Sponsor:

3M
St. Paul, Minnesota

PROTOCOL

Study Title:

Cell Proliferation Study with N-Ethyl Perfluorooctanesulfonamido Ethanol (N-EtFOSE; 3M T-6316.11), Perfluorooctane Sulfonic Acid Potassium Salt (PFOS; 3M T-6295.16), and N-Ethyl Perfluoroctanesulfonamide (PFOSA 3M T-7091.1) in Rats

Date:

January 12, 1999

Performing Laboratory

R.O.W. Sciences
15 Firstfield Road
Gaithersburg, Maryland 20878

Laboratory Study Identification:

Study Number: (1132-100)

PAI Project Number: (Histology number to be assigned by PAI by protocol amendment)

Study

Cell Proliferation Study with N-Ethyl Perfluorooctanesulfonamido Ethanol (N-EtFOSE; 3M T-6316.11), Perfluorooctane Sulfonic Acid Potassium Salt (PFOS; 3M T-6295.16), and N-Ethyl Perfluoroctanesulfonamide (PFOSA 3M T-7091.1) in Rats

Purpose

To assess cell proliferation and peroxisome proliferation in rats administered test material in the diet.

Sponsor

3M Corporate Toxicology
Building 220-2E-02, 3M Center
St. Paul, MN 55144-1000

Study Representative

Marvin T. Case, D.V.M, Ph.D.
3M Corporate Toxicology
Phone No.: 651 733-5180
Fax No.: 651 733-1773
Email: mtcase@mmm.com

Alternative Study Representative

Andrew M. Seacat, Ph.D.
3M Corporate Toxicology
Phone No.: 651 575-3161
Fax No.: 651 733-1773
Email: amseacat@mmm.com

Study Location

R.O.W. Sciences
15 Firstfield Road
Gaithersburg, Maryland 20878

Study Monitor

Sandra R. Eldridge, Ph.D.
Pathology Associates International
Phone No. 301 624-2036
Fax No. 301 663-8994
Email: srepaisaic@aol.com

Study Director

Gary W. Wolfe, Ph.D., D.A.B.T.
R.O.W. Sciences
Phone No.: 301 330-3723
Fax. No.: 301 330-3738
Email: gwolfe@lab.row.com

Principal Investigator

Sandra R. Eldridge, Ph.D.
Pathology Associates International
Phone No. 301 624-2036
Fax No. 301 663-8994
Email: SREPAISAIC@aol.com

Study Pathologist

Carolyn Moyer, D.V.M., Diplomate, A.C.V.P.
Pathology Associates International
Phone No. 301 624-2928
Fax No. 301 663-8994

Proposed Study Timetable

In-life Start Date: To be added by protocol amendment; Day 0
In life End Date: To be added by protocol amendment
Audited Draft Report Date: To be added by protocol amendment

Regulatory Compliance

This study will be conducted in the spirit of Good Laboratory Practice (GLP) regulations.

Animal Care and Use Statement

All procedures in this protocol are in compliance with the Animal Welfare Act Regulations, 9 CFR 1-4. In the opinion of the Sponsor and study director, the study does not unnecessarily duplicate any previous work.

Quality Assurance

Not applicable.

Test Materials

Test Material:	N-EtFOSE (completed by 3M)	PFOS (completed by 3M)	PFOSA (to be added by protocol amendment)	Wy-14,643 (to be added by protocol amendment)
Identification:	N-EtFOSE	PFOS	PFOSA	Wy
Lot Number:	FM 3929	217		
Purity:	99.2%	99%		
Stability:	> 5 years	> 5 years	> 5 years	
Storage Conditions:	room temp.	room temp.	room temp.	
Characteristics:	waxy solid	white powder	amber waxy solid	

Reserve (Archive) Samples

A reserve sample (approximately 5 g) of each lot will be taken and stored at room temperature. These samples will be transferred to the Sponsor after completion of the in-life phase to be retained in accordance with 40 CFR 792.195.

Disposition of Test Material

After authorization from the Sponsor, any remaining test material will be returned to:

Marvin Case, D.V.M., Ph.D.
3M Corporate Toxicology
Building 220-2E-02, 3M Center
St. Paul, Minnesota 55144-1000
Phone No.: 651-733-5180
Fax No.: 651-733-1773

Animals

Species:	Rat
Strain:	Crl:CD®(SD) IGS BR
Source:	Charles River Laboratories, Inc., Raleigh, NC
Age at Initiation of Treatment:	Preferable 6 weeks of age, but not more than 8 weeks of age
Weight at Initiation of Treatment:	150 to 300 g
Number and Gender:	males
Identification:	unique identification by individual car tags and cage cards

Husbandry

Housing:	Single housed in hanging stainless steel wire cages
Diet:	Teklad 7012 Certified Rodent Diet. Fresh food will be provided weekly. Feed is analyzed by the manufacturer for concentrations of specified heavy metals, aflatoxin, chlorinate hydrocarbons, organophosphates, and specified nutrients. Specified nutrients analyses are on file at R.O.W. Sciences.
Water:	Tap water, provided ad libitum via an automatic watering system or water bottles. The water is analyzed at least two times per year for contaminants and specific microbes. The results of these analyses are on file at R.O.W. Sciences.
Contaminants:	The study director and/or the Sponsor have considered possible interfering substances potentially present in animal feed and water, including the test material itself or possible structurally related materials as well as the items listed in (2) and (3) above. None of these contaminants are reasonably expected to be present in animal feed or water at levels sufficient to interfere with this study.
Environment:	The targeted temperatures are between 64 and 79°F with a relative humidity between 30% and 70%. Temperature and humidity are monitored continuously. A 12-hour light/12-hour dark cycle will be maintained. Ten or greater air changes/hour will be maintained.
Acclimation:	Animals will be acclimated to the facility for a minimum of 7 days prior to the start of dosing. Animals will be observed for general health and suitability for testing during this period. Animals that are diseased or unsuitable for testing will be removed from the study.
Randomization:	Using computer-generated random numbers wit assignment to groups, At the time of randomization, the weight variation of the animals of each sex used should not exceed ± 2 S.D. of the mean weight, and the mean body weights for each group of each sex will not be statistically different.
Justification:	Rats will be used because of the extensive historical data base, and the FDA requirements for a rodent species.

Group Designations, Dietary Levels and Scheduled Sacrifice Time Points

Group Number (Time Point)	Number of Male Rats							
	Control (0 ppm)	N-EtFOSE 300	100	30 ppm	PFOS 20 ppm	PFOSA 100 ppm	Wy-14,643 1000 ppm	Total No. of Animals
1 (48 hrs)	10	10	10	10	10	10	5	65
2 (7 days)	10	5	5	5	5	5	5	40
3 (14 days)	10	5	5	5	5	5	5	40
4 (1 wk recovery)	10	5	5	5	5	5	5	40
5 (4 wk recovery)	10	5	5	5	5	5	5	40
Total No. of Animals	50	30	30	30	30	30	25	225

Dosing Procedures

Method of Administration

Dietary. Animals in Groups 1 through 3 will receive test diet for 48 hours, 7 days, and 14 days, respectively.

Animals in Groups 4 and 5 will receive test diet for 14 days followed by a 1 or 4 week recovery period, respectively.

Reason for Dosing Route

The potential human exposure is by the oral route.

Dose Preparation

Before initiation of treatment, dose preparation of each test material will be mixed. All dose preparations will be stored at room temperature. Dose preparation will be documented and reported. See Attachment I for test diet preparation procedures.

Retention Sample

Samples (approximately 100 g) will be taken from the dose preparation and stored at room temperature. Unless used for analyses, these samples will be discarded at least 1 month after completion of the in-life phase.

Observation of Animals

Clinical Observations

Each animal will be observed twice daily (a.m. and p.m.) for mortality and moribundity; findings will be recorded as they are observed.

Body Weights

Prior to treatment (at randomization), weekly for Week 1 through 4 weeks of recovery.

Food Consumption

Weekly for Week 1 through 4 weeks of recovery.

Clinical Chemistry

Animals will be fasted overnight before animal's scheduled necropsy; blood will be collected from a jugular vein into an EDTA-coated tube. Serum enzyme levels of alanine aminotransferase (ALT), alkaline phosphatase, aspartate aminotransferase (AST), cholesterol and triglycerides will be determined.

Termination

Unscheduled Sacrifices and Deaths

Necropsies will be done. Animals to be sacrificed will be anesthetized with CO_2, weighed, and exsanguinated.

Scheduled Sacrifices

Interim Sacrifices

At 48 hrs, 7 days, and 14 days, animals will be fasted overnight, bled for serum samples, anesthetized with CO_2, weighed, and exsanguinated.

NOTE: Two serum samples will be needed, (1) a 0.5 ml sample for clinical chemistry and (2) a 1.5 ml sample for compound level analysis.

The abdominal cavity of each animal will be opened, the liver will be removed and weighed, and liver samples will be collected. Animals will be discarded after liver collection.

Terminal Sacrifices

After 1 and 4 weeks of recovery, animals will be fasted overnight, bled for serum samples, anesthetized with CO_2, weighed, exsanguinated, and necropsied.

NOTE: Two serum samples will be needed, (1) a 0.5 ml sample for clinical chemistry and (2) a 1.5 ml sample for compound level analysis.

Postmortem Procedures

Necropsy

The necropsy will include an examination of the external features of the carcass; all external body orifices; the abdominal, thoracic, and cranial cavities; organs; and tissues.

Cell Proliferation Tissue Collection and Immunohistochemical Evaluation

Representative samples of the left lateral lobe of the liver and any macroscopic lesions of the liver will be collected and preserved in zinc formalin.

After fixation, each sample of liver will be delivered to:

Sandra R. Eldridge, Ph.D.
Pathology Associates International
15 Worman's Mill Court, Suite I
Frederick, Maryland 21701

Proliferation cell nuclear antigen (PCNA) evaluation will be done on the samples. In addition, liver sections prepared from the same tissue block will be stained with hematoxylin and eosin and examined microscopically.

Palmitoyl-CoA Oxidase Tissue Collection and Analyses

A sample (approximately 500 mg) of the right lateral lobe of the liver will also be collected from select animals and flash-frozen in liquid nitrogen. See Attachment II for procedure. The liver tissue will be stored in a freezer set to maintain -60 to -80° C until analyzed by Covance for palmitoyl-CoA Oxidase activity. The liver samples to be analyzed will include all study animals, EXCEPT for the Wy-14,643 animals and all animals from the 4-week recovery groups. In addition to this study, samples from a previous 3M study will be analyzed for palmitoyl-CoA Oxidase activity; these samples consist of liver samples from 35 rats and 35 guinea pigs.

Tissue Collection for Electron Microscopic Evaluation

Sections of liver from all animals will be collected, minced to approximately one millimeter cubes and placed in a fixative appropriate for electron microscopy. The containers and fixative will be provided by PAI. Electron microscopy will be performed on one animal per treatment group exhibiting the highest cell proliferative response as well as one control animal at the discretion of the Sponsor, from one time point as well as the 4-week recovery. Thus, EM will be performed on one animal from the control, N-EtFOSE (one dose only to be determined), PFOS, PFOSA, and Wy groups at one of the time points, as well as the 4 week recovery, for a total of 10 animals.

Remaining Liver Tissue

The remaining liver tissue will be frozen and stored at -60 to -80° C for possible future analysis.

Organ Weights

At the scheduled sacrifices, the liver will be weighed.

Histopathology

Liver from each animal that is examined for cell proliferation will be stained with hematoxylin and eosin, and examined microscopically for histopathologic changes.

Reports

One copy of the draft report will be sent to the Sponsor. The report will include the following information:

Experimental Design and Methods

Results

dose analyses
mortality
clinical observations
body weights
body weight changes
food consumption
test material consumption
clinical pathology results
palmitoyl-CoA oxidase activities
macroscopic observations
microscopic observations
ultrastructural observations
cell proliferation assessments

Record Retention

All raw data, documentation, records, protocol, specimens, and final report generated as a result of this study will be archived in the storage facilities of PAI for a period of 1 year following submission of the final report to the Sponsor. One year after submission of the final report, all of the aforementioned materials will be sent to the Sponsor and a return fee will be charged. All raw data stored on magnetic media will be retain by PAI.

PROTOCOL APPROVAL

Marvin T Case — 13 Jan 1999

Marvin Case, D.V.M., Ph.D.
Study Representative
3M Corporate Toxicology

Date

Gary W. Wolfe — 1/12/99

Gary W. Wolfe, Ph.D., D.A.B.T
Study Director
R.O.W. Sciences

Date

1-12-99

Sandra R. Eldridge, Ph.D.
Principle Investigator
Pathology Associates International

Date

Attachment: I
Test Diet Preparation Procedures

1) Determine the amount of test diet (feed) that is to be prepared and weigh out that amount of feed.
2) Calculate the amount of test article that is needed to prepare the test diet at the desired concentration.
3) Accurately weigh out the necessary amount of test article.
4) Transfer the weighed test article to a container and add a small volume of acetone to container. Manually mix to dissolve the test material adding acetone as necessary (typical ratio of test material to acetone is 1 g: 15-20 ml acetone). Visually inspect test material/acetone for solubility of test material.
5) Prepare a pre-mix by transferring the dissolved test material into 4 kg of feed in a Hobart mixing bowl. Mix for 10 minutes. Transfer the premix to a larger mixer, add remaining amount of weighed diet, mix for 30 minutes.

Attachment: II

Collection of Tissue Samples for Biochemical and Molecular Analysis

Because of the extreme instability of certain enzymes and biomolecules, it is essential that tissues be harvested as soon after death as possible and flash frozen immediately in liquid nitrogen. Failure to follow these procedures may lead to loss of the entire sample and all the energies and resources that were invested into generating the samples. Therefore, make every effort to comply with the following:

1) Harvest the tissue samples as soon as possible after death. Delays may allow for biodegradation and/or inactivation of the desired endpoint.
2) Immediately submerse the tissue sample directly into liquid nitrogen. Dry ice or other alternatives will not suffice. It is important that the tissue be immersed directly in liquid nitrogen Transferring it to a dry vessel (or sample container) suspended in liquid nitrogen will not suffice. The tissue may freeze to the vessel wall and will then be impossible to remove without completely destroying the vessel (or sample container).
3) Be absolutely sure to maintain the tissue frozen. It should be stored in a sealed container at -70°C and shipped or transferred on dry ice. If needed, the frozen sample can be fractured (broken into portions for different applications) by placing in a crucible which contains liquid nitrogen to keep the sample frozen while grinding/fracturing.