COVANCE
THE DEVELOPMENT SERVICES COMPANY

Sponsors:

APME Ad-Hoc APFO Toxicology Working Group

PROTOCOL

Study Title:

26-Week Capsule Toxicity Study with Ammonium Perfluorooctanoate (APFO) in Cynomolgus Monkeys

Date:

September 23, 1998

Performing Laboratory:

Covance Laboratories Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704-2595

Laboratory Study Identification:

Proposal No. w6806a

Covance 6329-231

T- 68893

Study
26-Week Capsule Toxicity Study with Ammonium Perfluorooctanoate (APFO) in Cynomolgus Monkeys

Purpose
To assess the effect of the test material on critical enzyme levels, hormones, and other selected biochemical parameters when administered daily by capsule to cynomolgus monkeys for at least 26 weeks

Sponsors
APME Ad-Hoc APFO Toxicology Working Group

Study Monitor
Paul Lieder, PhD, DABT
3M
Toxicology Services
Building 220-2E-02, 3M Center
St. Paul, Minnesota, 55144-1000
Telephone No.: 612.737.2678
Facsimile No.: 612.733.1773

Alternate Study Monitor
John Butenhoff, PhD, DABT
3M
Telephone No.: 612.733.1962
Facsimile No.: 612.733.1733

Sponsor's Representative
David Farrar, PhD
ICI Chemicals & Polymers Limited
Occupational Health
The Heath, Runcorn, PO Box 13
Cheshire, WA7 4QF
Telephone No.: 01928.513953
Facsimile No.: 01928.515146

Study Location
Covance Laboratories Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Mailing Address: PO Box 7545-2595
Madison, Wisconsin 53707-7545

Study Director
Peter J. Thomford, PhD
Covance Laboratories Inc.
Telephone No.: 608.241.7207
Facsimile No.: 608.242.2736

Toxicologist
Dale Aldridge, BS
Covance Laboratories Inc.

Proposed Study Timetable
Experimental Start Date: September 29, 1998
In-Life Start Date: September 29, 1998
In-Life End Date: July 2, 1999
Audited Draft Report Date: October 22, 1999
Experimental Termination Date: To be determined

Regulatory Compliance
This study will be conducted in compliance with the Environmental Protection Agency Good Laboratory Practice Regulations as set forth in Title 40 of the US Code of Federal Regulations, Part 792, issued November 29, 1983 (effective December 29, 1983), and with any applicable amendments except that bile acid determinations done by University of Dundee will not be done in compliance with GLPs.

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
The protocol, study conduct, and final report will be audited by the Covance Quality Assurance Unit (QAU). The proliferation cell nuclear antigen evaluation data and report will be audited by the QAU of Pathology Associates International. The serum, urine, feces, and liver APFO analysis and report will be audited by the QAU of 3M Environmental Laboratories. The blood hormone determinations and report will be audited by the QAU of Ani Lytics Inc and DuPont, as appropriate. The receptor level determinations and report will be audited by the QAU of DuPont. Bile acid determinations done by the University of Dundee will not be audited.

Test Material

Identification
Ammonium Perfluorooctanoate (APFO)

Lot Number
The lot number will be maintained in the raw data.

Purity
Responsibility of the Sponsor

Stability
Responsibility of the Sponsor

Storage Conditions
At room temperature

Characteristics
Information on synthesis methods, composition, or other characteristics that define the test material is on file with the Sponsor.

Gelatin Capsules
Capsules (Size No. 2) obtained from Torpac, Inc. (Fairfield, New Jersey); lot number will be supplied by the manufacturer. Gelatin capsules will be stored at room temperature. A copy of the Certificate of Analysis provided by the manufacturer will be maintained in the data.

Reserve (Archive) Samples
A reserve sample of each lot of test material (1 g each) will be taken and stored at room temperature. These samples will be transferred to the Sponsor after completion of the in-life phase.

Disposition of Test Material
Any remaining test material will be returned after authorization from the Sponsor.

Animals

Species
Cynomolgus monkey

Source
Covance Research Products Inc.

Age at Initiation of Treatment
Young adult/adult

Weight at Initiation of Treatment
3 to 5 kg

Number and Sex
22 males

Identification
Collar tag

Husbandry

Housing
Individual; animals will be housed in suspended, stainless steel cages.

Diet
Certified primate diet (#8726C, Harlan Teklad) once or twice daily. The diet is routinely analyzed by the manufacturer for nutritional components and environmental contaminants. Results of specified nutrient and contaminant analyses are on file at Covance-Madison. Fruit, vegetables, or other supplements may be provided but will not require analysis.

Water
Ad libitum. Samples of the water are routinely analyzed for specified microorganisms and environmental contaminants. The results are on file at Covance-Madison.

Contaminants
There are no known contaminants in the diet or water at levels that might interfere with this study.

Environment
Environmental controls for the animal room will be set to maintain 18 to 29°C, a relative humidity of 30 to 70%, and a 12-hour light/12-hour dark cycle.

Acclimation
Minimum of 4 weeks

Randomization
Animals will be weighed, stratified by body weight, and allocated to the number of blocks equal to the number of animals to be selected for each group. Animals in each block will then be assigned to groups using computer-generated random numbers.

Justification
APFO is a known hepatic peroxisome proliferator (PP) in the rat. When exposed to PP, nonhuman primates (such as the cynomolgus monkey) respond similarly to humans (i.e., low to no hepatic response) and therefore are an appropriate human surrogate species.

Group Designations and Dosage Levels

Group	Dose Level (mg/kg/day)	Number of Males
1 (control)	0[a]	6[b]
2 (low-dose)	3	4
3 (mid-dose)	10	6[b]
4 (high-dose)	30	6[b]

a The control group (Group 1) will receive empty gelatin capsules.
b Two animals in Groups 1, 3, and 4 designated as recovery animals will be treated for 26 weeks, then treatment will be discontinued, and the animals will be observed for reversibility, persistence, or delayed occurrence of toxic effects for at least 13 weeks posttreatment. Based on plasma test material levels and clinical pathology findings, the recovery may be reduced or extended at the discretion of the study director after consultation with the Sponsors.

Dosing Procedures

Method of Administration
Orally by gelatin capsules, daily (7 days/week) for at least 26 weeks

Reason for Dosing Route
To compare with previously conducted toxicology studies using the oral route

Dose Preparation
Capsules will be prepared at least weekly. The size and number of capsules will depend on the physical characteristics of the test material, the dose level, and the weight of the monkey. Dose levels will be based on the test material as supplied. Individual daily doses will be based on the most recent individual body weights, with the exception of body weight collection days when the previous body weight will be used. All dose preparations will be stored at room temperature until dosed.

Dose Analysis
Because the test material is not being mixed with a vehicle, dose analyses are not required.

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.

Each animal will be observed daily and food consumption will be assessed qualitatively; abnormal findings will be recorded. Once weekly, each animal will be observed; abnormal findings, or an indication that the animal is normal will be recorded.

Additional findings will be recorded as they are observed.

Body Weights
Weekly before initiation of treatment, Day -1 (for the Day 1 dose calculations),on the first day of treatment, and weekly thereafter

Ophthalmic Examinations
Before initiation of treatment and before each scheduled sacrifice. The anterior portion of the eye, optic media, and ocular fundus will be examined using an indirect ophthalmoscope.

Clinical Pathology

Frequency

Unscheduled Collections
When possible, blood will be collected for clinical pathology tests from animals sacrificed at unscheduled intervals

Scheduled Collections
Before initiation of treatment; and (before the daily dose) after at least 30, 60, 90, and 180 days of treatment; and after at least 30, 60, and 90 days of recovery

Number of Animals
All

Method of Collection
Animals will be fasted overnight; blood will be collected from a femoral vein. Anticoagulants will be sodium citrate for coagulation tests and potassium EDTA for hematology tests. No anticoagulant is required for clinical chemistry tests. Urine will be collected on wet ice overnight (approximately 16 hours).

Tests

Hematology

red blood cell (erythrocyte) count	platelet count
hemoglobin	white blood cell (leukocyte) count
hematocrit	differential blood cell count
mean corpuscular volume	blood cell morphology
mean corpuscular hemoglobin	reticulocyte count
mean corpuscular hemoglobin concentration	

Coagulation

activated partial thromboplastin time	prothrombin time
	fibrinogen

Clinical Chemistry

glucose	aspartate aminotransferase
urea nitrogen	gamma glutamyltransferase
creatinine	sorbitol dehydrogenase
total protein	creatine kinase
albumin	calcium
globulin	inorganic phosphorus
total bilirubin	sodium
direct bilirubin (if total bilirubin is greater than 2.0 mg/dL)	potassium
	chloride
cholesterol	bile acids
triglycerides	amylase
alanine aminotransferase	lipase
alkaline phosphatase	pancreatic-specific amylase

Urinalysis

appearance	glucose
volume (approximately 16 hours)	ketones
specific gravity	bilirubin
pH	blood
protein	microscopic examination of sediment
urobilinogen	

Blood Hormone Determination

Frequency
Three times before initiation of treatment; after at least 30, 60, 90, and 180 days of treatment; and after at least 30, 60, and 90 days of recovery

Number of Animals
All

Method of Collection
Animals will not be fasted; blood will be collected from a femoral vein (an alternative vein may be used if necessary). Approximately 6 mL of blood for plasma samples will be collected into tubes with potassium EDTA as the anticoagulant. Approximately 6 mL of blood for serum samples will be collected without anticoagulant and allowed to clot. Blood samples will be maintained at room temperature until serum is harvested. Blood samples will be maintained chilled until plasma is harvested.

Serum Sample Handling
Samples for serum will be centrifuged within 1 hour after collection, and serum will be harvested. Serum will be divided into two approximately equal aliquots and stored in a freezer set to maintain -60 to -80°C until packed on dry ice and shipped to:

Dr. Saroj Das
Ani Lytics Inc.
200 Girard Street, Suite 200
Gaithersburg, Maryland 20877
Telephone No.: 301.921.0168
Facsimile No.: 301.977.0433

Ani Lytics Inc. will be notified regarding shipment of samples.

Serum Tests
Samples will be analyzed by Ani Lytics Inc., for estradiol, estrone, estriol, thyroid stimulating hormone, total and free triiodothyronine (T3), and total and free thyroxin (T4). Results will be provided for inclusion in the final report.

Plasma Sample Handling
Samples for plasma will be centrifuged within 1 hour after collection. Plasma will be harvested and stored in a freezer set to maintain -60 to -80°C until packed on dry ice and shipped to:

Jon C. Cook, PhD, DABT
E. I. du Pont de Nemours & Company, Inc.
Haskell Laboratory for Toxicology and Industrial Medicine
Building 11 Room 1325
1090 Elkton Road
Newark, Delaware 19711-0500
Telephone No.: 302.366.5495
Facsimile No.: 302.366.5003

DuPont will be notified regarding shipment of samples.

Plasma Analysis
Samples will be analyzed by DuPont Haskell Company for cholecystokinin. Results will be provided for inclusion in the final report.

Serum APFO Level Determination

Frequency
After 7 days of treatment and every 2 weeks thereafter during treatment and recovery

Number of Animals
All

Method of Collection
Animals will not be fasted; blood (approximately 2 mL) will be collected from a femoral vein (an alternative vein may be used if necessary) without an anticoagulant.

Sample Handling
Samples will be centrifuged within 1 hour after collection, and serum will be harvested. Serum samples will be stored in a freezer set to maintain -10 to -30°C.

Sample Shipping
Serum samples will be packed on dry ice and shipped to:

Kris J. Hansen, PhD
3M E.T. & S
Bldg. 2-3E-09
935 Bush Avenue
St. Paul, Minnesota 55106

Kris J. Hansen, PhD or her alternate will be notified regarding the shipment of the samples. Analysis of serum for APFO will be done on the samples. Results will be provided for inclusion in the final report.

Additional Blood Collection
At scheduled and unscheduled sacrifices, blood (as much as possible, up to 20 mL) will be collected from animals at the time of exsanguination. Approximately equally sized samples of serum (collected without anticoagulant) and whole blood and plasma (both collected using EDTA as an anticoagulant) from each animal will be transferred into containers (approximately 5 mL each). Whole blood, serum, and plasma samples will be stored in a freezer set to maintain -60 to -80°C until shipped to 3M (Kris Hansen) for possible future analysis.

Urine and Feces APFO Level Determination

Frequency
After 7 days of treatment and every 2 weeks thereafter during treatment and recovery (concurrent with serum APFO sample collection)

Number of Animals
All

Method of Collection
Animals will not be fasted; urine (collected on wet ice) and feces will be collected overnight.

Sample Handling
Samples of urine (at least 2 mL) and feces (at least 5 grams) will be stored in a freezer set to maintain -10 to -30°C.

Sample Shipping
Samples will be packed on dry ice and shipped to:

Kris J. Hansen, PhD
3M E.T. & S
Bldg. 2-3E-09
935 Bush Avenue
St. Paul, Minnesota 55106

Kris J. Hansen, PhD or her alternate will be notified regarding the shipment of the samples. Analysis of urine and feces for APFO will be done on the samples. Results will be provided for inclusion in the final report.

Termination

Unscheduled Sacrifices and Deaths
Necropsies will be done. Animals to be sacrificed will be anesthetized with ketamine and xylazine, weighed, and exsanguinated.

Scheduled Sacrifices
After at least 26 weeks of treatment, four animals/sex/group will be fasted overnight, then anesthetized with ketamine and xylazine, weighed, exsanguinated, and necropsied.

After at least 26 weeks of treatment and at least 13 weeks without treatment, the remaining animals will be fasted overnight, then anesthetized with ketamine and xylazine, weighed, exsanguinated, and necropsied.

Postmortem Procedures

Necropsy
The necropsy will include examination of:

all orifices
cranial cavity
external surface of the brain; the external surface of the spinal cord and cut surfaces of the brain and spinal cord will be examined whenever tissue trimming is performed
cervical tissues and organs
thoracic, abdominal, and pelvic cavities and viscera
external surface of the body
nasal cavity and paranasal sinuses

Organ Weights
From each animal at scheduled and unscheduled sacrifices, the following organs (when present) will be weighed; paired organs will be weighed separately:

adrenal (2)	liver
brain	pancreas
epididymis (2)	testis (2)
kidney (2)	thyroid (2) with parathyroid

Organ-to-body weight percentages and organ-to-brain weight ratios will be calculated.

Palmitoyl CoA Oxidase Determinations
The right lateral lobe of liver will be collected from each animal at the scheduled and unscheduled sacrifices, weighed, flash-frozen in liquid nitrogen, and stored in a freezer set to maintain -60 to -80°C until disposition of the samples is determined by the Sponsor.

Cell Proliferation Evaluation
Representative samples of left lateral lobe of the liver, left and right testes, and pancreas will be collected from each animal at the scheduled and unscheduled sacrifices, and preserved in zinc formalin.

After fixation, samples for proliferation cell nuclear antigen (PCNA) evaluation will be embedded in paraffin maintained at ambient temperature until disposition of the samples is determined by the Sponsor.

Bile Acid Determination
All available bile (up to 5 mL) will be collected from each animal at the scheduled and unscheduled sacrifices, flash-frozen in liquid nitrogen, and stored in a freezer set to maintain -60 to -80°C. Bile samples will be packed on dry ice and shipped to:

Biomedical Research Centre
Level 5
Ninewells Hospital and Medical School
University of Dundee
Dundee DD1 9SY
UK
Dr. Cliff Elcombe
Biomedical Research Centre
Ninewells Hospital and Medical School
University of Dundee
Dundee DD1 9SY, UK
Telephone: +44 (0)1382-632641
Fax: +44 (0)1382-425586

Results of bile acid determinations analyses will be provided for inclusion in the final report.

Receptor Level Determination
Samples (approximately 2 g) of liver and pancreas will be collected from each animal at the scheduled and unscheduled sacrifices, flash-frozen in liquid nitrogen, and stored in a freezer set to maintain -60 to -80°C. Liver and pancreas samples will be packed on dry ice and shipped to:

Jon C. Cook, PhD, DABT
E. I. du Pont de Nemours & Company, Inc.
Haskell Laboratory for Toxicology and Industrial Medicine
Building 11 Room 1325
1090 Elkton Road
Newark, Delaware 19711-0500
Telephone No.: 302.366.5495
Facsimile No.: 302.366.5003

DuPont will be notified regarding shipment of samples. Results of receptor level determinations will be provided for inclusion in the final report.

Tissue Preservation
Three samples (approximately 5 g each) of the liver and all remaining pancreas and left and right testes tissue, if any, (divided into three approximately equal samples) will be collected from each animal at the scheduled and unscheduled sacrifices, weighed, flash-frozen in liquid nitrogen, and stored in a freezer set to maintain -60 to -80°C for possible future analysis.

The following tissues (when present) from each animal will be preserved in 10% neutral-buffered formalin, unless otherwise specified, for possible future microscopic examination:

adrenal (2)
aorta
brain
cecum
colon
duodenum
epididymis (2)
esophagus
eye [(2) to be preserved in Davidson's fixative from sacrificed animals only]
femur with bone marrow (articular surface of the distal end)
gallbladder
heart
ileum
jejunum
kidney (2)
lesions
liver
lung
mammary gland
mesenteric lymph node
pancreas
pituitary
prostate
rectum
salivary gland [mandibular (2)]
sciatic nerve
seminal vesicle (2)
skeletal muscle (thigh)
skin
spinal cord (cervical, thoracic, and lumbar)
spleen
sternum with bone marrow
stomach
testis [(2) to be preserved in Bouins' solution from sacrificed animals only]
thymus
thyroid (2) with parathyroid
trachea
urinary bladder

Bone Marrow Smear
From the sternum of each animal at unscheduled and scheduled sacrifices (made and held for possible future examination)

Liver APFO Determination
A sample of liver (any non-formalin treated liver remaining after sampling for histopathology) will be collected from each animal at the scheduled and unscheduled sacrifices, weighed, flash-frozen in liquid nitrogen, and stored in a freezer set to maintain -60 to -80°C until shipped with serum samples to 3M (Kris Hansen) for analysis. Analysis of liver for APFO will be done on the samples. Results will be provided for inclusion in the final report.

Histopathology
Tissues from each animal in the control and high-dose groups and each animal that dies or is sacrificed at unscheduled interval will be embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically.

The adrenals, pancreas, liver, spleen, and testes from each animal in the low- and mid-dose groups will be embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically.

Suspected target organs noted at the high-dose will be embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically from all animals (at the Sponsor's request and added by amendment).

Reports
A draft report which includes the following information will be prepared and submitted:

Experimental Design and Methods

Results
mortality
clinical observations
body weights
body weight gains
food consumption
ophthalmic examination results
clinical pathology results
hormone analyses (provided by Ani Lytics, Inc. and the DuPont Haskell Company)
serum, urine, feces, and liver APFO levels (provided by 3M)
organ weights
organ-to-body weight percentages
organ-to-brain weight ratios
macroscopic observations
microscopic observations
palmitoyl CoA oxidase activities (provided by the Sponsor's designee)
cell proliferation evaluations (provided by Pathology Associates International)
bile acid determinations (provided by University of Dundee)
receptor determination (provided by DuPont)

Statistical Evaluation

Levene's test will be done to test for variance homogeneity. In the case of heterogeneity of variance at $p \leq 0.05$, transformations will be used to stabilize the variance. Comparison tests will take variance heterogeneity into consideration.

One-way analysis of variance (ANOVA) will be used (if applicable) to analyze initial body weights, body weight gains, continuous clinical pathology values, palmitoyl CoA oxidase activities, and organ weight data. If the ANOVA is significant, Dunnett's t-test will be used for control versus treated group comparisons.

One-way analysis of covariance (ANCOVA) will be used to analyze body weights, with initial body weights as the covariate. If the ANCOVA is significant, covariate-adjusted means will be used for control versus treated group comparisons.

Group comparisons (Groups 2 through 4 versus Group 1) will be evaluated at the 5.0% two-tailed probability level. Only data collected on or after the first day of treatment will be analyzed statistically. Data collected before the first day of treatment or during recovery will not be analyzed statistically.

At the end of 1 year after issuance of the audited draft report, if no requested revisions or instructions to finalize have been communicated by the Sponsor, then the audited draft report will be considered 'final' and issued as the final report, signed by the study director, and submitted to the Sponsor.

Any modifications or changes to the audited draft report requested 1 year after issuance will be performed at additional cost to the Sponsor.

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 Covance-Madison 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. The Sponsor may elect to have the materials retained in the Covance archives for an additional period of time, and Covance will charge a storage fee. If the Sponsor chooses to have Covance dispose of the materials, a disposal fee will be charged. All raw data stored on magnetic media will be retained by Covance.

Within one year after submission of the final report, all of the aforementioned materials from the Sponsor's designees (Ani Lytics Inc., E. I. du Pont de Nemours & Company, Inc., 3M E.T. & S, Pathology Associates International, and University of Dundee) will be sent to the Sponsor (Paul Lieder, PhD, DABT, 3M) to be maintained.

PROTOCOL APPROVAL

Paul W. Lieder — 9/25/98

Paul Lieder, PhD
Diplomate, ABT
Study Monitor
3M

Date

David Farrar, PhD
ICI Chemicals & Polymers Limited

Date

Peter J. Thomford — 9/23/98

Peter J. Thomford, PhD
Study Director
Department of Toxicology
Covance Laboratories Inc.

Date