ophthalmological abnormalities or clinical signs of disease or injury will be distributed by computerized, stratified randomization into study groups as designated in the Study Design, so that there are no statistically significant differences among group body weight means within a sex. The weight variation of selected rats will not exceed ± 20% of the mean weight for each sex.

Study animals will consist of three subgroups: 10 rats/sex/dose – subchronic toxicity, 5 rats/sex/dose – blood fluoride analysis, and an additional 10 rats/sex in control and high dose groups designated as recovery animals. After assignment to groups, each rat will be housed individually. Information on the cage labels will include the unique animal number and an individual identification number assigned to each rat. The last 3 digits of the animal number will be tattooed on the tail of each rat.

At study start (test day 0) the rats will be between 6 and 8 weeks of age. On test day 0, when possible, rats with body weights that are not within ± 20% of the mean within a sex will be removed from study and replaced with rats having body weights within that range (subject to the same selection criteria as the original rats).

Rats that have not been assigned to a test group, or which have been removed from study on test day 0 for out-of-range body weights, will be released for other laboratory purposes or be sacrificed by carbon dioxide asphyxiation and discarded without pathological evaluation, at the discretion of the study director.

F. Dosing Solution Preparation and Sampling

Dosing solutions of <CMPD> will be prepared <frequency> with <solvent>. The method of preparing the dosing solutions will be documented in the study records. <Samples will be analyzed to verify concentration and stability of <CMPD> in the dose solutions, as follows:

Type[a]	Dosing Solution Concentrations	Storage Conditions Until Analysis
Control	0 mg/ml	frozen
Concentration verification[b]	All	analyzed immediately or frozen
Stability:[c]		
5-hour room temperature	All	5 hours room temperature then frozen
7-day and 14-day refrigerated	All	7 or 14 days refrigerated then frozen

a Duplicate samples will be collected and analyzed.
b Samples will be collected at least 3 times during the study.
c Stability of <CMPD> in solution will be demonstrated by comparing concentrations of <CMPD> in baseline samples to those of the refrigerated samples and those stored at room temperature.

Extra samples may be taken at the discretion of the study director. Whenever extra samples are taken, a control sample will be collected and frozen the same day as dose-solution preparation.>

G. Administration of Test Substance

Dosing solutions will be administered daily by oral gavage at a dose volume of <10/20> ml/kg, based on the most recently recorded weight. Dose solutions will be kept <refrigerated> until used. Dosing solutions stored beyond <duration> after preparation will not be administered to animals. Control animals will be dosed with <solvent> only. All animals will be dosed for 90 days. Rats designated for recovery or fluoride analysis will not receive any dosing for the remainder of the recovery period.

H. Body Weights

During the study, all rats will be weighed once each week unless experimental findings or special scheduling situations warrant a change in the weighing schedule. In addition, rats undergoing neurobehavioral assessments will be weighed on the days of those observations.

I. Food Consumption and Food Efficiency

During the study, the amount of food consumed by each rat over the weighing interval will be determined by weighing each feeder at the beginning and end of the interval and subtracting the final weight and the amount of spillage from the feeder during the interval from the initial weight. From these measurements, mean daily food consumption over the interval will be determined. From the food consumption and body weight data the mean daily food efficiency will be calculated.

J. Detailed Clinical Observations and Mortality

During the study, cage-site examinations to detect moribund or dead rats and abnormal behavior and/or appearance among rats will be conducted at least twice daily throughout the study. Moribund rats will be sacrificed. At every weighing, each rat will be individually handled and examined for abnormal behavior and appearance in a standardized arena. The detailed clinical observations will include (but are not limited to) evaluation of fur, skin, eyes, mucous membranes, occurrence of secretions and excretions, autonomic nervous system activity (lacrimation, piloerection, and unusual respiratory pattern), changes in gait, posture, response to handling, presence of clonic, tonic, stereotypical, or bizarre behavior. Any abnormal clinical signs noted will be recorded.

Prior to sacrifice of moribund rats, if possible and at the discretion of the clinical pathologist and/or study director, blood samples for hematology and clinical chemistry measurements will be collected for clinical pathology evaluations.

K. Ophthalmology

Two ophthalmological examinations will be conducted by a veterinary ophthalmologist. The pretest examination will be performed on all rats received for the study, prior to assignment to groups. Any rats with preexisting ophthalmological abnormalities may be eliminated from consideration for use in the study. All surviving rats designated for subchronic toxicity evaluation and recovery will be examined at the end of the exposure phase of the study. Both eyes of each rat will be examined by focal illumination and indirect ophthalmoscopy. The eyes will be examined in subdued light after mydriasis has been produced.

A third ophthalmological examination may be conducted on rats designated for recovery, at the end of the recovery phase, if test substance-related effects are observed at the end of the exposure phase.

A report of findings from each ophthalmology examination will be submitted by the examining veterinarian.

L. Neurobehavioral Evaluation

For all of the following assessments, the experimenter will be unaware of the group designations of the animal. The abbreviated functional observational battery (FOB) and motor activity will be evaluated on all animals in the low and mid-dose group and in the rats designated for recovery (10 animals/group/sex) in control and high-dose groups. Spare rats will also be evaluated for the abbreviated FOB and motor activity prior to exposure.

1. Sensory Function

Prior to test substance administration and then during week 12 of test substance administration, simple assessments of sensory function will be made (grip strength, response to approach/touch, tail pinch, and sharp auditory stimulus [clicker]) and evaluation of motor activity (see Section L.2. Motor Activity). Fore- and hind-limb grip strength will be measured by a strain gauge device (Chatillon® Digital Force gauge). Due to technical difficulty of evaluating pupil size in albino rats under ambient lighting conditions, pupil size will be evaluated by assessment of pupillary response. Pupillary response will be conducted following motor activity. Pupillary response to a beam of light will be measured immediately prior to removing the rats from the motor activity chambers. Animals designated for recovery will be evaluated for sensory function during the last week of the recovery period, if effects are observed at the end of the exposure period.

2. Motor Activity

Motor activity (MA) will be assessed following the sensory function evaluation on the same day. Rats will be individually tested in one of 30 nominally identical, automated activity monitors (Coulbourn®). Group and gender will be counterbalanced across the monitors and time of day to the fullest extent possible. The infrared monitoring device enables measurement of 2 dependent variables, duration of movement and number of movements. A continuous movement is counted as 1 movement regardless of duration. Each test session will be 60 minutes in duration, and the results will be expressed for the total session as well as for 6 successive 10-minute blocks. Presence of defecation and urination on the cageboards below the motor activity cages will also be evaluated following each motor activity session. Animals designated for recovery will be evaluated for motor activity during the last week of the recovery period, if effects are observed at the end of the exposure period.

M. Clinical Pathology Evaluation

A clinical pathology evaluation will be conducted on all rats designated for subchronic toxicity at approximately 45 days and prior to necropsy on the last day of study. The day before collection of blood samples for the clinical pathology evaluation, the rats will be placed in metabolism cages. These rats will be fasted overnight and urine will be collected from each rat. Blood

samples for hematology and clinical chemistry measurements will then be collected from the orbital sinus of each rat while the rat is under light carbon dioxide anesthesia. Blood samples for coagulation parameters will be collected under carbon dioxide immobilization from the abdominal *vena cava* at sacrifice. Blood samples will be evaluated for quality by visual examination prior to analysis.

At the discretion of the study director or clinical pathologist, additional samples for selected clinical pathology tests may be collected from animals showing clinical evidence of toxicity or sacrificed *in extremis*.

1. Hematology

The following hematology parameters will be determined:

red blood cell count
hemoglobin
hematocrit
mean corpuscular volume
mean corpuscular hemoglobin
mean corpuscular hemoglobin concentration
red cell distribution width
absolute reticulocyte count
white blood cell count
differential white blood cell count
platelet count
microscopic blood smear examination

New methylene-blue-stained blood smears will be prepared from all hematology samples and will be evaluated, if required, to substantiate or clarify the results of hematology findings.

2. Coagulation Parameters

The following coagulation parameters will be determined:

prothrombin time
activated partial thromboplastin time

3. Serum Chemistry

The following serum chemistry parameters will be determined:

aspartate aminotransferase
alanine aminotransferase
sorbitol dehydrogenase
alkaline phosphatase
total bilirubin
urea nitrogen
creatinine
cholesterol
triglycerides
glucose
total protein
albumin
globulin
calcium
inorganic phosphorus
sodium
potassium
chloride
plasma fluoride

4. Urinalysis

The following urinalysis parameters will be determined:

quality	urobilinogen
color	blood
transparency	glucose
volume	protein
osmolality	bilirubin
specific gravity	ketone
pH	microscopic urine examination
	urine fluroide

5. Recovery Animals

At the discretion of the clinical pathologist and study director and if hematological or clinical pathology evaluations suggest systemic toxicity, clinical pathology evaluations including hematology, serum chemistry, and urinalysis, may be performed on animals designated for recovery (10 rats/sex in control and high dose groups) at the end of the recovery period.

N. Blood Analysis

The designated 5 animals/sex/group will be evaluated for <fluoride levels> during the 90-day dosing period. The same rats will be evaluated for <fluoride levels> during a recovery period, up to 90 days post-dosing, or until <fluoride levels> in exposed rats return to those of controls. Blood collection time points will be: day 1 (males only), and days 4, 10, 21, 35, 56, 77, 90, 93, 98, 105, 119, 133, 154, and 175 (males and females). Blood (approximately 0.5 – 1 mL) will be collected from the orbital sinus of designated animals while under light carbon dioxide anesthesia. On the day of blood collection, blood will be collected from the animals prior to dosing. The blood will be collected in glass tubes containing EDTA while on ice and stored in the <refrigerator> until they are sent to <> within <24 hours> for analysis. For each bleeding, blood should be collected at approximately the same time of day. The samples will be sent to <> in coolers containing ice.

Animals will be sacrificed at the end of the recovery period, or sooner if <fluoride levels> return to control levels.

O. Anatomic Pathology

1. Pretest

See Section D. Pretest Period.

2. Rats Designated for Subchronic Toxicity Evaluation

All rats found dead, accidentally killed, sacrificed *in extremis*, or sacrificed by design will undergo a gross and microscopic evaluation. All rats removed from study (except for out-of-range body weight on test day 0) will be sent to Pathology for gross evaluation and collection of tissues. Rats will be euthanatized by carbon dioxide anesthesia and exsanguination. Rats sacrificed by design will be fasted after 3 p.m. on the afternoon before their scheduled sacrifice. A final sacrifice will be performed on surviving rats following the final clinical pathology evaluation. The order of sacrifice for scheduled deaths will be random among all treatment groups within a sex. Blood samples for coagulation parameters will be collected under carbon dioxide immobilization from the abdominal *vena cava* of each rat at sacrifice and will be evaluated by Clinical Pathology.

The following tissues will be collected from rats which are found dead or accidentally killed (tissue integrity permitting), sacrificed *in extremis*, removed from study (except out-of-range body weight on test day 0), or sacrificed by design.

Digestive System
- liver
- esophagus
- stomach
- duodenum
- jejunum
- ileum
- cecum
- colon
- rectum
- salivary glands
- pancreas

Urinary System
- kidneys
- urinary bladder

Respiratory System
- lungs
- trachea
- nasal tissue
- larynx
- pharynx

Cardiovascular System
- heart
- aorta

Hematopoietic System
- spleen
- thymus
- mandibular lymph node
- mesenteric lymph node
- bone marrow[a]

Endocrine System
- pituitary gland
- thyroid gland
- parathyroid glands
- adrenal glands

Nervous System
- brain (including cerebrum, cerebellum, medulla/pons)
- spinal cord (cervical, mid-thoracic, lumbar)
- sciatic nerve

Musculoskeletal System
- skeletal muscle
- femur/knee joint
- sternum

Reproductive System
- Male
  - testes
  - epididymides
  - prostate
  - seminal vesicles
- Female
  - ovaries
  - uterus
  - mammary glands

Miscellaneous
- skin
- eyes (including optic nerve)
- gross observations[b]

a Bone marrow will be collected with the femur and sternum.

b Gross observations made at necropsy for which histopathology is not appropriate (e.g., fluid, ruffled fur, and missing anatomic parts) will generally not be collected.

All tissues will be placed in the appropriate fixative.

Rats sacrificed by design will have the following organs weighed: liver, kidneys, adrenal glands, thymus, brain, spleen, heart, and ovaries and uterus (female) or testes and epididymides (male). Relative organ weights (percent of final body weight; ratio to brain weight) will be calculated. Final body weights determined just prior to necropsy will be used in the assessment of organ weight changes. Organs from rats found dead, sacrificed *in extremis*, or accidentally killed may be weighed at the discretion of the pathologist or study director.

Bone marrow smears will be prepared at the final sacrifice from all surviving rats and will be evaluated if warranted by experimental findings.

All tissues collected from rats designated for subchronic toxicity evaluation in the high-concentration and control groups, and from rats that are found dead or accidentally killed (tissue integrity permitting), or are sacrificed *in extremis*, will be further processed to slides, stained with hematoxylin and eosin, and examined microscopically. Most gross lesions and any additional target organs from rats in the low- and intermediate-concentration groups will be evaluated microscopically. Selected gross observations for which a microscopic diagnosis would not be additive (e.g., osteoarthritis, pododermatitis, tail chronic dermatitis, calculus, and deformities of the teeth, toe, tail, or ear pinna) will be saved, but will generally not be processed for microscopic evaluation. Tissues from rats removed from study will not be processed for microscopic evaluation unless considered necessary by the study director or pathologist.

Additional procedures to identify and/or clarify histologic features of lesions may be performed at the discretion of the pathologist and will be documented in the final report.

3. Rats Designated for <Blood Fluoride>Analysis

Any rats designated for <blood fluoride> evaluations that are found dead, accidentally killed, or are sacrificed *in extremis* will be discarded without pathological evaluation. Rats designated for <blood fluoride> analysis that survive the exposure period will be sacrificed by carbon dioxide anesthesia and exsanguination approximately 90 days after the final dose of <CMPD>, or when plasma fluoride levels return to control values. These rats will be discarded without pathological evaluation.

4. Rats Designated for Recovery

Any rats designated for recovery evaluation that are found dead, accidentally killed, or are sacrificed *in extremis* will be sent to Pathology for a gross evaluation. Tissues will be collected and examined microscopically at the discretion of the study director or pathologist. The results will not be reported in the final report unless considered significant to the evaluation of the study. One month after the final dose, rats designated for recovery (10 rats/sex in control and high dose groups) will be sacrificed by carbon dioxide anesthesia and exsanguination, and will undergo a gross examination. Tissues (see section O.2.) will be collected and saved in the appropriate fixative. Gross lesions and target organs identified in rats sacrificed at the end of the 90-day exposure period will be examined microscopically.

STATISTICAL ANALYSES.

Except for Bartlett's test ($p < 0.005$), significance was judged at $p < 0.05$.

		Method of Statistical Analysis	
Parameter	Preliminary Test	If preliminary test is not significant	If preliminary test is significant
Body Weight Body Weight Gain Food Consumption Food Efficiency Organ Weight	Test for lack of trend[8]	Sequential application[9] of the Jonckheere-Terpstra trend test[10]	Preliminary tests for pairwise comparison
	OR[a]		
	Levene's test for homogeneity[11] and Shapiro-Wilk test[12] for normality[b]	One-way analysis of variance[13] followed with Dunnett's test[14]	Kruskal-Wallis test[15] followed with Dunn's test[16]
Motor Activity[c]	Levene's test for homogeneity[11] and Shapiro-Wilk test[12] for normality[b]	Repeated measures analysis of variance followed by Contrasts[17]	Sequential application[9] of the Jonckheere-Terpstra trend test[10]
Grip Strength Foot Splay	Bartlett's test[18] for homogeneity of variances	One-way analysis of variance[13] followed with Dunnett's test[14]	Kruskal-Wallis test[15] followed with Dunn's test[16]
Clinical Pathology[d] Fluoride	Levene's test for homogeneity[11] and Shapiro-Wilk test[12] for normality[b]	One-way analysis of variance[13] followed with Dunnett's test[14]	Kruskal-Wallis test[15] followed with Dunn's test[16]
Survival Incidence of Clinical Observations Incidence of FOB Descriptive Parameters	None	Cochran-Armitage test for trend[13][e]	
Incidence of Microscopic Lesions	None	None	

a Pairwise comparisons and associated preliminary tests are only conducted if the test for lack of trend is significant.

b If the Shapiro-Wilk test is not significant but Levene's test is significant, a robust version of Dunnett's test will be used.

c Test day and block (10-minute EPOCH) will be used as repeated-measure factors.

d When an individual observation is recorded as being less than a certain value, calculations are performed on half the recorded value. For example, if bilirubin is reported as <0.1, 0.05 is used for any calculations performed with that bilirubin data.

e If the incidence is not significant, but a significant lack of fit occurs, then Fisher's exact test[19] with a Bonferroni correction is used.

Other methods will be used, if appropriate, at the time of analysis. The statistical methods used will be described in the final report.

SAFETY AND HOUSEKEEPING

Good housekeeping procedures will be practiced to avoid contamination of diet preparation facilities and potential health hazards. To avoid skin contact, gloves will be worn when handling either the test substance or diets. In addition, the test substance will be handled in a chemical hood. Diets will be prepared in properly ventilated areas. Animal carcasses, feces, and unused diet will be incinerated.

RECORDS AND SAMPLE RETENTION

All original records will be retained at <facility, location>. Laboratory-specific or site-specific records such as personnel files and equipment records will be retained at the facility where the work was done. Preserved wet tissues, embedded tissues, histological slides, blood smears and bone marrow smears will be retained at <facility, location>. A sample of the test substance will be collected for archive purposes and retained at <facility, location>.

REFERENCES

1. EPA/FIFRA Good Laboratory Practice Standards (40 CFR 160). (1989).

2. OECD Principles of Good Laboratory Practice (as revised in 1997, published in ENV/MC/CHEM(98)17 (OCDE/GD(92)32).

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11. Levene, H. (1960). Robust test for equality of variances. *Contributions to Probability and Statistics* (J. Olkin, ed.), pp 278-292. Stanford University Press, Palo Alto.

12. Shapiro, S.S. and Wilk., M.B. (1965). An analysis of variance test for normality (complete samples). *Biometrika* **52**, 591-611.

13. Snedecor, G.W. and Cochran, W.G. (1967). *Statistical Methods*, 6th edition, pp 246-248 and 349-352. The Iowa State University Press, Iowa.

14. Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Amer. Statist. Assoc.* **50**, 1096-1121.

15. Kruskal, W.H. and Wallis, W.A. (1952). Use of ranks in one-criterion analysis of variance. *J. Amer. Statist. Assoc.* **47**, 583-621.

16. Dunn, O.J. (1964). Multiple contrasts using rank sums. *Technometrics* **6**, 241-252.

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18. Bartlett, M.S. (1937). Some examples of statistical methods of research in agriculture and applied biology. *J. Royal. Statis. Soc. Suppl.* **4**, 137-170.

19. Fisher, R.A. (1985). *Statistical Methods for Research Workers*, 13th edition. Haffner, New York.

PROTOCOL APPENDIX

Study Function	Study Personnel
Study Director:	<> <Senior> Research Scientist
Analytical Chemistry Evaluation:	<> Analytical Chemist
Neurobehavior Evaluation:	<> Neurotoxicologist
Clinical Pathology Evaluation:	<> Principal Research Scientist
Anatomic Pathology Evaluation:	«PP» Anatomical Pathologist

PROTOCOL APPENDIX (Continued)

Study Dates

Initiation of Test Substance Administration

<Analytical Chemistry Evaluations>
<Pretest>
<90-Day>

Ophthalmology Evaluations
Pretest
Week 13
<Week 17: optional>

Neurobehavioral Evaluations
Pretest
Week 13
<Week 17: optional>

Clinical Pathology Evaluations
90-Day

Blood Fluoride Analyses
Every 2 weeks of exposure phase
Every 2 weeks of recovery phase or
until values return to control values.

Scheduled Sacrifice
Week 13: exposure phase
Week 17: recovery phase
Week 26: total fluoride evaluation rats
(unless ended sooner)

SIGNATURES

Approved by: _____ _____

<> Primary Technician — Date

_____ _____

<> Analytical Chemist — Date

_____ _____

<> Neurotoxicologist — Date

_____ _____

<> Clinical Pathologist — Date

_____ _____

«PP» Pathologist — Date

_____ _____

<> Manager — Date

_____ _____

<> Study Director — Date

cc:

«Title»: Multigeneration Reproduction Study in Rats

Protocol

Contain NO CBI

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OBJECTIVE

The objective of this study is to evaluate the effect of «Title» («Compound») on the gonadal function, conception, parturition, and the growth and development of offspring of male and female Crl:CD®(SD)IGS BR rats over two generations involving the production of at least one set of litters in each generation.

This study will conform with applicable Good Laboratory Practice Standards[1-3] and Test Guidelines.[4-7]

SPONSOR AND TEST FACILITY

This study is sponsored by (name, sponsor) and will be conducted at <>. Sponsor approval of the study will be indicated by issuance of the work authorization form.

MATERIALS AND METHODS

A. Test Substance

1. Identification

The test substance will be identified in this protocol and in the study records by «Compound» or by the Sample Number.

2. Purity

<>%

3. Test Substance Stability

The stability of «Compound» will be confirmed by purity analyses conducted quarterly.

B. Animals

1. Species (Strain)

Crl:CD®(SD)IGS BR rats

2. Reason for Selection

The rat was selected for this study as it is a preferred species for reproductive toxicity testing as recommended by the guidelines. The Crl:CD®(SD)IGS BR strain was chosen because extensive background information is available from the literature, the supplier, and previous studies with other compounds. This strain is also considered suitable relative to longevity, hardiness, and incidence of spontaneous disease.

3. Source

Charles River Laboratories, Inc.

4. Description

Approximately 150 male and 150 female rats (no male-female siblings), requested to be approximately 49 days of age, and weigh approximately 180 to 220 grams and 151 to 175 grams, respectively.

5. Identification

a. Pretest

Temporary cage identification

b. After Assignment to Treatment Groups

Each rat will be assigned a unique six digit number and an identification number will be tattooed on the tail (see Appendix I).

C. Animal Husbandry

1. Environmental Conditions

Animal rooms will be maintained at an acceptable temperature of 20-26°C (targeted at 23°C) and relative humidity of 50 ± 10%. Animal rooms will be artificially illuminated (fluorescent light) on a 12-hour light/dark cycle (approximately 0600-1800 hours).

2. Caging

All male rats will be housed individually during non-mating periods in stainless steel, wire-mesh cages.

a. Pretest and Premating Periods

All rats will be housed individually in stainless steel, wire-mesh cages.

b. Cohabitation Period

All rats will be housed as breeding pairs in stainless steel, wire-mesh cages. At the end of the cohabitation period, females without evidence of copulation will be housed individually in polycarbonate pans.

c. Gestation Period

- **Days 0-20:** Females will be housed individually in stainless steel, wire-mesh cages.

- **Day 20-Delivery:** Females will be housed individually in polycarbonate pans.

d. Lactation Period

Adult females will be housed with their litters in polycarbonate pans.

The stainless steel, wire-mesh cages will be suspended above cageboard. Polycarbonate pans will contain bedding.

3. Food

PMI Nutrition International, Inc. Certified Rodent LabDiet® 5002 containing the appropriate dosage of «Compound» will be available *ad libitum*.

4. Water

Water from <> will be available *ad libitum*.

5. Animal Health Monitoring

<> Laboratory has an animal health monitoring program. The following procedures are performed periodically:

- Water samples are analyzed for total bacterial counts, and the presence of coliforms, lead, and other contaminants.
- Feed samples are analyzed for the presence of bacteria and fungi.
- Samples from freshly washed cages and cage racks are analyzed to ensure adequate sanitation by the cagewashers.
- Blood sera taken from nonstudy animals (sentinels) housed in the study room are analyzed for antibodies against common rodent pathogens.

Certified animal feed is used, guaranteed by the manufacturer to meet specified nutritional requirements and to be free of impurities which might influence the results of the study.

The animal health monitoring program is administered by the Laboratory Animal Veterinarian. Data are maintained separately from study records and will not be included in the final report unless warranted by the results of these evaluations.

6. Quarantine

Rats will be quarantined for at least six days, and then released for the study upon the approval of the Laboratory Animal Veterinarian or designee.

Rats that die or are sacrificed *in extremis* during the pretest period will be necropsied to check for the presence of disease. Dependent upon these findings, further diagnostic procedures may be employed at the discretion of the study director, the pathologist assigned to the study, or the laboratory veterinarian.

D. Experimental Design

Treatment Groups and Dietary Concentrations

Group Number					
Male		Female		#/Group	Concentration (ppm)[a]
P_1	F_1	P_1	F_1		
I-0	I-1	II-0	II-1	30	0 (Control)
III-0	III-1	IV-0	IV-1	30	<> (Low)
V-0	V-1	VI-0	VI-1	30	<> (Intermediate)
VII-0	VII-1	VIII-0	VIII-1	30	<> (High)

[a] Weight/weight concentration of «Compound», active ingredient, (adjusted for purity).

Treatment Schedule

Generation	Approximate Age at Start of Feeding(days)	Approximate Number of Study Days Before Mating	Duration of Feeding
P_1	63	70	Until sacrifice
F_1	21	70	Until sacrifice

Sacrifice Schedule

Animals	Generation	Schedule
Adult Males	P_1, F_1	After siring litters (at discretion of study director)
Pregnant Females	P_1, F_1	On day of weaning litters
Nonpregnant Females	P_1, F_1	With pregnant females
Weanlings	F_1, F_2	On day of weaning (except F_1's selected for parental rats)

E. Selection of Dose Levels

Dose levels selected for this study were based upon <>.

F. Randomization

1. P_1 Generation

Rats of each sex will be ranked by their most recently recorded body weight and randomly assigned to control and experimental groups. The randomization should result in a distribution in which the mean body weights for all groups within a sex are not statistically different ($p>0.05$). Animals that lose excessive weight or that are ill prior to the start of dosing will be removed from the study and replaced, if possible.

Rats not assigned to a test group, at the discretion of the study director,

- may be released for use in other studies, or
- will be sacrificed by carbon dioxide euthanasia and discarded without pathological evaluation.

2. F_1 Generation

On day 21 postpartum, offspring in the F_1 litters of each treatment level will be selected (one rat/sex/litter when possible) to serve as parents for the F_2 generation. For groups without sufficient litters, additional pups will be chosen from randomly selected litters within the group to achieve the required group size. Selection of rats within litters will be random.

G. Diet Administration, Preparation, and Sampling

1. Administration

«Compound» will be administered orally as it is a potential route for human exposure. During the test period, rats in each group will be fed a diet of PMI Nutrition International, Inc. Certified Rodent LabDiet® 5002 that contains 0, <>, <>, or <> ppm of «Compound».

2. Preparation and Sampling

«Compound» will be added to PMI Nutrition International, Inc. Certified Rodent LabDiet® 5002 and thoroughly mixed for a period of time that, by experience or pretest determination, is adequate to ensure homogeneous distribution in the diet. <The test substance will be dissolved/suspended in acetone prior to mixing. The same concentration of acetone will also be added to control diets. The manufacturer, lot number, and purity of the acetone used to dissolve the test substance prior to incorporation into the diet will be recorded in the study records. Neither the amount and

nature of the contaminants nor the use of acetone is expected to affect the integrity or validity of the study.> Diets will be prepared biweekly and refrigerated until used.

The following dietary analysis parameters will be evaluated. Stability of the test substance in diets was verified previously at <> and <> ppm. Other samples will be collected in duplicate and analyzed from the initial diet preparation prior to study start to verify homogeneity and concentration. In addition, samples will be collected in duplicate and analyzed for concentration at approximately 3, 6, and 9 months from study start.

Type	Collect From	Storage Conditions Until Analysis
Control	Diet Mixer	Frozen
Homogeneity:[a,b]	Diet Mixer	Frozen
Top	Top	Frozen
Middle	Middle	Frozen
Bottom	Bottom	Frozen
Stability:[c]		
7-day Room Temperature	Feed Jars	7 days at room temperature, then frozen
14-day Room Temperature	Feed Jars	14 days at room temperature, then frozen
21-day Room Temperature	Feed Jars	21 days at room temperature, then frozen
21-day Refrigerated	Diet Mixer	21 days refrigerated then frozen
Concentration Verification:[d]	As Apportioned	Analyzed immediately or frozen

[a] The mean value of samples will be used to determine concentration.
[b] Diet samples may also be collected to evaluate homogeneity if a new method of diet preparation is used.
[c] Stability of the test substance in the diet was established for <> and <> ppm from a 90-day rat feeding study.<>
[d] Diet samples will be collected at least 3 times during the study for each dietary level to verify concentration.

Stability of «Compound» in feed was determined by comparing concentrations of «Compound» in samples collected and frozen immediately to those stored at room temperature or refrigerated.

Extra diet samples may be taken at the discretion of the study director. Whenever extra diet samples are taken, a sample of control diet will be collected and frozen the same day as diet preparation.

H. Safety Precautions and Disposal of Waste Material

Good housekeeping procedures will be practiced to avoid potential health hazards and contamination of diet preparation facilities. To avoid skin contact, gloves will be worn when handling either the test substance or diets. In addition, the test substance will be handled in a chemical hood. Diets will be prepared in properly ventilated areas. Animal carcasses, feces, and unused diet will be incinerated.

I. Body Weight Measurements for Parental Rats

1. Premating Feeding Period

All P_1 and F_1 rats will be weighed once a week. All animals being evaluated for developmental landmarks (vaginal patency, preputial separation) will be weighed the day criteria are achieved.

2. Gestation and Lactation Periods

P_1 and F_1 females will be weighed on days 0, 7, 14, and 21 of each period.

Females without evidence of copulation, that copulate and do not deliver a litter, and males will continue to be weighed on a weekly schedule.

J. Food Consumption and Food Efficiency for Parental Rats

1. Premating Feeding Period

Individual food consumption will be determined weekly throughout the period, ending test day 70.

2. Gestation Period

Individual food consumption of pregnant P_1 and F_1 females will be recorded on gestation days 0, 7, 14, and 21.

From these determinations and body weight data, individual daily food consumption and food efficiency will be calculated.

K. Clinical Observations for Parental Rats

Cage-site examinations will be conducted at least once daily throughout the study. Moribund rats will be sacrificed. At least once weekly throughout the premating feeding, gestation, and lactation periods, each of the P_1 and F_1 parental rats will be individually handled and carefully examined for abnormal behavior and/or appearance.

L. Breeding

1. Start of Cohabitation

After approximately 10 weeks of exposure to the test substance

2. Duration of Cohabitation Period

Until evidence of copulation is observed (designated as day 0 of gestation), or two weeks have elapsed.

3. Evidence of Copulation

Intravaginal or extruded copulation plug

4. Cohousing

Each female will be continually housed on a 1:1 basis with a randomly selected, nonsibling male of the same dietary concentration level, in the male's cage. On the day copulation is confirmed, the female will be transferred back to individual cage housing.

Based on the outcome of the first mating and at the discretion of the study director, the females may be cohoused again in the manner outlined above, except with different males, to produce a second set of litters.

M. Estrous Cycle Evaluation

Vaginal smears will be collected daily from all P_1 and F_1 female rats in order to determine the stages of the estrous cycle. Vaginal smears will be collected beginning three weeks prior to mating, and continuing until copulation is confirmed, or the cohabitation period has ended.

Vaginal smears will also be collected from all P_1 and F_1 parental female rats at the time of sacrifice to determine the stage of estrous cycle.

N. Gestation Procedures

After being transferred into polycarbonate pans (on day 20 of gestation for mated females, or at the end of the cohabitation period for females without evidence of copulation), female rats will be observed at least twice daily for signs of delivery and offspring.

O. Lactation Procedures

The day when delivery is complete is designated day 0 postpartum.

1. Day 0 Postpartum

Live and dead pups in each litter will be counted as soon as possible after delivery is completed. Live pups in each litter will be individually weighed. Anogenital distance in

F_2 pups will be recorded, if treatment-related effects are observed in sex ratio or sexual maturation of F_1 pups.

2. Day 4 Postpartum

Litters will be culled randomly to eight (four/sex when possible). Extra offspring will be euthanized (by decapitation) and discarded without pathological examination. Litters of eight offspring or less will not be reduced. Litter counts and individual pup weights will be determined prior to and after culling.

3. Days 7 and 14 Postpartum

Pups in each litter will be counted by sex and individually weighed.

4. Day 21 Postpartum (Weaning)

Pups in each litter will be counted by sex and individually weighed. Offspring from the F_1 litters will be randomly selected to serve as parents for the F_2 generation. Randomly selected offspring from the litters of both the F_1 and F_2 generations will undergo gross pathological evaluation. Unselected weanlings will be euthanized by carbon dioxide euthanasia and discarded without pathological evaluation.

At each examination period, offspring will be individually handled and examined for abnormal behavior and appearance; any dead, missing, or abnormal pups will be recorded.

P. Developmental Landmarks

Developmental landmarks in the F_1 generation male and female rats (designated for mating) will be monitored on a daily basis until criterion is achieved.

1. Vaginal Patency

Female rats will be examined beginning on test day 0 (Postnatal day 21).

2. Preputial Separation

Male rats will be examined beginning on test day 14 (Postnatal day 35).

Q. Euthanasia and Postmortem Examinations

1. P_1 and F_1 Adults

a. Method of Euthanasia

Carbon dioxide euthanasia and exsanguination

b. Postmortem Examinations

All P_1 and F_1 parental generation rats will be subjected to gross pathological examination, including those that die or are sacrificed *in extremis* prior to the end of feeding and those for which mating did not result in production of offspring. The following table lists tissues to be collected and/or weighed from all parental generation rats (tissue integrity permitting):

Organ Weights from Parental Rats

Male	Female	Both Sexes
Testes	Uterus (with oviducts and cervix)	Brain
Epididymides	Ovaries	Pituitary
Seminal Vesicles (with coagulating glands and their fluids)		Liver
		Kidneys
		Adrenal Glands
Prostate		Spleen

Tissues Collected from Parental Rats[a]

Male	Female	Both Sexes
Testis	Ovaries	Adrenal Glands
Epididymis	Uterus[b] (with oviducts)	Pituitary
Prostate	Vagina	Gross Observations[c]
Seminal Vesicles	Cervix	
Coagulating Gland		

[a] Reproductive tissues collected from male parental rats for possible histopathological evaluation will be placed in Bouin's solution. Reproductive tissues collected from female parental rats and all non-reproductive tissue will be placed in formalin.

[b] The uteri of all cohabited females will be examined for the presence and number of implantation sites.

[c] Gross lesions observed at necropsy for which histopathology is not appropriate (e.g., fluid, ruffled fur, no milk spot, fetus in birth canal, and missing anatomic parts) will generally not be collected.

c. Histopathological Examination

Histopathological examination of the tissues will be conducted for ten randomly chosen high dose and control animals per sex from both the P_1 and F_1 generations. Target organs and organs demonstrating treatment-related changes will be examined for the remainder of the high-dose and control animals and for all parental animals in

the low- and mid-dose groups in order to further delineate or establish a "no-observable-adverse-effect level". Reproductive organs of animals in low and intermediate dose groups with impaired reproductive performance (e.g., failure to mate, conceive, sire, or deliver healthy offspring; or effects in estrous cyclicity or sperm parameters) will be evaluated. In addition, a quantitative evaluation of primordial and growing follicles will be conducted on the F_1 females selected for histopathological evaluation. Five ovarian cross sections (6 µm thick) will be taken at least 100 µm apart from the inner third of each ovary. Primordial and growing follicles (up to but not including antral follicles) will be enumerated for each of 10 ovarian sections per animal. Most gross lesions and target organs from low and intermediate levels will also be evaluated microscopically. Selected gross observations for which a microscopic diagnosis would not be additive (e.g., osteoarthritis, pododermititis, tail chronic dermatitis, calculus, and deformities of the teeth, toe, tail, or ear pinnae) will be saved, but will generally not be processed for microscopic evaluation.

2. Offspring

Offspring that are found dead during the lactation period will undergo gross pathological evaluation.

3. F_1 and F_2 Weanlings

a. Method of Euthanasia

Carbon dioxide euthanasia and exsanguination

b. Postmortem Examinations

Three F_1 and three F_2 weanlings/sex/litter, litter size permitting, will undergo gross pathological evaluation. One weanling/sex/litter will be designated for organ weight and histopathological evaluation. The weanlings selected for organ weight and histopathological evaluation will have brain, spleen, and thymus weights recorded and all gross lesions and target organs (<>) will be preserved for possible future histopathological examination.

c. Histopathological Examination

Microscopic evaluation of the preserved gross lesions will be conducted at the discretion of the study director or pathologist. Tissues collected from both male and female weanlings for possible histopathological evaluation will be placed in formalin.

R. Sperm Assessment

Sperm parameters for all P_1 and F_1 parental males will be evaluated. The right epididymis will be removed, and the right cauda epididymis will be weighed. Sperm will be collected from the right cauda epididymis and percent motility and morphology will be determined. The left

epididymis and testis will be frozen in liquid nitrogen and stored between -65°C and -85°C for sperm and spermatid counts, respectively.

DATA ANALYSES

The following table lists the indices of reproductive function that will be calculated for the P_1 and F_1 adults.

Reproductive Function Calculations

Mating Index (%)	=	Number Copulating[a] / Number Cohabited	x 100
Fertility Index (%)	=	Number Bearing Litters[b] / Number Cohabited[a]	x 100
Fecundity Index (%)	=	Number Bearing Litters[b] / Number Copulating[a]	x 100
Gestation Index (%)	=	Number of Litters with at Least One Live Pup / Number of Litters	x 100
Implantation Efficiency (%)[c]	=	Number of Pups Born / Number of Implantation Sites	x 100
Pups Born Alive (%)[c]	=	Number of Pups Born Alive / Number of Pups Born	x 100
Viability Index (%)[c,d]	=	Number of Pups Alive Day 4 Preculling / Number of pups born alive	x 100
Lactation Index (%)[c,d]	=	Number of pups alive at weaning (21 postpartum) / Number of pups alive Day 4 Postculling	x 100

[a] Evidence of copulation = copulatory plug, found dead pregnant, or delivery of a litter.
[b] Including those found dead pregnant during gestation.
[c] To be determined for each litter. Mean and standard deviation for each dose level will be calculated.
[d] Excluding litters sacrificed due to death of dam during lactation.

Statistical Methods

Parameter	Method of Statistical Analysis
Body Weight Body Weight Gain Food Consumption	One-way Analysis of Variance[8]; followed, when significant, with Dunnett's test[9]

Gestation Length	
Organ Weight	Same as above; or nonparametric tests if Bartlett's test for homogeneity of variances is significant (alpha = 0.005)
Hematology	Same as above; or nonparametric tests if Levene's test for homogeneity and Shapiro-Wilk test[10] for normality are significant ($p<0.05$). If the Shapiro- Wilk test is not significant but Levene's test is significant, a robust version of Dunnett's test will be used.
Implantation Site Numbers Implantation Efficiency Mean Number of Pups Per Litter Percent Born Alive Sex Ratio Viability Index Lactation Index Precoital Interval Estrous Cycle Length Sperm Parameters Vaginal Patency Preputial Separation	Jonckheere's test[11]
Mean Pup Weights (Covariates: litter size, sex ratio) Anogenital Distance (Covariate: pup weight)	Linear contrast of the least square means[12]
Incidence of Microscopic Lesions Incidence of Clinical Observations Number of Females Cycling Normally Mating Index Fecundity Index Fertility Index Gestation Index	Cochran-Armitage test for trend or, if not all groups are compared, Fisher's Exact test[13]

For each parameter analyzed with a trend test, the test will be applied to the data sequentially. If a significant dose-response is detected, data from the top dose group will be excluded and the test repeated until no significant trend is detected.[14] For litter parameters, the proportion of affected fetuses per litter or the litter mean will be used as the experimental unit for statistical evaluation.[15] The level of significance selected is $p < 0.05$. Additional statistical tests will be used, and other parameters analyzed, if deemed necessary.

Where the data are tied and the standard large sample version of Jonckheere's test is not applicable, exact p values will be calculated using permutation methodology.[16]

RECORDS AND SAMPLE STORAGE

<>

CRITICAL DATES

In-Life Study Start Date:	<>	
P_1 Adult Sacrifice:	<>	(Approximate)
F_1 Weanling Sacrifice:	<>	(Approximate)
F_1 Adult Sacrifice:	<>	(Approximate)
F_2 Weanling Sacrifice:	<>	(Approximate)
In-Life Completion Date:	<>	

REFERENCES

1. EPA/FIFRA Good Laboratory Practice Standards (40 CFR 160). (1989).

2. OECD Principles of Good Laboratory Practice (C(81)30(Final), Annex 2).

3. MAFF Japan Good Laboratory Practice Standards (59 NohSan No. 3850).

4. EPA/FIFRA Pesticide Assessment Guidelines, Subdivision F; Hazard Evaluation: Human and Domestic Animals. Series 83-4 (PB 86-108958). (1984).

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PROTOCOL APPENDIX I

Animal Identification Marking System (AIMS)

Rats will be identified by a tail tattoo number according to the following numbering system:

P_1 GENERATION

Groups		Tattoo Number[a]		Dietary Concentration
Male	Female	Male	Female	of «Compound» (ppm)
I-0	II-0	101-130	201-230	0 (Control)
III-0	IV-0	301-330	401-430	<> (Low)
V-0	VI-0	501-530	601-630	<> (Intermediate)
VII-0	VIII-0	701-730	801-830	<> (High)

F_1 GENERATION

Groups		Tattoo Number[a,b]		Dietary Concentration
Male	Female	Male	Female	of «Compound» (ppm)
I-1	II-1	101×-130×	201×-230×	0 (Control)
III-1	IV-1	301×-330×	401×-430×	<> (Low)
V-1	VI-1	501×-530×	601×-630×	<> (Intermediate)
VII-1	VIII-1	501×-530×	801×-830×	<> (High)

[a] Each rat will be assigned a unique Animal Number.

[b] F_1 rats will be assigned an ID number based on their P_1 dams' ID number followed by one more digit. For male pups the initial digit will be changed to the odd number corresponding with their group number. The final digit will indicate if it is the first or second pup of a sex selected from a litter. For example, if one male and two females are selected from dam 401 their ID numbers will be 3011, 4011 and 4012, respectively. Each F_1 pup selected will also receive its own animal identification number.

«Title»: Multigeneration Reproduction Study in Rats

Protocol

Prepared by:

«SD», «SDDegree» «SDTitle» Study Director	Date
<> Chemist	Date
<> Staff Pathologist	Date

Approved by:

<> Management	Date

cc:

***Test Substance Name*: Rat Bone Marrow Erythrocyte Micronucleus Test**

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of the test substance as measured by its ability to induce micronucleated polychromatic erythrocytes in rat bone marrow.

2.0 SPONSOR

2.1 Name: <>

2.2 Address: <>

2.3 Representative: <>

2.4 Sponsor Project #: <>

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Substance: <>

3.2 Controls: Vehicle: Test substance vehicle
Positive: Cyclophosphamide (CP)

3.3 Test Substance Characterization

Unless alternate arrangements are made, the testing facility will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test substance, and the stability and strength of the test substance in the solvent (or vehicle).

3.4 Test Substance Retention Sample

The retention of a reserve sample of the test substance will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: <>

4.2 Address: <>

4.3 Study Director: <>

Contain NO CBI

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5.0 PROPOSED STUDY DATES

5.1 Experimental Start Date: <>

5.2 Experimental Termination Date: <>

5.3 Draft Report Date: <>

5.4 Final Report Date <>

6.0 TEST SYSTEM

Closed-colony, random-bred rodents are acceptable models for mutagenicity studies. Sprague-Dawley rats are selected because of the availability of historical control data.

6.1 Source: Harlan Sprague Dawley, Inc., Frederick, MD or
Charles River Breeding Laboratories, Kingston, NY or Raleigh, NC or
other approved alternates

6.2 Age at initiation of study: 6-8 weeks

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The assay will be conducted according to established procedures (Heddle, 1973; Mavournin *et al.*, 1990; Hayashi *et al.*, 1994). Following the administration of three concentrations of test substance as well as positive and negative (vehicle) controls to male and female rats, bone marrow cells will be collected at 24 and 48 hours and examined for the presence of micronucleated polychromatic erythrocytes. The clastogenic potential of the test substance will be measured by its ability to increase micronucleated polychromatic erythrocytes in treated animals as compared to vehicle control animals. The study design will be as follows:

	Number per Sex to be Sacrificed After Dose Administration	
	24 Hr	48 Hr
Vehicle Control		
Vehicle alone	5	5
Test Substance		
Low Dose	5	-
Mid Dose	5	-
High Dose	5	5
Positive Control		
Cyclophosphamide	5	-

7.1 Selection of Test Substance Vehicle

Unless the Sponsor has indicated the test substance vehicle, a solubility determination will be conducted to measure the maximum soluble concentration or workable suspension of test substance in vehicle. Vehicles compatible with this test system, in order of preference, include, but are not limited to, distilled water or saline, 1% carboxymethylcellulose in water, and corn oil. The vehicle of choice will be that allowing preparation of dosing solutions required to achieve targeted doses.

7.2 Dose Selection

Selection of doses for the micronucleus assay will be based on the toxicity of the test substance but will not exceed 2000 mg/kg. In the absence of toxicity data, a pilot study will be performed at a dose of 2000 mg/kg using up to five male and five female rats. Three or more lower doses will be tested using two male rats each. If dose administration produces no treatment-related mortality, the high dose for the micronucleus test will be 2000 mg/kg. In the event of mortality in excess of 50% at 2000 mg/kg in the pilot study, an extensive toxicity study will be performed using up to four test substance doses, each containing up to five male and five female rats. Rats will be observed after dose administration and each working day thereafter for 3 days for clinical signs of chemical effect. Body weights will be recorded prior to dose administration and at 1 and 3 days after dose administration.

Unless specified otherwise by the Sponsor, the high dose for the micronucleus test will be 50% to 80% of the $LD_{50/3}$ (the dose required to kill 50% of the animals within 3 days after administration) but will not exceed 2000 mg/kg. Two additional doses will be tested, one-half and one-fourth of the high dose.

7.3 Route and Frequency of Administration

Animals will be dosed by intraperitoneal (IP) injection. IP injection was selected to maximize delivery of the test substance to the target system. IP injection is an acceptable method for administration of test substance concentrations to laboratory animals. Animals will receive the test substance as a single administration.

7.4 Controls

7.4.1 Vehicle control

The solvent vehicle for the test substance will be used as the vehicle control.

7.4.2 Positive control

Cyclophosphamide monohydrate (CP, CAS number 6055-19-2) will be administered as the positive control at a dose of 30-60 mg/kg. CP will be administered by the same route as the test substance.

7.5 Animal Receipt and Quarantine

Virus antibody-free (VAF) rats will be quarantined for no less than 5 days prior to dose administration. The animals will be observed each working day for signs of illness, unusual food and water consumption, and other general conditions of poor health. All animals will be judged to be healthy prior to utilization in the study.

7.6 Animal Care

Animals will be housed in an AAALAC-accredited facility with a controlled environment of 50 ± 20% relative humidity and 72 ± 3°F with a 12 hour light/dark cycle. Rats of the same sex will be housed up to three per cage in plastic autoclavable cages. Heat-treated hardwood chips will be used for bedding. Animals will have free access to a certified laboratory rodent chow which has been analyzed for environmental contaminants and to tap water.

7.7 Randomization

The animals will be assigned to seven groups of five males and five females each using a randomization procedure based on equalization of group mean body weights. At the time of randomization, the weight variation of animals will not exceed ±20% of the mean weight. Additional animals may be designated and dosed as replacement animals in the high dose group to be used in the event of mortality prior to the scheduled sacrifice. This will be done at the discretion of the Study Director after evaluation of the toxicity data. Each animal will be given a sequential number and identified by ear tag.

7.8 Dose Preparation and Administration

The test substance-vehicle mixture, the vehicle alone and the positive control (CP) will be given as a single administration. The rate of administration will be 10 ml/kg body weight unless larger volumes, up to 20 mL/kg, are required to deliver the targeted dose. All rats in the experimental groups will be weighed and the dose volume will be based on individual body weight.

7.9 Bone Marrow Collection

Twenty-four and 48 hours after dose administration, animals will be sacrificed by carbon dioxide asphyxiation. The positive control group will be sacrificed 24 hours after dose administration. Immediately following sacrifice, the femurs will be exposed, cut just above the knee and the bone marrow will be aspirated into a syringe containing fetal bovine serum. The bone marrow cells will be transferred to a capped centrifuge tube containing approximately 1 mL fetal bovine serum.

The bone marrow cells will be pelleted by centrifugation and the supernatant will be drawn off, leaving a small amount of fetal bovine serum with the remaining cell pellet. The cells will be resuspended by aspiration with a capillary pipette and a

small drop of the bone marrow suspension will be spread onto a clean glass slide. Each slide will be identified by the experiment and animal number. At least two slides will be prepared from each animal, air dried, fixed by dipping in methanol, stained with nucleic acid-specific stain, acridine orange.

7.10 Scoring for Micronuclei

Slides will be coded using a random number table by an individual not involved with the scoring process. Using medium magnification, an area of acceptable quality will be selected such that the cells are well spread and stained. Using oil immersion, 2000 polychromatic erythrocytes will be scored per animal for the presence of micronuclei. The number of micronucleated normocytes in the field of 2000 polychromatic erythrocytes will also be enumerated. The proportion of polychromatic erythrocytes to total erythrocytes will also be recorded per 1000 erythrocytes. The proportion of polychromatic erythrocytes to total erythrocytes in test substance-treated animals should not be less than 20% of the control value.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The mean incidence of micronucleated polychromatic erythrocytes must not exceed 3/1000 (6/2000) polychromatic erythrocytes (0.3%) in the negative (vehicle) control. The incidence of micronucleated polychromatic erythrocytes in the positive control group must be significantly increased relative to the negative control ($p \leq 0.05$, Kastenbaum-Bowman Tables).

9.0 EVALUATION OF TEST RESULTS

The incidence of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes will be presented for each animal and treatment group. Statistical significance will be determined using the Kastenbaum-Bowman tables which are based on the binomial distribution.

In order to quantify the test substance effect on erythropoiesis, as an indicator of bone marrow toxicity, the proportion of polychromatic erythrocytes to total erythrocytes will be presented for each animal and treatment group.

All conclusions will be based on sound scientific judgement; however, as a guide to interpretation of the data, the test substance will be considered to induce a positive response if a dose-responsive increase in micronucleated polychromatic erythrocytes is observed and one or more doses are statistically elevated relative to the vehicle control ($p \leq 0.05$, Kastenbaum-Bowman Tables) at any sampling time. If a single treatment group is significantly elevated at one sacrifice time with no evidence of a dose-response or if there is evidence of a dose response with no evidence of a significant increase in any treated group relative to the control group, the Sponsor will be contacted. The test substance will be judged negative if no statistically significant increase in micronucleated polychromatic erythrocytes above the concurrent vehicle control values and no evidence of dose response are observed at any sampling time.

10.0 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. The report will include:

- Test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of the test substance, if known.

- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of the test substance in the solvent/vehicle, if known.

- Test animals: species and strain of animals used; number, age and sex of animals; source, housing conditions, diet, etc.

- Test conditions: positive and negative (vehicle/solvent) control data; data from range-finding study, if conducted; rationale for dose level selection; details of test substance preparation; details of the administration of test substance; rationale for route of administration; methods for verifying that the test substance reached the general circulation or target tissue, if applicable; details of food and water quality; description of treatment and sampling schedules; method of slide preparation; methods for measurement of toxicity; criteria for scoring micronucleated immature erythrocytes; number of cells analyzed per animal; criteria for considering study as positive, negative or equivocal.

- Results: signs of toxicity; proportion of polychromatic erythrocytes among total erythrocytes; number of micronucleated polychromatic erythrocytes per animal; mean±standard deviation of micronucleated polychromatic erythrocytes per group; dose-response relationship, where possible; statistical analyses; concurrent negative control data; historical negative control data with ranges, means and standard deviations; concurrent positive control data.

- Discussion of results.

- Conclusion.

11.0 RECORDS AND ARCHIVES

All raw data, the protocol and all reports will be maintained according to Standard Operating Procedure by <facility, address>.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline 474 (Genetic Toxicology: Mammalian Erythrocyte Micronucleus Test), adopted 1997, and with the

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (1996 and 1997).

This study will be performed in compliance with the provisions of the EPA (TSCA), OECD, Japanese (MAFF) and EEC Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test substance will be disposed of following finalization of the report.

13.0 REFERENCES

Heddle, J.A. 1973. A rapid in vivo test for chromosomal damage. Mutation Res. 18:187-190.

Hayashi, M., R.R. Tice, J.T. Macgregor, D. Anderson, D.H. Blakey, M. Dirsch-Volders, F.G. Oleson Jr., F. Pacchierotti, F. Romagna, H. Shimada, S. Sutou and B. Vannier. 1994. *In vivo* rodent erythrocyte micronucleus assay. Mutation Res. 312: 293-304.

Mavournin, K.H., D.H. Blakey, M.C. Cimino, M.F. Salamone and J.A. Heddle. 1990. The *in vivo* micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Res. 239:29-80.

M.A. Kastenbaum and K.O. Bowman. 1970. Tables for determining the statistical significance of mutation frequencies. Mutation Res. 9:527-549.

14.0 APPROVAL

_____ _____
Sponsor Representative Date

_____
(Print or Type Name)

_____ _____
Study Director Date

_____ _____
Study Management Date

***Test Substance Name: In Vitro* Mammalian Chromosome Aberration Test**

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of a test substance based upon its ability to induce chromosome aberrations in human peripheral blood lymphocytes (HPBL).

2.0 SPONSOR

2.1 Name: <>

2.2 Address: <>

2.3 Representative: <>

2.4 Sponsor Project #: <>

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Substance Name: <>

3.2 Controls: Solvent: Test Substance Solvent or Vehicle
Positive: Mitomycin C (MMC)
Cyclophosphamide (CP)

3.3 Test Substance Characterization

Unless alternate arrangements are made, the testing facility will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test substance and the stability and strength of the test substance in the solvent (or vehicle).

3.4 Test Substance Retention Sample

The retention of a reserve sample of the test substance will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: <>

4.2 Address: <>

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4.3 Study Director: <>

5.0 PROPOSED STUDY DATES

5.1 Proposed Experimental Start Date: <>

5.2 Proposed Experimental Termination Date: <>

5.3 Proposed Report Date: <>

5.4 Final Report Date: <>

6.0 TEST SYSTEM

Peripheral blood lymphocytes will be obtained from healthy adults without a recent history of either radiotherapy, viral infections or the administration of drugs. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (Preston *et al.*, 1981).

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The test will be conducted using standard procedures, by exposing human lymphocytes to a minimum of four concentrations of the test substance as well as to positive and solvent controls. In the non-activated test system, treatment will be for 4 hours and for 20 hours; in the S9 activated test system, exposure will be for 4 hours (Swierenga et al., 1991). The dividing cells will be arrested in metaphase and harvested for microscopic evaluation of chromosome aberrations at approximately 20 hours (1.5 normal cell cycles) after the initiation of treatment in order to ensure evaluation of first-division metaphase cells (Galloway et al., 1994). The clastogenic potential of the test substance will be measured by its ability to increase chromosome aberrations in a dose-responsive manner when compared to a control group. The 4 hour non-activated and S9-activated studies will be scored initially. In the event of a positive response in the 4 hour non-activated study, the prolonged exposure non-activated study may not be scored. The test substance will also be assessed for its ability to induce numerical chromosome aberrations.

7.1 Solubility Determination

Unless the Sponsor has indicated the test substance solvent, a solubility determination will be conducted to determine the solvent and the maximum soluble concentration up to a maximum of 500 mg/ml. Solvents compatible with this test system, in order of preference, include but are not limited to sterile water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5), and acetone (CAS 67-64-1). The solvent will be the test substance solvent, selected in order of preference, that permits preparation of the highest soluble stock concentration, up to 500 mg/ml.

7.2 Preliminary Toxicity Assay to Select Dose Levels

Selection of the dose levels for the cytogenetics assay will be based upon inhibition of mitosis after treatment as determined in a cytotoxicity study. Cells will be exposed to solvent alone and to at least nine concentrations of test substance, the highest concentration being 5000 µg/ml or 10 mM whichever is lower. The pH of the highest test substance dosing solution will be measured, and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The osmolality of the highest dosing solution, lowest precipitating dose level (where applicable) and the highest soluble dose level (where applicable) will also be measured. Peripheral blood cells will be cultured in RPMI-1640 containing 15% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin and 100 µg streptomycin/ml and 1% phytohemagglutinin. Cells seeded approximately 46 hours earlier will be exposed for 4 hours in the absence and presence of S9 and for 20 hours in the absence of S9. After exposure the cultures will be grown in complete medium for 16 hours. Eighteen hours after treatment initiation Colcemid® (0.1 µg/ml) will be added to the cultures. Cells will be collected at 20 hours after the initiation of treatment by centrifugation, treated with hypotonic KCl solution and fixed with methanol-glacial acetic acid. Metaphase preparations will be made and stained. The percentage of cells in mitosis per 500 cells scored (mitotic index) will be determined for each treatment group.

Whenever possible, the high dose for the chromosome aberration assay will be selected to give at least 50% toxicity (mitotic inhibition relative to the solvent control). At least two additional dose levels, demonstrating minimal or no toxicity, will be evaluated in the chromosome aberration assay. In the event the test substance cannot be dissolved at a high enough concentration in an appropriate solvent to be toxic, then the highest dose to be tested will be the concentration resulting in minimum precipitation in test medium. Precipitation will be determined with the unaided eye. In the event the test substance demonstrates a dose-responsive increase in toxicity (mitotic inhibition relative to the solvent control) at concentrations that exceed solubility in treatment medium, then the highest dose to be evaluated for chromosome aberrations will be the concentration resulting in minimum precipitation in test medium. In the event that neither cytotoxicity nor insolubility is observed in the preliminary test, the highest dose in the chromosome aberration assay will be 5 mg/ml or 10 mM whichever is lower. If excessive precipitation of the test substance-solvent solution occurs upon addition to treatment medium, or if the osmolality of the treatment medium is excessive, the Sponsor will be consulted.

7.3 Frequency and Route of Administration

Target cells will be treated for 4 hours in the absence and presence of S9, and for 20 hours in the absence of S9, by incorporation of the test substance-solvent mixture into the treatment medium. This technique has demonstrated to be an effective method of detection of chemical clastogens in this test system (Evans, 1975).

If the Sponsor is aware of specific metabolic requirements, then this information will be utilized in the preparation of the study design. Verification of a clear positive response is not required. Negative results will not be confirmed when justification can be provided. Equivocal results may be confirmed, upon consultation with the Sponsor, and may employ a modification of the study design. This guidance is based on the OECD Guideline 473 (adopted July 1997) and ICH Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals (1996).

7.4 Controls

7.4.1 Solvent (or Vehicle) Control

The solvent for the test substance will be used as the solvent control. For solvents other than water, physiological buffer, or medium, the final concentration in treatment medium will not exceed 1%.

7.4.2 Positive Controls

Mitomycin C will be used at two concentrations within the range of 0.1-0.50 µg/ml as the positive control for the non-activated test system. For the S9-activated system, cyclophosphamide will be used at two concentrations within the range of 10-75 µg/ml. One dose level of each positive control will be evaluated microscopically for chromosome damage.

7.5 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used. Each batch preparation of S9 will be assayed for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool. The final concentration of the cofactors and S9 in the reaction vessel will be 2 mM $MgCl_2$, 6 mM KCl, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate and 20 µl S9 per ml RPMI-1640 serum-free medium.

7.6 Preparation of Target Cells

Peripheral blood lymphocytes will be cultured in complete medium (RPMI-1640 containing 15% fetal bovine serum, 2mM L-glutamine, 100 units penicillin and 100 µg streptomycin/ml, and 1% phytohemagglutinin) by adding 0.6 ml heparinized blood to a centrifuge tube containing 9.4 ml complete medium. The tubes will be

incubated upright at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air for 44-48 hours.

7.7 Test System Identification

Using a permanent marking pen, the test system will be identified by the BioReliance study number, treatment condition and date.

7.8 Treatment of Target Cells

Forty-four to 48 hours after culture initiation, duplicate centrifuge tubes will be refed with approximately 10 ml complete medium for the non-activated exposure or 10 ml S9 reaction mixture for the activated exposure to which will be added 100 µl of dosing solution of test or control substance in solvent. Larger volumes of dosing solution may be used if water or medium is used as the solvent.

For the S9-activated exposure, the cells will be treated for 4 hours in the presence of an S9 reaction mixture, washed free of chemical and cultured for an additional 16 hours with Colcemid® (0.1 µg/ml) present for the last 2 hours. For the non-activated exposure, treatment will be for 4 hours followed by a 16 hour recovery period and for 20 hours continuously with Colcemid® (0.1 µg/ml) present for the last 2 hours.

7.9 Collection of Metaphase Cells

Cells will be collected approximately 20 hours after initiation of treatment (about 64-68 hours after culture initiation). This time is selected to represent the first-division metaphase after initiation of test substance treatment. Two hours prior to harvest, Colcemid® will be added to the cultures at a final concentration of 0.1 µg/ml. The cells will be collected by centrifugation, treated with 0.075M KCl, washed with two changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately 2-8°C. To prepare slides, the cells will be collected by centrifugation and resuspended in fresh fixative. An aliquot of fixed cells will be applied dropwise onto a microscope slide and air-dried. The slide will be identified by the experiment number, treatment condition and date. At least two slides will be prepared from each treatment tube. The slides will be stained with Giemsa and permanently mounted.

7.10 Scoring for Metaphase Aberrations

Slides will be coded using random numbers by an individual not involved with the scoring process. At least 3 dose levels in each harvest will be evaluated in the aberration assay and will be selected according to the criteria described in section 7.2. The 4 hour non-activated and S9-activated studies will be scored initially. In the event of a positive response in 4 hour non-activated study, slides from the extended non-activated exposure may not be scored. Metaphase cells will be examined under oil immersion without prior knowledge of treatment groups.

Whenever possible, a minimum of 200 metaphase spreads containing 46 centromeres from each dose level (100 per duplicate treatment tube) will be examined and scored for chromatid-type and chromosome-type aberrations (Scott et al., 1990). The number of metaphase spreads that will be examined and scored per duplicate flask may be reduced if the percentage of aberrant cells reaches a statistically significant level before 100 cells are scored. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥10 aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with a structural aberration or gap will be recorded using a calibrated microscope stage. The mitotic index will be recorded as the percentage of cells in mitosis per 500 cells counted. The percent polyploid and endoreduplicated cells will be evaluated per 100 metaphase cells for each dose level analyzed for structural aberrations.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Solvent Controls

The frequency of cells with structural chromosome aberrations in the solvent controls must be within the range of the historical solvent control.

8.2 Positive Controls

The percentage of cells with aberrations must be statistically increased ($p \leq 0.05$, Fisher's exact test) in the positive control relative to the solvent control.

9.0 EVALUATION OF TEST RESULTS

The toxic effects of treatment are based upon inhibition of mitosis and will be reported for the cytotoxicity and chromosome aberration study. The number and types of aberrations (structural and numerical) found, the percentage of structurally damaged cells in the total population of cells examined (percent aberrant cells), the percentage of numerically damaged cells in the total population of cells examined, and the average number of structural aberrations per cell (mean aberrations per cell) will be calculated and reported for each treatment group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the average number of aberrations per cell.

Statistical analysis of the percentage of aberrant cells will be performed using the Fisher's exact test. The Fisher's test will be used to compare pairwise the percent aberrant cells of

each treatment group with that of the solvent control. In the event of a positive Fisher's exact test at any test substance dose level, the Cochran-Armitage test will be used to measure dose-responsiveness.

All conclusions will be based on sound scientific judgement; however, as a guide to interpretation of the data, the test substance will be considered to induce a positive response if the percent aberrant cells is increased in a dose-responsive manner with one or more concentrations being statistically significant ($p \leq 0.05$). A reproducible significant increase at the high dose only with no dose response or a reproducible significant increase at one dose level other than the high dose with no dose response will be considered positive. Test substances not demonstrating a statistically significant increase in aberrations will be concluded to be negative.

10.0 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.
Results presented will include, but not be limited to:

- Test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test substance, if known.

- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test substance in solvent/vehicle, if known.

- source of cells and time the cells were obtained, karyotype features (modal chromosome number) and suitability of the cell type used.

- test conditions: composition of medium; CO_2 concentration; incubation time; solvent and solvent selection rationale; concentration of test substance and concentration selection rationale; composition and acceptability criteria for the metabolic activation (S9) system; duration of treatment; duration of treatment with and concentration of Colcemid®; type of metabolic activation system used; positive and solvent controls; methods of slide preparation; number of cell cultures; criteria for scoring aberrations and criteria for considering studies positive, negative or equivocal.

- results: description of precipitation; pH and osmolality of the treatment medium; mitotic inhibition relative to the solvent control; mitotic index and number of metaphases analyzed; type and number of aberrations (structural and numerical) given separately for each treated and control culture; concentration-response relationship; statistical analysis; historical control data

11.0 RECORDS AND ARCHIVES

All raw data, the protocol and all reports will be maintained according to Standard Operating Procedure by the <facility, address>.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline 473 for Testing of Chemicals (Genetic Toxicology: *In Vitro* Mammalian Chromosome Aberration Test), adopted 1997, and with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (1996 and 1997).

This study will be performed in compliance with the provisions of the EPA (TSCA), OECD, Japanese (MAFF) and EEC Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test substance will be disposed of following finalization of the report.

13.0 REFERENCES

Evans, H.J. and M.L. O'Riordan. 1975. Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. Mutation Res. 31:135-148.

Galloway, S.M., M.J. Aardema, M. Ishidate Jr., J.L. Ivett, D.J. Kirkland, T. Morita, P. Mosesso and T. Sofuni (1994) Report from working group on in vitro tests for chromosomal aberrations, Mutation Research 312(3):241-261.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. S2A document recommended for adoption at step 4 of the ICH process on July 19, 1995. Federal Register 61:18198-18202, April 24, 1996.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. S2B document recommended for adoption at step 4 of the ICH process on July 16, 1997. Federal Register 62:16026-16030, November 21, 1997.

OECD Guideline for the Testing of Chemicals, Guideline 473 (*In Vitro* Mammalian Chromosome Aberration Test), Revised Draft Document, Adopted July 1997.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom. 1981. Mammalian *in vivo* and *in vitro* cytogenetics assays: A report of the US EPA's Gene-Tox Program. Mutation Res. 87:143-188.

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14.0 APPROVAL

_____	_____
Sponsor Representative	Date
_____	
(Print or Type Name)	
_____	_____
Study Director	Date
_____	_____
Study Management	Date

Test Substance Name: Bacterial Reverse Mutation Test with an Independent Repeat Assay

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of Salmonella typhimurium and at the tryptophan locus of Escherichia coli WP2 uvrA in the presence and absence of S9 activation.

2.0 SPONSOR

2.1 Name: <>

2.2 Address: <>

2.3 Representative: <>

2.4 Sponsor Project No.: <>

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Substance Name: <>

3.2 Test Substance I.D.: <> (to be used in the report title and text)

3.3 Controls:

Negative:	Test substance vehicle
Positive:	9-aminoacridine
	2-aminoanthracene
	methyl methanesulfonate
	2-nitrofluorene
	sodium azide

3.4 Test Substance Characterization

Unless alternate arrangements are made, the testing facility will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test substance, and the stability and strength of the test substance in the solvent (or vehicle).

Contain NO CBI

3.5 Test Substance Retention Sample

The retention of a reserve sample of the test substance will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

Name: <>

Address: <>

Study Director: <>

5.0 PROPOSED STUDY DATES

5.1 Experimental Start Date: <>

5.2 Experimental Termination Date: <>

5.3 Draft Report Date: <>

5.4 Final Report Date: <>

6.0 TEST SYSTEM

The tester strains will include the S. typhimurium histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames et al. (1975) and the E. coli tester strain WP2 uvrA as described by Green and Muriel (1976).

Genotype of the Strains Used for Mutagen Testing						
Histidine Mutation			Tryptophan Mutation	Additional Mutations		
hisG46	hisC3076	HisD3052	TrpE	LPS	Repair	R-factor
TA1535	TA1537	-	-	Rfa	ΔuvrB	-
TA100	-	TA98	-	Rfa	ΔuvrB	+R
-	-	-	WP2 uvrA	-	ΔuvrA	-

Each S. typhimurium tester strain contains, in addition to a mutation in the histidine operon, additional mutations that enhance sensitivity to some mutagens. The rfa mutation results in a cell wall deficiency that increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded. The deletion in the uvrB gene results in a deficient DNA excision-repair system. Tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R-factor). It has been suggested that the plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA100 is reverted by both frameshift and base substitution mutagens and TA1535 is reverted only by mutagens that cause base substitutions.

The E. coli tester strain has an AT base pair at the critical mutation site within the trpE gene (Wilcox et al., 1990). Tester strain WP2 uvrA has a deletion in the uvrA gene resulting in a deficient DNA excision-repair system. Tryptophan revertants can arise due to a base change at the originally mutated site or by a base change elsewhere in the chromosome causing the original mutation to be suppressed. Thus, the specificity of the reversion mechanism is sensitive to base-pair substitution mutations (Green and Muriel, 1976).

The S. typhimurium tester strains were received directly from Dr. Bruce Ames, University of California, Berkeley. The E. coli tester strain was received from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom).

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The test substance will be tested at a minimum of five dose levels along with appropriate negative and positive controls with tester strains TA98, TA100, TA1535, TA1537 and WP2 uvrA with and without S9 activation. All dose levels of test substance, negative controls and positive controls will be plated in triplicate.

7.1 Solubility Determination

A solubility determination will be conducted to determine the maximum soluble concentration or workable suspension up to a maximum of 500 mg/mL. Vehicles compatible with this test system, in order of preference, include but are not limited to: deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice will be the solvent, selected in order of preference, which permits preparation of the highest workable/soluble stock concentration, up to 500 mg/mL.

7.2 Preliminary Toxicity Assay to Select Dose Levels

Selection of dose levels for the mutagenicity assay will be based upon the toxicity and precipitation profile of the test substance assessed in a preliminary toxicity assay. This preliminary assay will be conducted by exposing TA98, TA100, TA1535, TA1537 and WP2 *uvr*A to negative controls and to at least eight concentrations of test substance, one plate per dose level, in both the presence and absence of S9 activation. Unless indicated otherwise by the Sponsor, the highest dose will be the highest workable concentration in the vehicle of choice but not to exceed 5 mg/plate. In selecting dose levels for the mutagenicity assay the following guidelines will be employed. Doses will be selected such that precipitate

does not interfere with manual scoring. Whenever possible, the highest dose for the mutagenicity assay will be selected to give some indication of toxicity without exceeding 5 mg/plate. For freely soluble, nontoxic test substances, the highest dose level will be 5 mg/plate. For precipitating, nontoxic test substances, the highest dose level will be selected in an attempt to yield precipitate at only the top one or two dose levels. The Sponsor will be consulted regarding dose selection if (1) the maximum dose level is selected based on precipitation and this dose level is less than 5 mg/plate or (2) the maximum achievable test substance dose level is less than 5 mg/plate and this dose level is nontoxic.

7.3 Frequency and Route of Administration

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

After the data generated in the first assay have been evaluated, the mutagenicity assay will be repeated. The dose levels used in the second assay will be the same as those used in the first assay unless the Study Director determines that the dose levels should be changed due to an equivocal response, excessive cytotoxicity or excessive precipitate. If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay. (e.g., activation system or treatment method). This guidance is based on the OECD Guideline 471 (adopted July 1997) and ICH Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals (1997).

7.4 Controls

7.4.1 Positive Controls

All combinations of positive controls and tester strains plated concurrently with the assay are listed below:

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Strain	S9 Activation	Positive Control	Concentration (µg/plate)
Salmonella Strains	Rat	2-aminoanthracene	1.0
WP2 uvrA			10
TA98	None	2-nitrofluorene	1.0
TA100, TA1535		sodium azide	1.0
TA1537		9-aminoacridine	75
WP2 uvrA		methyl methanesulfonate	1,000

7.4.2 Negative Controls

Appropriate negative controls will be plated for each tester strain with and without S9 activation. The negative control will be the vehicle alone, unless there is no historical basis for use of the selected vehicle. In the latter case, both untreated and vehicle controls will be used.

7.4.3 Sterility Controls

The most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

7.5 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 homogenate will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used. Each batch of S9 homogenate will be assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenzanthracene to forms mutagenic to *S. typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 10% S9 homogenate, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. This mixture is referred to as S9 mix. Sham mix will be 100 mM phosphate buffer at pH 7.4.

7.6 Preparation of Tester Strains

Overnight cultures will be inoculated from the appropriate master plate or from the appropriate frozen stock. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. At the end of the working day, each inoculated flask will be placed in a resting shaker/incubator at room temperature. The shaker/incubator will be programmed to begin shaking at

approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

7.7 Test System Identification

Each plate will be labeled with a code system that identifies the test substance, test phase, dose level, tester strain and activation type as described in standard operating procedures.

7.8 Test Substance Preparation

Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under yellow light.

7.9 Treatment of Test System

One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45 ± 2°C. When necessary to achieve the target concentration or eliminate toxic vehicle effects, aliquots of other than 50 µL of test substance/vehicle/positive control will be plated. The mixture will be vortex mixed and overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay has solidified, the plates will be inverted and incubated for approximately 48 to 72 hours at 37 ± 2°C. Plates that are not counted immediately following the incubation period will be stored at 2 - 8°C.

7.10 Scoring

The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the negative control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification.

7.11 Tester Strain Verification

On the day of use in the mutagenicity assay, all tester strain cultures will be checked for the appropriate genetic markers cited in §6.0.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the mutagenicity assay to be considered valid:

8.1 Tester Strain Integrity

To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

8.2 Spontaneous Revertant Background Frequency

Based on historical control data, all tester strain cultures must exhibit characteristic number of spontaneous revertants per plate in the negative controls (vehicle). The mean revertants per plate must be within the following ranges (inclusive): TA98, 10 - 50; TA100, 80 - 240; TA1535, 5 - 45; TA1537, 3 - 21; WP2 *uvr*A, 10 - 60.

8.3 Tester Strain Titers

To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

8.4 Positive Control Values

Each mean positive control value must exhibit at least a three fold increase over the respective mean negative control value (vehicle) for each tester strain.

8.5 Toxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean negative control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

9.0 EVALUATION OF TEST RESULTS

For a test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

9.1 Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than three times the mean negative control value (vehicle).

9.2 Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean negative control value (vehicle).

10.0 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. The report will include:

- Test Substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test substance, if known.
- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test substance in solvent/vehicle, if known.
- Strains: strains used; number of cells/mL per culture; strain characteristics.
- Test conditions: amount of test substance per plate with rationale for dose selection and number of plates per concentration; media used; type and composition of metabolic activation system, including acceptability criteria; treatment procedures.
- Results: signs of toxicity; signs of precipitation; individual plate counts; the mean number of revertant colonies per plate and standard deviation; dose-response relationship, where possible; statistical analysis, if any; concurrent negative and positive control data means and standard deviations; historical negative and positive control data with ranges, means and standard deviation.
- Discussion of results.
- Conclusion.

11.0 RECORDS AND ARCHIVES

All raw data, the protocol and all reports will be maintained according to Standard Operating Procedure by the <facility name, address>.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline 471 for Testing of Chemicals (Genetic Toxicology: Bacterial Reverse Mutation Assay), adopted 1997 and

with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (1996 and 1997).

This study will be performed in compliance with the provisions of the EPA (TSCA), OECD, Japanese (MAFF) and EEC Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test substance will be disposed of following finalization of the report.

13.0 REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp^+ reversion in *Escherichia coli*. Mutation Research 38:3-32.

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McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

OECD Guideline 471 for Testing of Chemicals (Genetic Toxicology: Bacterial Reverse Mutation Assay), Adopted July 1997.

Wilcox, P., Naidoo, A., Wedd, D.J. and Gatehouse, D.G. (1990). Comparison of *Salmonella typhimurium* TA102 with *Escherichia coli* WP2 tester strains. Mutagenesis 5:285-291.

14.0 APPROVAL

_____	_____
Sponsor Representative	Date
_____	
(Print or Type Name)	
_____	_____
Study Director	Date
_____	_____
Study Management	Date

«Title»: Developmental Toxicity Study in Rats

Protocol

Contain NO CBI

TABLE OF CONTENTS

OBJECTIVE

The purpose of this study is to evaluate the developmental toxicity of «Title» («Compound»), administered by gavage to pregnant rats from the time of implantation to the end of gestation.

This study will conform with applicable Good Laboratory Practice Standards[1-4] and Test Guidelines.[5-8]

SPONSOR AND TEST FACILITY

This study is sponsored by <sponsor name> and will be conducted at <laboratory, location>. Sponsor approval of the study will be indicated by issuance of the work authorization form.

MATERIALS AND METHODS

A. Test Substance

The test substance will be identified in this protocol and in the study records by «Compound» or by the Sample Number. The test substance will be supplied by <sponsor>. <Sponsor> is responsible for characterization of the test substance including the items cited in CFR 40, Part 792, Subpart F, section 792.105(a). Available information on the purity, composition, contaminants, synonyms, CAS registry number, basic physical properties, hazards, and hazardous material classification(s) will be provided by <sponsor> on the sample evaluation form. The original form will be retained in the official file for the study.

B. Vehicle

The vehicle will be 0.5% methyl cellulose, U.S.P., 4000 centipoises, C.A.S. Reg. No. 9004-67-5.

C. Animals

The Crl:CD®(SD)IGS BR rat was selected for this study because it is a preferred species for developmental toxicity testing as recommended by the guidelines. The Crl:CD®(SD)IGS BR strain was chosen because extensive background information is available from the literature, the supplier, and previous studies with other compounds. This strain is also considered suitable relative to hardiness and incidence of spontaneous disease.

One hundred thirty nulliparous, time-mated females, will be received on <> and <>, <year> from Charles River Laboratories, Inc. Body weights on the day the rats are mated, day 0 of gestation (day 0G) will be supplied by the vendor. The rats for this study will be requested to be at either 1, 2, or 3 days of gestation upon receipt, and between 51 to 70 days old.

Each animal is assigned a unique number and identified with an ear punch/notch by the supplier prior to shipping. Upon receipt, each animal will be assigned a unique animal number. Both the animal number and the ear punch/notch number will be recorded on each cage card. A master list of unique animal numbers and corresponding unique ear punch/notch numbers will be maintained with the study records.

D. Husbandry

Animal rooms will be maintained at an acceptable temperature of 20-26°C (targeted at 23°C) and a targeted relative humidity of 50% ±10%. Animal rooms will be artificially illuminated (fluorescent light) on a 12-hour light/dark cycle (approximately 0600-1800 hours). Animals will be housed individually in suspended, wire-mesh, stainless steel cages. Nesting material will not be provided because the dams will be euthanized prior to parturition. PMI Nutrition International, Inc. Certified Rodent LabDiet® 5002 will be available *ad libitum*. Water from <> will be available *ad libitum*.

<> Laboratory has an animal health monitoring program. The following procedures are performed periodically:

- Water samples are analyzed for total bacterial counts, and the presence of coliforms, lead, and other contaminants.
- Food samples are analyzed for the presence of bacteria and fungi.
- Samples from freshly washed cages and cage racks are analyzed to ensure adequate sanitation by the cagewashers.

Certified animal feed is used, guaranteed by the manufacturer to meet specified nutritional requirements and to be free of impurities which might influence the results of the study.

The animal health monitoring program is administered by the Laboratory Animal Veterinarian. Data are maintained separately from study records and will not be included in the final report unless warranted by the results of these evaluations.

Rats will be quarantined for at least 3 days, and then released for the study upon approval of the Laboratory Animal Veterinarian or a designee.

E. Experimental Design

The experimental design is shown below:

Group	Dose[a] (mg/kg/day)	Concentration[b] (mg/mL)	Mated Females[c]
I	0[d]	0.0	25
II	<>	<>	25
III	<>	<>	25
IV	<>	<>	25
V	<>	<>	25

[a] «Compound», administered once daily, by gavage, on days 6-20 of gestation, at a dosage volume of <> mL/kg

[b] To achieve these concentrations of active ingredient, the solutions will be adjusted for a purity of <> %.
[c] Copulation confirmed
[d] Vehicle only (0.5% methyl cellulose)

F. Selection of Dose Levels

The dose levels for the current study were based on <>.

G. Randomization

Dams will be assigned to lots according to their gestation day. Then, they will be ranked on the basis of day 0 gestation body weights and assigned to control and experimental groups by random sampling from the ranked list. The distribution should result in mean body weights for all groups that are not statistically different ($p>0.05$). Dams that lose excessive weight or appear ill prior to the start of dosing will be removed from the study.

H. Preparation, Administration, and Analysis of Test Solutions

Solutions of the test material in the vehicle will be prepared daily. The method of mixing the test material with the vehicle will be documented in the study records.

«Compound» will be administered by gavage because the oral route is the route which is recommended by regulatory agencies for this type of study. The volume administered will be based on the most recent body weight.

Samples (~3 mL each) of each test suspension will be taken three times during the study: near the beginning, the middle, and the end of the dosing period. Analysis of the first sampling will address homogeneity, concentration, and stability. For the second and third samplings, analyses will address concentration. Additional samples may be taken and/or analyzed at the discretion of the study director. Samples will be submitted, shortly after preparation, to the Analytical Group. On days samples are taken, the solutions remaining after dosing will be frozen for additional analysis, if required. Frozen solutions will be discarded if no additional analysis is necessary. The analytical method used will be documented in the study records.

I. Safety Precautions and Disposal of Waste Material

Good housekeeping procedures will be practiced to avoid potential health hazards and contamination of formulation facilities. To avoid skin contact, gloves will be worn when handling either the test substance or test solutions. In addition, the test substance will be handled in a chemical hood. Dosing solutions will be prepared in properly ventilated areas. Animal carcasses, feces, and unused dosing solutions will be incinerated.

J. Animal Euthanasia

Females will be euthanized by carbon dioxide inhalation. Fetuses will be decapitated before proceeding with visceral examinations; those to be viscerally examined without decapitation will be injected with sodium pentobarbital. All other fetuses also will be injected with sodium pentobarbital before fixation.

K. Parameters to be Studied

1. In-life Observations of Females

Body weights will be recorded at least twice prior to dosing to provide data for quarantine release, and on days 6-21G. Food consumption will be measured on days 4, 6, 8, 10, 12, 14, 16, 18, 20, and 21G. Clinical signs will be recorded at least twice prior to dosing to provide data for quarantine release; signs will be recorded once daily on days 4-21G and twice daily on days 6-20G. Clinical signs observed at other times will be recorded by exception.

2. Postmortem Observations of Females Dying Prior to Scheduled Euthanasia

A gross external and visceral examination will be performed within 24 hours after the female is found dead. Rats found dead will be refrigerated until necropsied.

Pregnancy status will be determined by the presence or absence of implantation sites. If possible, the sites will be counted and classified as apparently alive or resorbing at the time of the dam's death.

3. Postmortem Observations of Dams Delivering Early

Dams that deliver before scheduled euthanasia will be euthanized within 24 hours of detection and a gross external and visceral examination will be performed. Lesions noted will be retained for further examination at the discretion of the study director or a designee. Corpora lutea counts and the number of visible implantation sites will be recorded.

Fetuses remaining in the uterus and those delivered early will be examined to the extent possible, but the collected data will be recorded with the maternal gross postmortem findings so as to easily permit exclusion of the data from all calculations performed for live fetuses.

4. Postmortem Observations of Females Surviving to Scheduled Euthanasia (Day 21G)

Viscera will be examined grossly immediately after euthanasia. Lesions noted will be retained for further examination at the discretion of the study director or a designee. The intact and the empty uterus of each dam having at least one viable fetus will be weighed to permit calculation of maternal body weight adjusted to exclude the products of conception. The corpora lutea count for each ovary of females with viable fetuses will be recorded.

For each female with visible implantation sites, the types of implants (live and dead fetuses, and resorptions) and their relative positions will be recorded. The uterus of each apparently "nonpregnant" female will be stained with ammonium sulfide[9] to detect very early resorptions; counts will be recorded. Data from females determined to be pregnant by staining will be used only to calculate the incidence of pregnancy and the number of females with total resorptions.

5. Fetuses of Females Surviving to Scheduled Euthanasia

The intrauterine location of each fetus will be recorded. The sex and body weight of each fetus will be recorded. The external alterations detected for each live fetus will be recorded. For each litter, the first live fetus and every other live fetus thereafter will be examined for visceral alterations.[10] Retarded renal development will be classified using the scheme of Woo and Hoar.[11] In addition, all live fetuses with malformations visible at external examination will be examined for soft tissue alterations; decapitation of these fetuses will be at the discretion of the study director or a designee. After fixation in Bouin's fixative, the heads of decapitated fetuses will be examined and alterations will be recorded. Examinations will be based on the method of Barrow and Taylor.[12] After alcohol fixation, the alizarin-stained skeletons will be examined and skeletal alterations will be recorded for each live fetus.

Those fetuses classified as dead will be examined externally, viscerally, and skeletally. Data for these fetuses will be excluded from all calculations performed for live fetuses.

Fetal organs or tissues will be retained if deemed necessary for obtaining a definitive diagnosis. Bilateral organs will be retained when only one is affected. Sufficient tissues from control fetuses will be fixed to provide comparison with suspect tissues, if needed; tissues will be discarded if not needed.

L. Control of Bias

In addition to random assignment to groups, all females will be coded before scheduled euthanasia and will remain coded during the collection of postmortem and fetal data.

M. Statistics

Sequential two-tailed trend testing[13] will be applied to the data for each parameter as tabulated below. If a significant dose-response is detected, data from the top dose group will be excluded and the test repeated until no significant trend is detected. For litter parameters, the proportion of affected fetuses per litter or the litter mean will be used as the experimental unit for statistical evaluation.[14] The level of significance selected is $p < 0.05$. Additional statistical tests may be used, and other parameters analyzed, if deemed necessary.

Where the data are tied and the standard large sample version of Jonckheere's test is not applicable, exact p values will be calculated using permutation methodology.[15]

Parameter	Trend Test
Maternal weight Maternal weight change Maternal food consumption	Linear contrast of means[16]
Live fetuses Dead fetuses	Jonckheere's test[17]

Resorptions Implantations Incidence of fetal alterations	
Incidence of pregnancy Clinical observations Maternal mortality Females with total resorptions Early deliveries	Cochran-Armitage test[16]
Fetal weight (Covariates: litter size, sex ratio) Sex ratio (Covariate: litter size)	Linear contrast of least square means[18]

N. Absorption of Test and Control Substances

Determination of the degree of absorption of the test and control substances by the test system is not necessary to achieve the objectives of the study.

O. Records and Sample Storage

Specimens (if applicable), raw data, and the final report will be retained at <>.

CRITICAL DATES

Study Start (first day 6G):	<>
Completion (last fetal evaluation):	<> (approximate)
Final Report:	<>

REFERENCES

1. Good Laboratory Practice Standards, U.S. Environmental Protection Agency (EPA), Federal Register 54, No. 158 (Toxic Substances Control; 40 CFR Part 792), pp 34043-34050 (1989).

2. U.S. Environmental Protection Agency (EPA), Good Laboratory Practice Standards (Federal Insecticide, Fungicide and Rodenticide Act), 40 CFR 160 (1989).

3. Organisation for Economic Cooperation and Development, Good Laboratory Practice Standards. ISBN 92-64-12367-9, Paris (1982).

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«Title»: Developmental Toxicity Study in Rats

Protocol

Prepared by:

«SD», «SDDegree»
«SDTitle»
Study Director

Date

<>
Chemist

Date

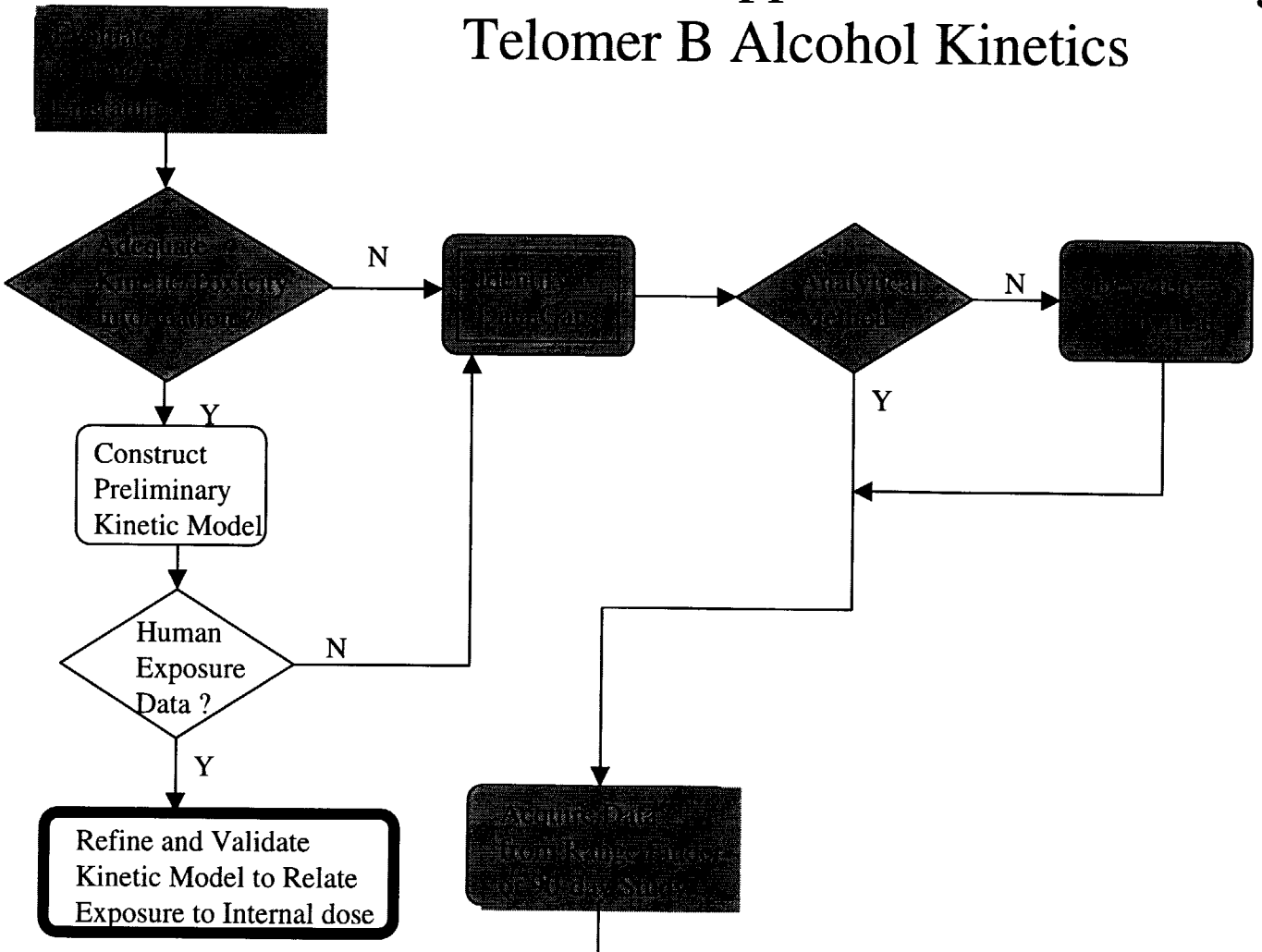
Approved by:

<>
<Director>

Date

cc:

Modified Approach for Describing Telomer B Alcohol Kinetics



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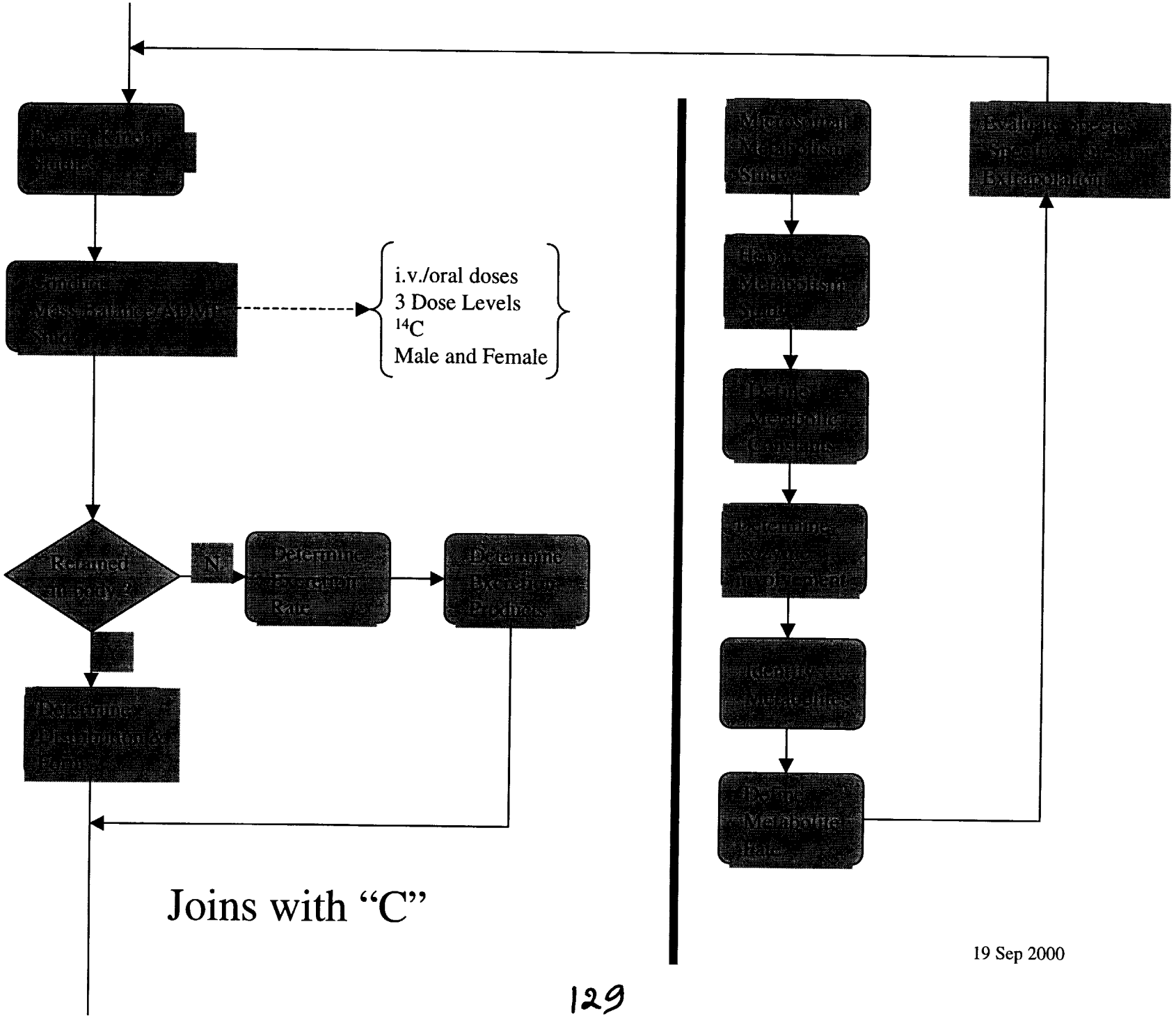

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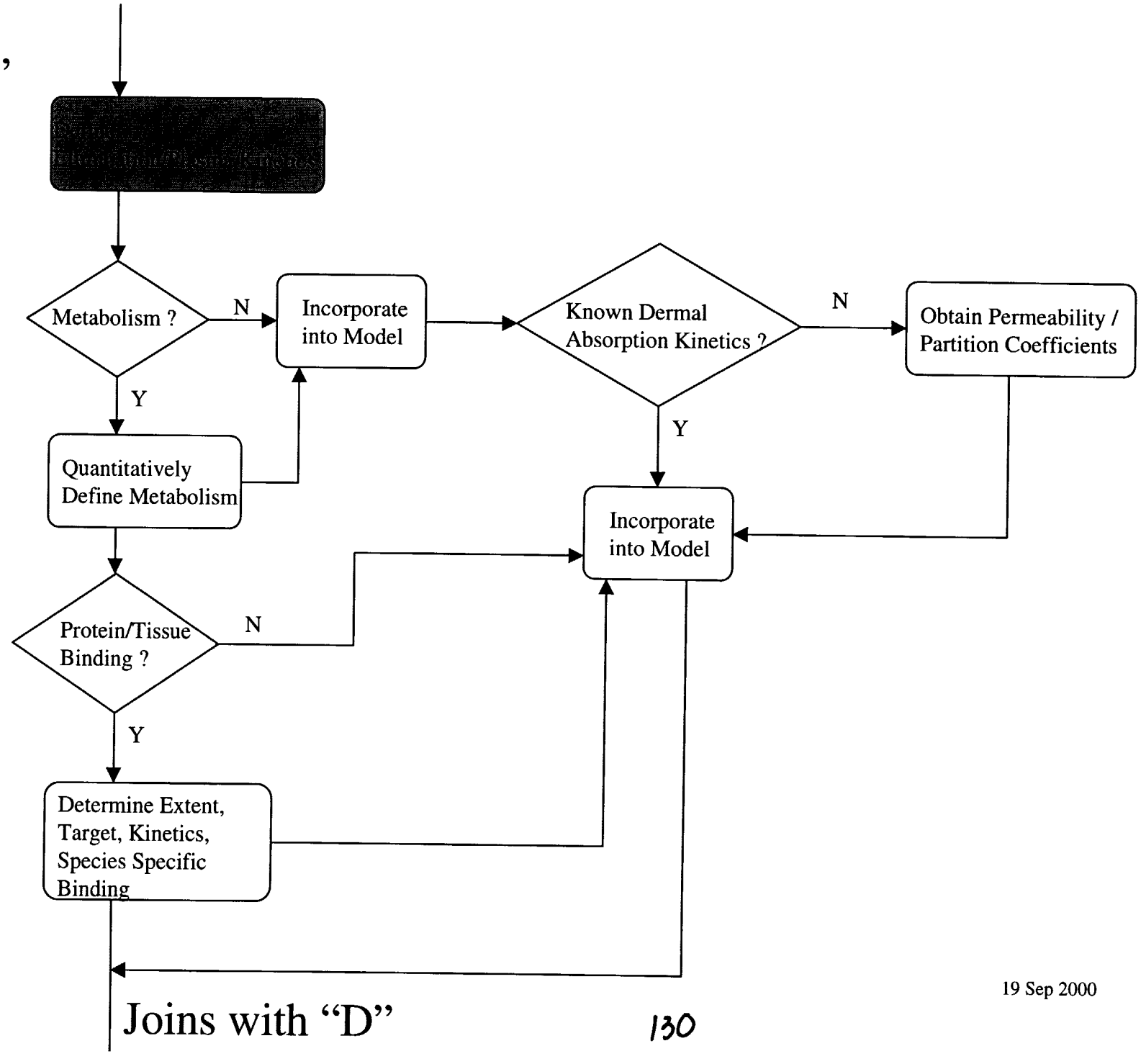

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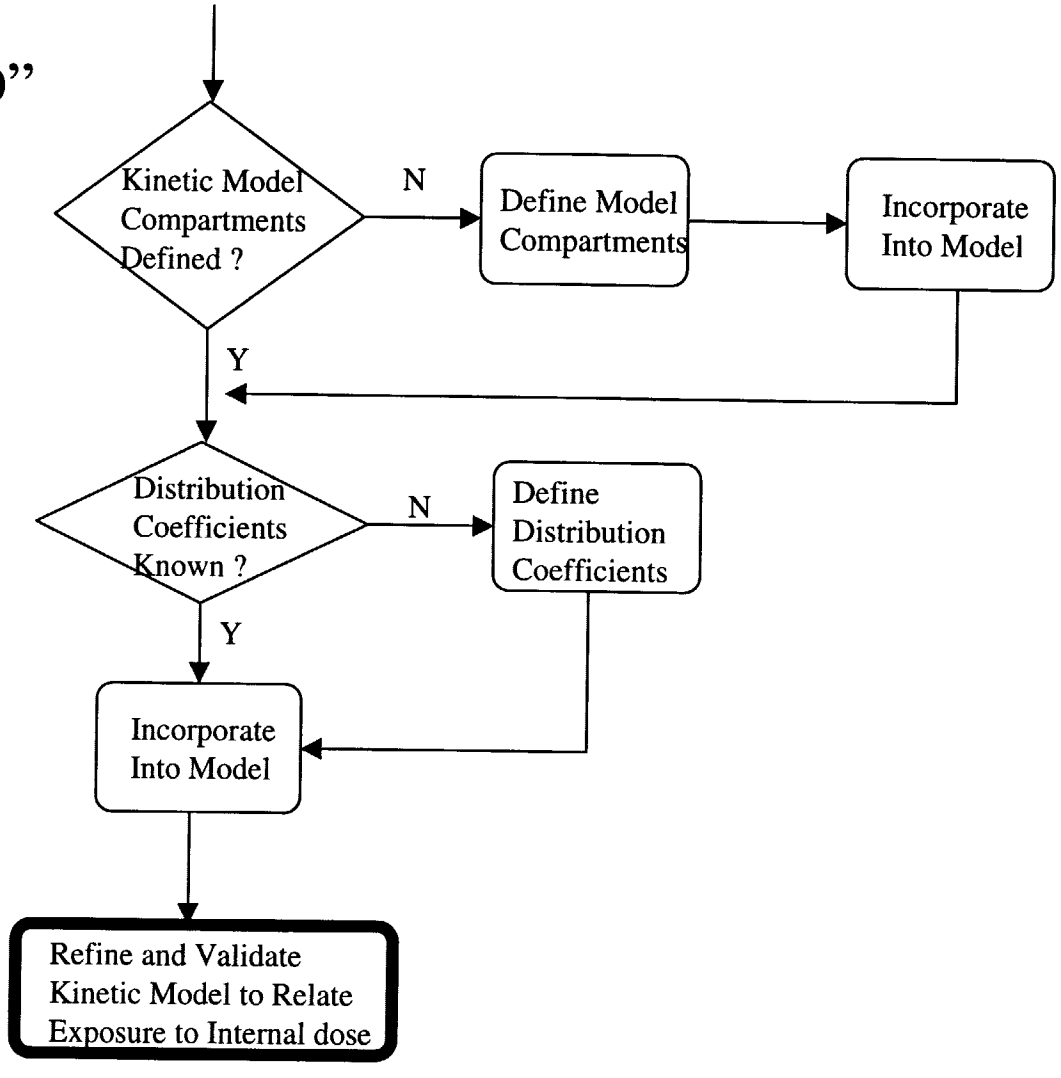
Kinetic Model Compartments Defined ?
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Define Model Compartments
Incorporate Into Model
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Distribution Coefficients Known ?
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Define Distribution Coefficients
Y
Incorporate Into Model
Refine and Validate Kinetic Model to Relate Exposure to Internal dose

In Vitro Microsomal Metabolism Study

Objective:

The objective of this study type is to determine the rate of metabolism of a test compound in microsomes prepared from livers of a number of species (normally rat, rabbit, dog, monkey, and human) and to identify the metabolites produced in each species.

Methods:

First, it is necessary to determine linearity of reaction rate with protein concentration. To do this, reactions are run with protein concentrations in the range of 0.5-3 mg/mL protein while holding the starting concentration and reaction time constant for each species. Reactions are carried out at 37° C. The reaction medium consists of an NADPH regeneration system, cofactors, microsomes, and phosphate buffer, pH 7.4. Test compound is added to the reaction medium to achieve a final concentration of 1-100 uM, determined by pilot studies. The reaction is started by the addition of NADP solution and incubated for a constant time, determined in pilot studies. The reaction is terminated by the addition of either organic solvent or strong acid. The test samples are then partially purified by centrifugation, solid phase extraction, or both. The samples are then analyzed by HPLC-MS.

Next, the kinetics of the test compound are determined. To do this, reactions are run at a constant initial concentration of test substance and a constant protein concentration. Reactions are carried out at 37° C. The reaction medium consists of an NADPH regeneration system, cofactors, microsomes, and phosphate buffer, pH 7.4. Test compound is added to the reaction medium to achieve a final concentration of 1-100 uM, determined by pilot studies. The reaction is started by the addition of NADP solution and the reactions are terminated at various time points, determined in pilot studies. The reaction is terminated by the addition of either organic solvent or strong acid. The test samples are then partially purified by centrifugation, solid phase extraction, or both. The samples are then analyzed by HPLC-MS.

The concentration of each unknown sample is calculated using a standard curve. The resulting concentrations (in units of μM) are plotted on a semi-logarithimic plot against time in minutes. An exponential regression line with the general equation $y = ce^{bx}$ where c is the y intercept and b is the first order rate constant for disappearance of the test compound is fitted to the data. *In vitro* half –life was calculated as $t_{1/2} = 0.693/b$. Intrinsic clearance (CL_{int}) was calculated by two different methods. The first method requires dividing the initial amount of test compound (in nmol) by the total area under the curve (AUC, in nmol•min/mL) from a plot of concentration versus time. The quotient is then divided by the total amount of protein (in mg) in the reaction vial, giving CL_{int} in units of mL/min/mg protein. Multiplying this value by the grams of liver weight per kg of body weight and mg of protein per gram of liver gives CL_{int} in units of mL/min/kg.

The second method of calculating CL_{int} involves dividing the first order rate constant by the protein concentration in units of mg/mL, which gives CL_{int} in terms of mL/min/mg protein. As in method 1, multiplying this value by the grams of liver weight per gram of body weight and mg of protein per gram of liver gives CL_{int} in units of mL/min/kg. The CL_{int} values were further compared with estimates of hepatic blood flow to allow some inferences to be made regarding first pass elimination effects in the various species.

Metabolites are identified by HPLC-MS, and their identity is confirmed with authentic standards, if available.

METABOLISM OF TELOMER B BY MAMMALIAN HEPATOCYTES

Principle: Isolated hepatocyte suspensions provide a high capacity *in vitro* metabolic system of somewhat greater biological complexity compared to sub-cellular fractions. Hepatocytes express all of the enzymes and cofactors associated with normal hepatic chemical metabolism and generally provide a realistic approximation of a given chemical's *in vivo* metabolite profile.

Test Species: Hepatocytes from males and females of the following species will be used for Telomer B metabolism studies:

Sprague-Dawley Rat
CD-1 Mouse
New Zealand White Rabbit
Cynomolgus Monkey
Beagle Dog
Human

Hepatocytes from rodent species are prepared in house by the two-stage collagenase perfusion method. Cryopreserved non-rodent hepatocytes are available commercially.

Study Design: The basic design for a hepatocyte metabolism experiment for a given species/sex is illustrated in the following table:

Group	Role	N	Cells (x 10^6)	Substrate
1	Experimental	5	5	Telomer B
2	Pos. Control	5	5	Ethoxycoumarin
3	Neg. Control	5	5	Vehicle
4	Neg. Control	5	5*	Telomer B

* Heat inactivated

Group 2 assures the metabolic activity of the hepatocyte suspensions, Group 3 provides an analytical background sample and Group 4 controls for non-enzymatic degradation of the test compound. Hepatocytes are suspended in Liebovitz L-15 medium at a concentration of approximately 1 x 10^6 cells/mL. Reactions are incubated at 37 °C for 2 hrs, then terminated by cooling, acidification, or addition of organic solvent as appropriate. Following sample clean-up, extracts are analyzed by LC/MS for parent and metabolites.

DRAFT PROTOCOL

Toxicokinetics of Telomer B Alcohol in the Rat

Project Number: DuPont-<>

Work Request Number: <>

Service Code Number: <>

DuPont Animal Welfare Committee No. <>

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INTRODUCTION

Telomer B alcohol (TBA) is a fluorinated alcohol used in the manufacture of repellents and surfactants. TBA is similar in structure to perfluorooctanoic acid (PFOA), an 8-carbon, straight chain fatty acid analogue in which the aliphatic carbons are saturated with fluorine.

In metabolism studies with PFOA, excretion was found to be more rapid in female than male rats following oral dosing.[1] This sex difference was apparently not due to differential metabolism of PFOA, as only parent was detected in excreta and tissues, but rather to the presence of testosterone, which was correlated with a reduction in the elimination rate of PFOA in male rats. Binding to a male-specific testosterone-inducible protein in serum or tissue may explain the excretion differences observed; female rats with less PFOA binding protein apparently have increased renal elimination, via the organic acid transport system, which is active in females but not male rats.[2]

The mechanism to explain the observed sex differences may also be structure-activity based. Perfluorodecanoic acid (PFDA), a 10-carbon perfluorinated chemical, was eliminated more slowly than the shorter 8-carbon PFOA in female compared to male rats, and lacks the observed sex differences in renal excretion and toxicity.[3,4]

OBJECTIVES

The objectives of this study are to determine the absorption, distribution, excretion and metabolism of TBA in male and female rats following oral and dermal dosing.

SPONSOR AND TEST FACILITY

This study is sponsored by the Telomer Research Group. The sponsor's approval was effective the date the sponsor authorized the work on the Work Authorization Form.

The study will be conducted at Haskell Laboratory for Toxicology and Industrial Medicine, E. I. du Pont de Nemours and Company, Newark, Delaware, in accordance with all applicable Good Laboratory Practice Standards.[5,6,7] Areas of noncompliance will be documented in the final report.

REGULATORY TEST GUIDELINES

This study has been designed to meet the testing requirements of (1) OECD Guidelines for Testing of Chemicals, Toxicokinetics, 417 (April 1984), and (2) US EPA Health Effects Test Guidelines, OPPTS 870.7485, Metabolism and Pharmacokinetics.

MATERIALS AND METHODS

A. Test Substance

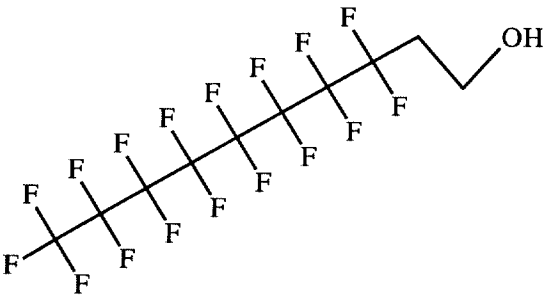
The test substance, Telomer B alcohol (TBA), and an analytical standard of TBA of known purity will be supplied by the sponsor and will be assigned a Haskell Laboratory Number, which will be documented in the study records.

CAS Registry Number: <>

Molecular Weight: 464

Molecular Formula: $C_{10}H_5F_{17}O$

Structure:



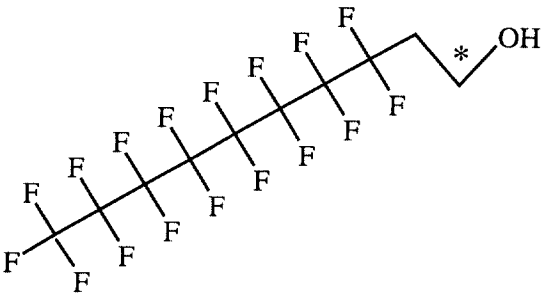
Purity: >95%

B. Radiolabeled Test Substances

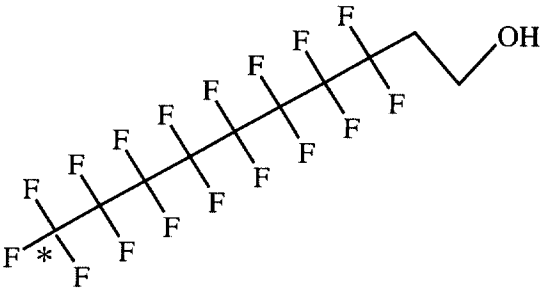
Specific Activity: To be determined

Radiochemical Purity: >95%

[1-C^{14}] Telomer B alcohol



[10-C^{14}] Telomer B alcohol



[*] denotes the position of the radiolabel

C. Test Species

Male and female Crl:CD®(SD)IGS BR rats will be used and obtained from Charles River Laboratories, Inc., Raleigh, North Carolina. The rat was selected for use in the study, as it is the principal toxicology animal model. The Sprague-Dawley rat has been used for toxicokinetic testing of other fluorinated test materials.[1,3]

At the time of dosing, rats should be sexually mature and the weight variation should not exceed ±20% of the mean weight by dose group. Each animal will be assigned a unique identification number to be used throughout the study. The last 3 digits of the animal identification number will be marked on the tail of each animal in indelible ink.

D. Animal Husbandry

Upon arrival at Haskell Laboratory, rats will be removed from shipping cartons and housed in appropriate cages, according to Standard Operating Procedures LA003-P-006 unless specified in the study records. Animals will be maintained under quarantine for at least six days unless approved otherwise by the site veterinarian, have three recorded weight gains and no abnormalities detected. After the quarantine period, rats will be selected for study.

Animal rooms are targeted at a temperature of 23±1°C and a relative humidity of 40-60%. Animal rooms are artificially illuminated (fluorescent light) on a 12-hour light/dark cycle.

Throughout the dosing period with test compound, the rats will be housed individually in appropriate cages according to the SOP.

PMI Nutrition International, Inc. Certified Rodent LabDiet® 5002 and tap water will be provided *ad libitum*. Animals will not be fasted before dosing with test substance.

E. Animal Health Monitoring

Haskell Laboratory has an animal health monitoring program. The following procedures are performed periodically to assure that contaminants are not present at levels that would be expected to impact the scientific integrity of the study.

- Water samples are analyzed for total bacterial counts and the presence of coliforms, lead, and other contaminants.
- Feed samples are analyzed for the presence of bacteria and fungi.
- Samples from freshly washed cages and cage racks are analyzed to assure adequate sanitation by the cagewashers.

Haskell Laboratory uses certified animal feed. The feed is guaranteed by the manufacturer to meet specified nutritional requirements and to be free of a list of specified contaminants.

Certified animal feed is used, guaranteed by the manufacturer to meet specified nutritional requirements and to be free of impurities which might influence the results of the study.

The animal health monitoring program is administered by the laboratory animal veterinarian. Data are maintained separately from study records and will not be included in the final report at the discretion of the study director.

F. Dose Preparation, Analysis and Rates

Telomer B alcohol and/or radiolabeled TBA will be mixed with an appropriate dosing vehicle. Water or other solvent may be added as required to achieve the target low and high dose concentrations of Telomer B alcohol.

Dose preparations for the single dose experiments may be prepared on the day of use or prior to the day of use. Dose solution for the repeat dose experiments may be prepared in bulk, stored appropriately, and used as needed.

HPLC-MS or another suitable analytical method will be used to analyze dose solutions for chemical concentration. Dose solutions containing radiolabeled TBA will be assayed in triplicate for radioactive concentration by liquid scintillation counting (LSC), and radiochemical purity will be determined by appropriate mean using radiometric detection.

Oral doses will be administered at a rate of 4 mL/kg. Dermal doses will be administered at a rate of 10 $\mu L/cm^2$.

G. Sacrifice

Rats will be sacrificed by CO_2 asphyxiation and exsanguinated by cardiac puncture.

H. Pilot Studies

The following pilot studies will be conducted to determine the plasma kinetics of TBA and to resolve how TBA is eliminated following oral administration. The data from these experiments may be useful in setting the whole blood serial collection time-points, determining the principal routes and rates of elimination, and establishing the need for one or two radiolabeled forms of TBA to describe elimination/metabolism.

1. Plasma kinetics following oral dosing with non-radiolabeled TBA

Two male and two female rats will have cannula surgically implanted in the jugular vein and allowed to recover for at least one day before dosing. Rats will be administered the test compound (non-radiolabeled) by the oral route and housed in suspended wire-mesh cages.

Following dosing, serial blood samples will be drawn from the jugular vein cannula and placed into Microtainer tubes containing EDTA. Serial sampling time-points will be documented in the study record. Alternatively, whole blood may be collected from the tail vein.

Whole blood samples will be maintained on wet ice. Plasma will be separated from red cells by centrifugation. Plasma will be stored frozen at approximately –15°C until processed for analysis.

2. Excretion and metabolism following oral dosing with radiolabeled TBA

In separate experiments for each radiolabeled form of TBA, two male and two female rats will be administered the test material formulated with radiolabeled TBA at the high dose level by oral gavage. Following dosing, rats will be housed individually in glass metabolism units suitable for the collection of urine, feces, expired air and volatile organics.

Urine and feces will be collected (on dry ice) at approximately 12 hours and every 24 hours after dosing until sacrifice.

Expired air will be passed through two scrubbing towers, one containing 2 N NaOH and the second containing ethylene glycol or other suitable solvent. Scrubbing towers will be replaced every 24 hours, post-dose, until sacrifice.

Animals will be sacrificed after at least 95% of administered radioactivity has been excreted or after 7 days post-dosing, whichever takes place first.

The following tissues will be collected at sacrifice:

Blood	Fat	Liver
Kidney	Muscle	heart
Lung	Testes	Ovaries/uterus
Bone	Brain	Spleen
G.I. tract and contents	Adrenals	Skin sample

After collection, tissue samples will be stored at approximately -15°C until processing and analysis. The remaining carcass will be homogenized and assayed so that total material balance can be determined.

If less than 2% of the total dose is eliminated during the duration of the study as either $^{14}CO_2$ or volatile organics, collection of expired air will not be performed in the main study experiments. If greater than 20% of the dose is eliminated via the feces, bile will be collected in the main study experiments.

If results suggest that either radiolabeled form of TBA will suffice in establishing the excretion and metabolism of TBA, one form will be selected and used in the main study distribution, excretion and metabolism experiments.

I. Main Study

1. Absorption Experiments

The principal objective of the absorption experiments is to determine the rate and extent of absorption of TBA following oral and dermal dosing using plasma kinetics.

Dose Routes and Frequency

The absorption experiments will be comprised of five dose groups.

- Low dose, single oral gavage
- High dose, single oral gavage
- Low dose, single 8-hour dermal exposure
- High dose, single 8-hour dermal exposure
- Low dose, intravenous

Each dose group will be composed of 4 male and 4 female rats. Each dose group will receive non-radiolabeled test compound.

The intravenous dose will provide a reference for estimating bioavailability of TBA by the oral and dermal exposure routes.

Experimental Design

Rats will have a cannula surgically implanted in the jugular vein one or two days prior to dosing.

Rats designated to receive a dose via the dermal route will have the dorso-thoracic region shaved free of hair the day prior to dosing. After shaving the site will be washed with a dilute soap solution and covered with gauze and a body wrap to prevent contamination. Rats will be then fitted with an Elizabethan-style collar to prevent grooming of the application site. The collar will be worn for the duration of the experiment. On the day of dosing, the protective body wrap and gauze cover will be removed and a silicone O-ring with an internal area of 10 cm^2 will be glued to the shaved dorso-thoracic region using an adhesive. Following dosing, the O-ring will be covered with a rigid nylon mesh and a non-occlusive body wrap. At the end of the exposure period, the non-occlusive cover will be removed and the site washed, rinsed and dried with natural sponges.

Following dosing, serial whole blood samples will be drawn from the cannula and placed into Microtainer tubes containing EDTA. Sampling times will be documented in the study records. Alternatively, whole blood may be collected from the tail vein. For rats in the dermal exposure groups, whole blood will be collected during after the exposure phase (8 hours). The application site will be washed free of excess test material at 8 hours.

Whole blood will be placed on wet ice, and plasma will be separated by centrifugation and frozen at approximately –15°C until analyzed.

2. Distribution Experiments

The principal objective of the distribution experiments is to determine the concentration and clearance of TBA in selected tissues and organs at plasma T_{max} and $T_{max/2}$ for TBA following oral and dermal dosing. Tissues should be selected based upon previous toxicology data or results of the pilot excretion/metabolism experiment.

Dose Routes and Frequency

The distribution experiments will be comprised of four dose groups, for each of two radiolabeled forms of TBA. The need to use both radiolabeled forms of TBA will be established in pilot experiments.

- Low dose, single oral gavage
- High dose, single oral gavage
- Low dose, single 8-hour dermal exposure
- High dose, single 8-hour dermal exposure

Each dose group will be composed of 4 male and 4 female rats. Each dose group will receive dose formulated with radiolabeled TBA.

Experimental Design

Rats will be dosed via the oral and dermal exposure routes at the low and high dose levels. The pre-dosing procedures for rats in the dermal exposure groups will be as described under the absorption experiments. The exposure period for the dermal exposure groups will be 8 hours. Following dosing rats will be killed at plasma T_{max} and $T_{max/2}$ for TBA.

Tissues will be collected at sacrifice and stored at approximately -15°C until processing and analysis. Total radioactivity in tissues will be determined. Selected tissues may be extracted and analyzed to determine the metabolite profile. Methods will be documented in the study records.

3. Excretion and Metabolism Experiments

The objectives of the excretion and metabolism experiments are to determine the rates and routes for elimination of TBA and/or its metabolites, to establish the principal pathways for metabolism of TBA and to determine if daily (repeat) low oral dose exposure alters the excretion kinetics or metabolism of TBA. A mass balance for total radioactivity administered will also be determined.

Dose Routes and Frequency

The excretion/metabolism experiments will be comprised of five dose groups, for each of two radiolabeled forms of TBA. The need to use both radiolabeled forms of TBA will be established in pilot experiments.

- Low dose, single oral gavage
- High dose, single oral gavage
- Low dose, single 8-hour dermal exposure
- High dose, single 8-hour dermal exposure
- Low dose, daily (repeat) oral gavage

Each dose group (single dose) will be composed of 4 male rats and 4 female rats. Each dose group will receive dose formulated with radiolabeled TBA.

For the daily (repeat) low-dose oral gavage experiment, four male and four female rats will be administered non-radiolabeled test compound at the low dose rate for a total numbers of days necessary to achieve steady-state plasma levels of TBA. The exposure time for the dermal experiments will be 8 hours. Once steady state has been achieved, as determined by periodic sampling of whole blood for TBA and/or selected metabolites, rats will be administered a dose of the test material formulated with radiolabeled TBA.

Experimental Design

Pre-dose procedures for rats in the dermal exposure groups will be as described for the absorption experiments. Following administration of the radiolabeled dose, urine and feces will be collected (on dry ice) at approximately 12 hours and 24 hours following dosing, and each 24

hours post-dosing until sacrifice. Animals will be sacrificed after at least 95% of the administered radioactivity has been excreted as determined in pilot experiments. The excreta may be analyzed to determine the metabolite profile.

If significant amounts of $^{14}CO_2$ or volatile organics were observed in the pilot study, traps will be used to collect any $^{14}CO_2$ and/or volatile organics that may be liberated. If required, the expired air will be passed through two traps connected in series, the first contains 2N NaOH and the second containing ethylene glycol or other suitable solvent. Solvent traps will be collected and replaced every 24 hours post-dosing throughout the study. In the case of the Low Dose Multiple dose groups, expired air will only be collected following administration of the radiolabeled dose.

The tissues collected at sacrifice will be determined by the results of previous toxicology experiments and/or pilot studies. After collection, tissue samples will be stored at approximately -15°C until processing and analysis. The remaining carcass will be homogenized and assayed so that total material balance can be determined.

Selected tissues may be analyzed to determine the metabolite profile.

At the conclusion of the radiolabeled dose experiments, cages will be rinsed with detergent and water (50:50 v/v), water and acetone. Cages may be wipe-tested to determine efficiency of cage rinse. A second cage rinse may be carried out at the study director's discretion. Cage wash will be stored at room temperature.

If the feces contains greater than 20% of the administered dose, as determined in pilot experiments, two additional groups of four male and four female rats (oral and dermal) will be used to establish the origin of material in the feces. Rats, will have a cannula surgically implanted in and bile duct and provided with a bile maintenance drinking solution (isotonic dextrose/KCl/NaCl) as the sole source of drinking fluid for the duration of the experiment. Following dosing, urine, feces and bile will be collected (on dry ice) at approximately 12 hours and 24 hours. Animals will be sacrificed after 24 hours. The excreta may be analyzed to determine the metabolite profile.

J. Quantitation of Radioactive Residues

1. Plasma

Plasma samples will be thawed, and aliquots analyzed in duplicate for ^{14}C by liquid scintillation counting (LSC). Plasma may also be analyzed for parent compound and metabolites by LC-MS.

2. Urine

Urine samples from each collection interval will be thawed, and aliquots analyzed in triplicate for ^{14}C by LSC. Urine may also be analyzed for parent compound and metabolites by LC-MS.

3. Feces

Feces from each collection interval will be homogenized in water and aliquots will be combusted. The CO_2 liberated from combustion will be trapped and analyzed in triplicate for ^{14}C by LSC to determine total fecal radioactivity for individual rats. Feces may also be extracted and analyzed for parent compound and metabolites by LC-MS.

4. Bile

Bile from each collection period will be thawed and aliquots will be analyzed in triplicate for ^{14}C by LSC. Bile may also be extracted and analyzed for parent compound and metabolites by LC-MS.

5. Cage rinses

Aliquots of cage rinses will be analyzed in triplicate for ^{14}C by LSC.

6. Feed residue

Feed residue will be homogenized and aliquots will be combusted. The CO_2 liberated from combustion will be trapped and analyzed in triplicate for ^{14}C by LSC.

7. Carcass

Individual carcasses will be analyzed for residual ^{14}C only if the recovery of total radioactivity in excreta and plasma for that animal is <98%. Carcasses will be ground on dry ice and homogenized, and aliquots of the resulting homogenate will be combusted. The CO_2 liberated from combustion will be trapped and analyzed in triplicate for $^{14}CO_2$ by LSC.

8. Samples from dermal experiments

In addition to the samples normally collected and analyzed from experiments using radiolabeled TBA, the following samples unique to dermal experiments will also be collected and analyzed.

The application skin site and sponges used for washing and drying the application skin site will be digested in 1N potassium hydroxide and aliquots analyzed by LSC.

The protective appliance used to contain and protect the dose site will be soaked in an appropriate solvent. Aliquots will be taken and analyzed by LSC.

K. Material Balance

Total recovery for rats receiving radioactive TBA should average 100 ± 10% of the amount administered.

L. Clinical Observations and Mortality

Cage-site examinations to detect moribund or dead rats and abnormal behavior and/or appearance among rats will be conducted at least once daily throughout the study. Moribund rats will be sacrificed.

M. Statistical Analyses

Group data will be represented as a mean ± SD. Other statistical evaluations may be performed, if necessary.

N. Measurement of Radioactivity

Radioactivity in selected liquid samples will be assayed by LSC. Total radioactive residues in solid samples will be determined by combustion, trapping liberated $^{14}CO_2$ followed by LSC.

Samples for LSC will be analyzed for 10 minutes, or until 160,000 disintegrations (0.5% 2σ) are accumulated, whichever comes first.

SAFETY AND HOUSEKEEPING

All chemicals used during this study will be handled according to the procedures specified in the Chemical Catalog and/or the accompanying MSDS and disposed of according to the Stine-Haskell Waste Disposal Guidelines (Procedure 100). Radiochemicals will be handled and disposed of in accordance with the guidelines outlined in the Stine-Haskell Radiation Safety Manual and Radioactivity Users Guide.

RECORDS AND SAMPLE STORAGE

Specimens (if applicable), raw data, and the final report will be retained at Haskell Laboratory, Newark, Delaware, or at Iron Mountain Records Management, Wilmington, Delaware.

CRITICAL DATES

Date of Approval:	Date Study Director signed Protocol
Proposed Experimental Start:	<first date test substance is applied to test system>
Proposed Experimental Termination:	<last date on which data are collected directly from the study>
Proposed Study Completion:	<date final report will issue>

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SIGNATURES

Issued by Study Director: _____ _____
<name, credentials> Date
<title>

Approved by: _____ _____
<name, credentials> Date
<title>

CC: QA

Summary of study experiments

1) Pilot experiments
   a) Test material: Telomer B alcohol (TBA) and [^{14}C]TBA
   b) Number of animals per treatment group: 2 male and 2 female
   c) Treatment groups:
      i) Plasma kinetics following a single high oral dose of TBA
      ii) Excretion and metabolism following a single high oral dose of [^{14}C]TBA
         (1) [10-C^{14}]TBA
         (2) [1-C^{14}]TBA

2) Absorption experiments
   a) Test material: TBA
   b) Number of animals per treatment group: 4 male and 4 female
   c) Treatment groups:
      i) Single low oral dose
      ii) Single high oral dose
      iii) Single low dermal exposure (8 hours)
      iv) Single high dermal exposure (8 hours)
      v) Single low intravenous dose

3) Distribution experiments
   a) Test material: [^{14}C]TBA
   b) Number of animals per treatment group: 4 male and 4 female
   c) Treatment groups:
      i) Single low oral dose, sac at T_{max}
      ii) Single low oral dose, sac at $T_{max/2}$
      iii) Single high oral dose, sac at T_{max}
      iv) Single high oral dose, sac at $T_{max/2}$
      v) Single low dermal exposure (8 hours), sac at T_{max}
      vi) Single low dermal exposure (8 hours), sac at $T_{max/2}$
      vii) Single high dermal exposure (8 hours), sac at T_{max}
      viii) Single high dermal exposure (8 hours), sac at $T_{max/2}$

4) Excretion and metabolism experiments
   a) Test material: [^{14}C]TBA
   b) Number of animals per treatment group: 4 male and 4 female
   c) Treatment groups:
      i) Single low oral dose
      ii) Single high oral dose
      iii) Single low dermal exposure (8 hours)
      iv) Single high dermal exposure (8 hours)
      v) Single low daily oral dosing with TBA, last dose with [^{14}C]TBA