R.J. REYNOLDS TOBACCO COMPANY
PRODUCT DEVELOPMENT AND ASSESSMENT
TOXICOLOGY DIVISION
EXPERIMENTAL PROTOCOL

Research Protocol

Protocol Identifier: TOX-060

Protocol Title: Comparative 30-Week Skin Painting Promotion Evaluation of Cigarette Smoke Condensate From a Reference Cigarette and a Test Cigarette in Female SENCAR Mice

Date

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Anticipated Start Date:
Anticipated Finish Date:

xc: S. Reynolds (for distribution to Animal Care Committee)

PROTOCOL FOR THE COMPARATIVE 30-WEEK SKIN PAINTING PROMOTION EVALUATION OF CIGARETTE SMOKE CONDENSATE FROM A REFERENCE CIGARETTE AND A TEST CIGARETTE IN FEMALE SENCAR MICE

1.0 Summary of Experimental Design

This study will consist of dermal administration of cigarette smoke condensate obtained from either a reference cigarette (1R4F) or a novel test cigarette. A total of 10 treatment groups, each containing 40 female SENCAR mice, will be used. Treatment groups will receive a single initiating dose of either DMBA or acetone on the first day of the study (initiation phase). During all subsequent weeks, the treatment groups will be administered their designated test or control articles three times per week (promotion phase). At study termination (Week 31), surviving mice will be necropsied and designated tissues will be collected. End-points will include body weights, organ weights, tumor tracking data and histopathology.

2.0 Facilities and Administration

2.1 Sponsor: R. J. Reynolds Tobacco Company
Bowman Gray Technical Center
Winston-Salem, NC 27102

2.2 Evaluation Facility: R.J. Reynolds Tobacco Company
Toxicology Division
Building 630-2
Shorefair Drive and 32nd Street
Winston-Salem, NC 27102

2.3 Contractors:

a. Veritas Labs: Veterinary, Necropsy, Histopathology
b. Charles River Labs: Serology
c. DynCorp/P.R.I.: Animal Care, Quality Assurance

2.4 Study Administration:

a. Study Director:
Daniel R. Meckley, B.A., DABT
b. Manager, Toxicology Division:
Arnold T. Mosberg, Ph.D., DABT
c. Study Veterinarian:
John Sagartz, DVM, ACVP
d. Study Pathologist:
Kent Van Kampen, DVM, ACVP

3.0 Proposed Study Schedule:

Cigarette Receipt: February 2, 1994
Quarantine Start:
First Day of Dosing:
Last Dose Day:
Terminal Necropsy:
Report Dates:
In-Life Memo Report:
Draft CSC Chem Report:
Draft Pathology Report:
Draft Final Report:
Final Report:

4.0 Objective:

The objective of this study is to compare the potential toxicity and tumor promotion characteristics of cigarette smoke condensate (CSC) from a test cigarette to condensate from a reference cigarette, when administered to female SENCAR mice by the dermal route for 29 weeks.

5.0 Test and Control Articles:

5.1 Cigarettes: Reference cigarettes and test cigarettes will be supplied by R. J. Reynolds Tobacco Company. The test cigarette will be identified as R345 or 3-102E. The reference cigarette will be the University of Kentucky 1R4F. This reference cigarette was selected for comparison because its blend and design are representative of an average market cigarette. The configurations and blend specifications of the test and reference cigarettes will be included in the study file.

1R4F reference cigarettes will be stored refrigerated (approximately 4°C) until removed for conditioning. R345 cigarettes will be stored, as received, in the ambient laboratory environment.

5.2 Dose Preparation:

5.2.1 Generation of Cigarette Smoke Condensate: All cigarettes will be conditioned to the laboratory environment (approximately 70°F and 50% relative humidity) for approximately 18-hours prior to being smoked. Cigarettes will be "smoked" on AMESA Type 1300C smoke generators operated under Federal Trade Commission (FTC) standard conditions of a 2-second puff with a volume of 35-ml, taken

once per minute. The 1R4F reference cigarette will be smoked to a mean butt length of 3 mm above the filter overwrap. The test cigarettes, which do not get shorter while being smoked, will be smoked for 8 puffs before being automatically replaced on the smoke generator.

Condensate will be collected via cold trap (SOP #MSP-004) and pooled. The pooled condensate will be prepared and stored as a concentrated solution of CSC in Nanograde® acetone (SOP #BSP-001).

Dosing solutions will be prepared by dilution of the concentrated condensate solution with Nanograde® acetone. The concentrated condensate solution and dosing solutions will be stored in amber glass bottles in Room 34 (approximately -20°C, freezer) for up to 8 weeks.

5.2.2 7,12-Dimethylbenz(a)anthracene (DMBA): This chemical will be used as the initiator for the appropriate test and control groups. The bulk chemical will be obtained from Aldrich Chemical Company (Milwaukee, WI) and stored according to the manufacturers specifications. Dosing solutions will be prepared by placing a weighed amount of chemical in a volumetric flask and adding Nanograde® acetone until the desired concentration (volume) is obtained. The dosing solution will be prepared once, approximately one week prior to use, and will be stored in an amber glass bottle in Room 34.

5.2.3 Acetone (Mallinckrodt, Nanograde®): This chemical will be used as the vehicle control material. It will also be used as the diluent for DMBA and cigarette smoke condensates. The bulk chemical will be obtained from Baxter, Scientific Products (Charlotte, NC) and stored according to the manufacturers specifications. Smaller quantities used to dose the vehicle control animals will be removed from the bulk supply and stored in amber glass bottles in Room 34 for up to 8 weeks.

5.3 Chemical Analysis:

5.3.1 Cigarette Parameters:
The chemical characterization of mainstream smoke from the test and reference cigarettes will be determined by FTC method prior to the start of the study and documented. These could include, but may not be limited to the following analyses; puffs/cigarette, total particulate matter (TPM)/cigarette, nicotine, water, "tar", ammonia, carbon monoxide, pH, benzo(a)pyrene, formaldehyde, acetaldehyde, acetone, acrolein, hydrogen cyanide, hydroquinone, catechol, phenol, cresol, nitrogen oxides and nitrosamines.

5.3.2 Cigarette Smoke Condensate:
The chemical characterization of the concentrated condensate solutions will be determined for each batch (before application to the animals) and will be documented. The following components will be determined for the concentrated condensate solution; water and nicotine (SOP #BSP-002), pH (SOP #BSP-003), benzo(a)pyrene (SOP #BSP-004), and phenolics (SOP #BSP-005). Each set of dosing solutions prepared from the concentrated condensate solution will be analyzed for nicotine and water (before application to the animals) and documented. The stability of a high dose condensate solution will be initiated before the date of first dose administration and will continue throughout the course of the study. Reserve samples from each batch of concentrated condensate solution and each dosing solution will be stored in amber glass bottles and retained in Room 34 for as long as the quality of the preparation affords evaluation but no longer than 2 years following study completion.

5.3.3 7,12-Dimethylbenz(a)anthracene:
The manufacturers analysis of the bulk chemical will be retained with the study records. The purity of the bulk chemical will be confirmed prior to study initiation. The bulk chemical will not be utilized if the purity is not within 5% of the manufacturers analysis. The concentration of the dosing solution will be determined (before application to the animals) and documented. A reserve sample of bulk chemical and the dosing solution will be stored in an amber glass bottle and retained in Room 34.

5.3.4 Acetone (Mallinckrodt, Nanograde®):
The manufacturers analysis of the bulk

chemical will be maintained with the study records. The purity of the bulk chemical will be confirmed prior to study initiation. The bulk chemical will not be utilized if the purity is not within 5% of the manufacturers analysis. A reserve sample of the bulk chemical will be stored in amber glass bottles and retained in Room 34.

5.4 Route of Administration: The dermal route of administration has been selected because it has been reported to show positive results utilizing cigarette smoke condensate (National Cancer Institute, 1980) and is the best available means by which dose may be exaggerated to elicit responses in a limited population.

5.5 Hazardous Materials and Safety:

5.5.1 Hazardous Materials: The following hazardous materials will be used during this study; Cigarette Smoke Condensate; DMBA and acetone. Material Safety Data Sheets for DMBA and acetone are provided as attachments to this protocol. Only the smallest quantity needed for a particular procedure will be used. Excess material will be disposed of as described below.

5.5.2 Safety Procedures: Safety procedures will be employed for personnel protection, due to the use of materials of known and unknown carcinogenic potential. These procedures adhere to the provisions of the R. J. Reynolds Tobacco, Chemical Hygiene Plan (developed to comply with the OSHA Laboratory Standard, 29 CFR 1910.1450) and will include protective clothing covering the body, protective eyewear, respirators with particulate or vapor/particulate filter cartridges, room ventilation system, and a container-within-a-container transport system for the dosing solutions (SOP #MSP-003).

5.5.3 Disposal of Contaminated Wastes: Excess materials and/or materials contaminated or potentially contaminated with the above hazardous materials will be disposed of via a North Carolina-certified hazardous waste or medical waste disposal firm.

6.0 <u>Experimental Use of Animals</u>:

6.1 Justification for Use of Animals: Animals (mice) are the model of choice for evaluating complete and

two-stage tumorigenesis and also because they have been reported to show positive results utilizing cigarette smoke condensate (National Cancer Institute, 1980).

Several short-term in vitro bacterial/mammalian cell assays have been developed to aid in the screening of chemicals for tumorigenicity potential but most are based on a mutagenicity end-point. Although there is circumstantial evidence supporting mutations as an integral event in tumorigenesis, this cannot be considered to be proven. Even the simplest two-stage model of tumorigenesis cannot be totally explained on the basis of mutation. In vitro tests for toxicity evaluation also have inherent disadvantages, particularly the unrepresentative metabolic system and the absence of complex immunological and physiological processes present in the whole animal (Purchase, 1983). For these reasons there is not currently an adequate alternative to the use of animals for evaluating the tumorigenic potential of chemicals.

The results of a literature search for alternative in vitro assays for tumor promotion characteristics is contained in the study file.

6.2 Justification for Use of Species and Strain: SENCAR mice were selected for this study because they have been selectively bred for susceptibility to skin tumorigenesis and this is the strain of mice used for previous cigarette smoke condensate promotion evaluations conducted at R.J. Reynolds Tobacco Company.

6.3 Justification for Number Required: This initiation-promotion study design represents a reduction in the number of animals needed to conduct a chronic tumorigenicity study when compared to the standard National Toxicology Program protocol utilizing 50-60 animals of each sex. For this study, a total of 400 female SENCAR mice will be required for this study. 420 female SENCAR mice will be ordered. The number of animals in each group was selected to provide the appropriate statistical power for differentiating dermal tumor development from background [85%, based on an ArcSin Transformation of Proportion, Type I and Type II Error=0.05 each, Level of Significant difference detection approximately 15%, Mosberg, 1988)] and for comparison with other similar studies. The 20 additional animals are needed for serology (10 total, see section 6.6), to have sufficient animals to replace any that do not

survive the quarantine period and to have a sufficient number to perform allocation to experimental groups. Mice that remain after allocation into experimental groups or any mice that are shipped in excess of the 420 number by the vendor may be used for methods development studies approved by the Institutional Animal Care and Use Committee or will be killed by first anesthetizing with 70% CO_2 in air and then cervical dislocation (SOP # TOX002 and TOX005). Records of the fate of each animal received will be kept.

6.4 Duplication of Experiment: This study is being conducted because the design and mainstream smoke components of the test cigarette are different from conventional cigarettes and a "Level of Concern" and/or the overall weight of the evidence requires testing as outlined in the R.J. Reynolds Tobacco Company's "Draft, Data Requirements and Strategies for Chemical and Toxicological Testing of Changes in Cigarette Technology" (Rees, 1992). The results of a literature search (maintained with the study records) found no previous or published data for testing cigarette smoke condensate from the test cigarette on SENCAR mice.

6.5 Animal Supplier: Weanling SENCAR mice, 3-5 weeks old, will be ordered from Harlan Sprague-Dawley Inc., Indianapolis, IN.

6.6 Quarantine and Serological Evaluation: The animals will be quarantined in Room 39 for approximately 14 days under conditions simulating those of the study (SOP #AC-016). These animals will be assigned a pre-study identification number which will be indicated on it's cage card.

Within 48-hours after delivery, 10 animals will be randomly selected for health screening. These animals will be anesthetized with 70% CO_2 in air and blood will be drawn from either the vena cava or the heart. While still under anesthesia, the animals will then be killed by exsanguination (SOP # TOX004). Only personnel approved by the laboratory veterinarian will perform these procedures. Sera will be processed for routine measurement of the following antibodies to disease: Pneumonia virus of Mice, Sendai, Mouse Hepatitis, Minute Virus of Mice, Mouse Polio (GDVII), Reo Type 3, Polyoma, Ectromelia, Mouse Adenovirus FL/K87, Epizootic Diarrhea of Infant Mice, Mouse cytomegalovirus, Mouse Pneumonitis, Mouse Thymic virus, Hantaan virus, Lymphocytic Choriomeningitis, Mycoplasma pulmonis, Encephalitozoon cuniculi and

Ciliated Associated Respiratory Bacillus.

These animals will then be necropsied and the lungs examined microscopically, to ascertain health status. The utility of the study is dependent upon negative serology data being obtained on the pre-study samples, and upon a suitable written statement from the veterinarian/pathologist on the animals killed at delivery, releasing the animals from quarantine.

6.7 Allocation of Animals to Study Groups: Near the end of the quarantine period, animals will be assigned to dose groups, by body weight, using the "A" module of the Xybion software (Version 4.1.7,; Xybion Medical Systems, Cedar Knolls, NJ) (SOP #AC-010). To insure groups of similar mean starting weight, all groups will be compared by ANOVA and least significant difference criteria and demonstrated to be not significantly different at a 5 percent two-sided risk level.

6.8 Animal Identification: After allocation into experimental groups, animals will be identified by tail tattoo (SOP #AC-017). This procedure will only be performed by individuals who have been trained and certified in tattooing techniques by the manufacturer of the equipment or in-house certified and trained personnel. Animals will be numbered consecutively with a unique identification number.

6.9 Animal Husbandry: The animals will be housed and cared for in accordance with the Animal Welfare Act of 1970 and amendments (Public Law 91-579), as set forth in CFR Title 9, Part 3, Subpart F, "Specifications for the humane handling, care, treatment and transportation of warm-blooded animals other than dogs, cats, rabbits, hamsters, guinea pigs, non-human primates and marine mammals."

The mice will be housed in Room 39. Mice will be individually housed in stainless steel wire bottomed cages (3 3/4"W x 9"L x 5"H) suspended on stainless steel racks. Animals will be transferred to clean housing once per week.

The environmental conditions of the animal room will be monitored, and controls will be set to meet the following conditions:

a. A 12 hours on/12 hours off fluorescent (low UV) light cycle will be maintained. Lights will come on at 06:00 and go off at 18:00 hours via an

automatic timer monitored by study technicians.

b. The temperature of the room will be 72 ± 5°F and will be recorded continuously (SOP #AC-018).

c. The relative humidity will be 55 ± 15 percent and will be recorded continuously (SOP #AC-018).

d. Filtered (HEPA and charcoal) air will be provided to insure a minimum of ten changes of room air per hour.

Upon initiation of the study the location of each treatment group within a caging rack will be rotated every other week in an orderly fashion to insure a balanced contribution to study variance; likewise, the location of each caging rack within the room will be rotated every other week in an orderly fashion. Comprehensive records of these activities will be maintained (SOP #MSP-022).

Animals will have ad libitum access to Certified Purina Rodent Chow 5002 pelleted feed (Purina Mills, Inc., St. Louis, MO). Clean feeders will be provided weekly. Analyses of each feed lot will be obtained from the vendor and reviewed to determine (documented in the raw data) the potential effect on the study of any contaminants. It is unlikely that contaminants other than those analyzed for would be present in the feed. Feed lots will be monitored to assure that no feed is used if it is more than 180 days from date of milling.

Water will be provided to animals on an ad libitum basis via an automatic system. The water source is the municipal supply of the City of Winston-Salem which has been distilled (Meuller/Barnstead TD50 Thermodrive Still; Springfield, MO) in-house. This water is analyzed annually. No known contaminants are present that would interfere with the purpose or conduct of this study.

7.0 Experimental Design and Dose Regimen: This study will consist of dermal administration of cigarette smoke condensate obtained from the 1R4F reference cigarette and the test cigarette.

A total of 10 treatment groups will be used. Each treatment group will contain 40 female SENCAR mice. Three groups of each condensate will be initiated with a single 200 ul application of 75 ug DMBA in acetone and promoted with either 10, 20 or 40 mg dry condensate in acetone/200 ul/application of test or reference CSC. The rationale for selecting these dose levels is based

on the results of previous promotion studies conducted at R.J. Reynolds where dose-responses have been observed with other CSC's demonstrating increasing tumor production with increased CSC concentration. Two additional groups (one of each condensate) will be initiated with a single 200 ul application of acetone and promoted with 40 mg of dry condensate in acetone/200 ul/ application. These two CSC groups provide data on the complete tumorigenic potential of the CSC's. Two control groups are included in the study design. Vehicle control animals will receive acetone only (200 ul/application). A DMBA control group will be initiated with a single 200 ul application of 75 ug of DMBA in acetone and promoted with acetone (200 ul/application).

Experimental Groups

Group Number	Treatment Group (Initiator/Promoter)
	Control Groups
1	Acetone/Acetone
2	DMBA[a]/Acetone
	Test Groups
3	DMBA/1R4F CSC (10 mg/appl)
4	DMBA/1R4F CSC (20 mg/appl)
5	DMBA/1R4F CSC (40 mg/appl)
6	DMBA/R345 CSC (10 mg/appl)
7	DMBA/R345 CSC (20 mg/appl)
8	DMBA/R345 CSC (40 mg/appl)
9	Acetone/1R4F CSC (40 mg/appl)
10	Acetone/R345 CSC (40 mg/appl)

a. 75 Micrograms DMBA

Appropriate groups will receive a single 200 microliter (ul) application of either acetone or 75 micrograms (ug) DMBA in acetone on Study Day 1 (Monday, 10:00 ± 2 hours), initiation phase. Beginning on the following Monday and continuing for all subsequent weeks of the study (Weeks 2-30), each control (Groups 1 and 2), low dose CSC (Groups 3 and 6) and mid dose CSC (Groups 4 and 7) treated groups will be administered their designated test or control article (in acetone, 200 ul/application) three times per week [Mondays, Wednesdays and Fridays (excluding holidays), promotion phase]. Because the high dose CSC solutions are

anticipated to contain levels of nicotine which are acutely toxic (based on FTC results of mainstream smoke analysis), Groups 5, 8, 9 and 10 will be administered their respective mid-dose concentration (20 mg "tar"/application) three time per week during Weeks 2 and 3. The dose regimen for these animals will then be adjusted to twice daily 100 ul application of their respective high dose concentration (40 mg "tar") for Weeks 4 and 5. At Week 6 and for the remainder of the study, the dose regimen for these high dose CSC groups will be single 200 ul applications 3 days per week at 40 mg "tar"/application. This regimen is provided to slowly acclimate the animals to the higher dose concentration and yet minimize the acute nicotine effects. Except for the first 2 weeks of CSC application, this regimen maintains the total daily dose of "tar" administered to the high dose animals.

At study termination (Week 31), surviving mice will be necropsied and designated tissues (see Section 7.7) will be collected.

7.1 Clipping Animals: An electric clipper will be used to remove hair from the back of each animal. An area larger than the application site will be clipped to allow homogenous application of test and control materials and clear observation of the dosed area (SOP #MSP-005). Control animals will be clipped at the same frequency as dosed animals. Clipping will be performed on Tuesdays or Thursdays (excluding holidays) with each animal being clipped once per week.

7.2 Dose Application Procedure (SOP #MSP-010): A volumetric pipet equipped with a disposable tip will be used for this study. The polyethylene tip will be used to spread the dose over the application site. A new tip will be used for each concentration. The dose will be applied to the mid-back of the animal and spread evenly over the clipped dorsal skin.

7.3 Observations: The following parameters will be monitored during the in-life portion of this study:

7.3.1 Moribundity/Mortality Checks (SOP #MSP-002). Twice daily observations of all study animals for dead or moribund animals, once in the morning and once in the afternoon, will be made seven days per week (including holidays). Records of observations will be placed in the AOLOG module of the Xybion computer system. Animals whose conditions makes it unlikely that they will survive to the next observation period or appear to be

in pain, based on criteria established by the Study Director and the veterinary staff, will be killed and necropsied; tissues (see Section 7.6) will be retained in 10% neutral buffered formalin for histopathologic evaluations. Clinical observations will be recorded shortly before euthanasia. If necropsy cannot be performed immediately, the carcass will be stored at approximately 4°C to minimize autolysis. Animals will be necropsied no later than 16 hours after death.

7.3.2 Body Weight (SOP #AC-008). Animals will be weighed at delivery, at the allocation to study groups prior to the start of the study, weekly for the first 10 weeks, during week 12, and then at 4-week intervals thereafter. Data will be entered into the A-module of the Xybion computer system.

7.3.3 Clinical Observations (SOP #MSP-011). Except for weekends and holidays, daily observation will occur during the procedures of dosing, clipping and checking for dead animals. All positive findings will be recorded as unscheduled clinical observations using the AINPUT module of the Xybion computer system. Negative findings (Normal-no significant findings) will not be recorded.

In addition, more detailed (scheduled) clinical observations will be performed and the results (both positive and negative) will be recorded at weekly intervals during this study.

7.3.4 Tracking of Skin Masses (SOP #MSP-013). The dosed area will be carefully examined every week, usually at the time of scheduled clinical observations, and all new masses and abnormalities or changes to a previously observed mass will be recorded using the A or M module of the Xybion computer system. Skin masses will be described, measured (length, width and height) and recorded on the individual computer diagram. There will be a diagram for each animal exhibiting a mass.

7.4 Necropsy (SOP 117B and 117E): Animals from all dose groups, including control groups, which die early or are killed due to moribundity or according to study schedule will receive a complete necropsy examination. Animals will be killed by first

anesthetizing with 70% CO_2 in air and then cervical dislocation (SOP # TOX003 and TOX005). Tissues to be collected and preserved in 10% neutral buffered formalin are described in the gross pathology section of this protocol. The N module of the Xybion software will be used for data acquisition at necropsy.

7.5 Organ Weights. The non-fasted terminal body weight and the absolute wet weight of liver (with gall bladder incised), lungs (complete with trachea and associated mediastinal tissue), spleen, brain, heart (excluding major vessels), adrenals (pair) and kidneys (pair) will be obtained from animals killed at final necropsy. Organ weights and the terminal body weight will be used to calculate organ:body weight and organ:brain weight ratios. No measurements will be made of organ weights in animals which die early or are killed due to moribundity.

7.6 Gross Pathology: At necropsy, previously observed masses/lesions will be verified and any new masses/lesions identified at necropsy will be entered into the Presumptive Tumor tracking section of the N-Module by a qualified person.

Color photographic slides (with linear scale) of both the ventral and dorsal surface of each animal killed at final necropsy will be obtained.

Tissues to be collected at necropsy and preserved in formalin are denoted below:

Skin(treated and untreated)
Mandibular and mesenteric lymph nodes
Mammary glands with adjacent skin
Salivary gland
Tongue
Thigh muscle
Sciatic nerve
Sternebrae, femur and vertebrae including marrow
Small intestine (duodenum, jejunum, ileum)
Large intestine (cecum, colon, rectum)
Tissue masses or suspect tumors and regional lymph nodes
Harderian glands
Liver
Oral cavity, larynx and pharynx
Trachea
Lungs and bronchi
Heart and aorta
Thyroid
Parathyroids
Esophagus
Stomach
Ovaries
Uterus
Nasal cavity and nasal turbinates
Brain
Pituitary
Eyes
Clitoral glands
Zymbal glands (auditory sebaceous glands)
Spinal cord(thoracic)
Urinary bladder

Gall bladder
Pancreas
Spleen
Thymus

Kidneys
Adrenals
Tail and Carcass

The skin from the application site will be excised and its orientation clearly indicated (by leaving the ears attached). The skin will be placed on an index card so that it will be fixed flat. A sample of skin will also be obtained from a non-mass section of the application site; this non-mass section is important for diagnosing non-neoplastic changes (dermatitis, hyperplasia). Undosed skin will also be taken from the inguinal region. Care will be taken upon removing sections from the excised skin for histology to retain the remainder of the excised skin intact. All masses and lesions will be included for histopathology.

7.7 Microscopic Pathology: All skin masses and skin lesions, skin exhibiting no apparent lesions in the dosed area and undosed control skin from the inguinal region will be designated for histopathologic evaluation. The following tissues will also be examined for the DMBA/Acetone control (Group 2) and high dose CSC treated (Groups 5, 8, 9 and 10) animals: brain, liver, lungs, kidneys, spleen, adrenals, gonads and gross lesions. Evaluation of these tissues from the mid- and low-dose groups and other tissues may be requested from the examining pathologist if a review of necropsy data indicates the presence of lesions. The P module of the Xybion software will be used for data acquisition in histopathology.

7.8 Experimentally Induced Pain or Distress: Pain is associated with the tail tattooing procedure (see section 6.8). The procedure is a recommended identification method for rodents by the American Association for Laboratory Animal Science and will be performed by certified or trained technicians without anesthesia or analgesics in accordance with the tattoo-equipment-manufacturer's procedures.

There will be no experimentally induced pain during this study. Minimal distress may be experienced during the procedures of fur clipping, dosing, clinical observations and mass tracking. These procedures will only be performed by qualified individuals who have been trained by the laboratory animal veterinarian. No post-procedure pain is expected so no analgesic agent will be administered.

7.9 Survival Surgery: No survival surgery will be

conducted in this study.

7.10 Invasive Techniques: Two invasive techniques are required during this study. The first is the collection of blood from the vena cava or heart of randomly selected animals prior to study initiation. This procedure is presented in section 6.6 and is needed to document the health of the animals being used on this study. The second is tail tattooing. This procedure is presented in sections 6.8 and discussed in 7.8

7.11 Euthanasia: The methods of euthanasia are detailed in sections 6.3, 6.6 and 7.4. Only personnel trained by the laboratory animal veterinarian will perform these procedures. All animals that die or are killed while on study will be necropsied and the entire carcass fixed in formalin. The carcasses of animals killed for serological evaluation (no prior experimental treatment) will be stored frozen in air-tight plastic bags until they are disposed of via a North Carolina-certified medical waste disposal firm.

8.0 Statistics: Data will be analyzed using statistical tests available through the Xybion system. Statistical procedures could include but are not limited to the following. For body weights, body weight and absolute body weight gains, absolute organ weights, % organ to body weight and % organ to brain weight ratios; means and standard deviations, Bartlett's test of homogeneity of variance, Dunnett's T-test, Fisher's least significant difference (LSD) test of significance and Cochran and Cox's modified t-test of significance. The one-way analysis of variance is given only on reports that present the data by dose group. Bartlett's test of homogeneity of variance is performed whenever group comparisons are made. When the data are homogeneous, either Fisher's LSD or Dunnett's test is performed at fixed 0.05 and 0.01 levels. When the data are nonhomogeneous, Cochran and Cox's modified t test is performed at fixed 0.05 and 0.01 levels. Cumulative percentage of tumor bearing animals and median and mean time to tumor are calculated for tumor tracking data.

Gross tumor data will be transcribed from the XYBION system and survival (time to first tumor and mean number of tumors/tumor bearing animal) will be analyzed by using the SAS statistical procedures PROC LIFETEST and PROC CATMOD respectively. Fisher's Exact test (GraphPAD InStat, 1990 GraphPAD Software, Version 2.04) may also be utilized.

9.0 Records to be Maintained: Records that would be required to reconstruct the study and to demonstrate

adherence to the protocol will be maintained. These will include, but not be limited to the following:

- o The study protocol and any amendments
- o The XYBION protocol and any amendments
- o The names, signatures and initials for study personnel
- o Deviations from the study protocol and SOP's
- o Pertinent correspondences
- o Animal receipt and quarantine records
- o Health screening results
- o Records of allocation of animals to study groups
- o Animal identification (tattooing)
- o Cigarette inventory/utilization records
- o Mainstream smoke characterization records
- o Feed and water analysis records
- o Temperature and humidity records of the animal room
- o Documentation of animal room light cycle
- o Animal housing and care
- o Equipment maintenance and calibration records
- o Mortality, dose administration, clipping, body weight, clinical observation and mass tracking records
- o Necropsy records
- o Histopathology records
- o Condensate collection records
- o Dose solution preparation
- o Purity, stability and concentration analysis results
- o Statistical analysis results

10.0 Quality Assurance: This study will not be listed as a regulated study and the results will not be submitted to any regulatory agency. The study will be subject to periodic inspections, and the data and final report will be reviewed by PRI's quality assurance unit according to the Food and Drug Administration's Good Laboratory Practice Regulations for nonclinical laboratory studies (21 CFR, Part 58, 9/87).

11.0 Reports:

In-Life Data Summary: A memo report of the study will be prepared within 5 weeks of the date of the last animal necropsied. The memo report will include, but not necessarily limited to the following:

- o Summary of the study design
- o Review of the study objectives
- o Presentation/discussion of group mean body weights, clinical observations, grossly observed "tumor" counts, terminal body weights, organ weights and gross observations noted at final necropsy.
- o Preliminary conclusions based on in-life observations

Final Report: A written draft final report of the study

will be prepared within 30 weeks of the date of the last animal necropsied. The report will include, but not be limited to the following:

- Name and address of the facility performing the study and the dates on which the study was initiated and completed
- Objectives and Procedures as stated in the approved protocol
- Test and control substances identified by name, code number, strength, purity, and composition.
- Stability of the test and control substances under the conditions of administration
- Materials and Methods
- Description of the test system used including the number of animals used, sex, body weight range, source of supply, species, strain and substrain, age, and procedure used for identification.
- Description of the dosage, dosage regimen, route of administration, and duration
- Description of all circumstances that may have affected the quality, outcome of the study or integrity of the data
- Name of the study director, the names of other scientists or professionals, and the names of all supervisory personnel involved in the study
- Description of the transformations, calculations or operations performed on the data, a summary and analysis of the data and a statement of the conclusions drawn from the analysis
- Signed and dated reports of each of the individual scientists or other professionals involved in the study. These will include the study director, pathologist, analytical toxicologist and veterinarian.
- Location where specimens, raw data, and the final report are to be stored
- Statement prepared and signed by the quality assurance unit

12.0 Statement of the Study Director: The study director assumes the responsibility for assuring that all phases of this experiment are conducted according to the stated protocol. Any deviation from the protocol will be documented and any necessary changes in the protocol will be documented by protocol amendments. If the protocol amendments involve changes in animal care or use, then prior approval of the Institutional Animal Care and Use Committee will be obtained. The study director assures that this study does not represent any unnecessary duplication of experimental studies using animal resources. The study director also assures that all use of experimental animals conforms to the Animals Welfare Act of 1970 and amendments thereof, and the "Code of Ethics" as set forth by the Society of

Toxicology (SOT January, 1985).

13.0 References:

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