UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

MAR 6 1996

OFFICE OF PREVENTION, PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Review of Mammalian Toxicology Studies Submitted by AgriDyne Technologies, Inc. in Support of the Registration of Azadirachtin End-Use Products, Azatin EC, Azatin-Plus EC, and Azatin Technical (ID# 62552-1, 62552-2, and 62552-3, respectively; DP Barcodes D199083, D201475, D201498, D201500, D201502, D201481, D201485; Submissions S454609, S458922, S462493, S462496, S462493, S458927, S458928)

TO: Paul L. Zubkoff, Ph.D.
Regulatory Action Leader
Biopesticides and Pollution Prevention Division (7501W)

FROM: Sheryl K. Reilly, Ph.D., Reviewer SKR 2/1/96
Biopesticides and Pollution Prevention Division (7501W)

THRU: J. Thomas McClintock, Ph.D., Team Leader JTMc 3/6/96
Biopesticides and Pollution Prevention Division (7501W)

ACTION REQUESTED: Review of mammalian toxicology studies submitted to support the registration of Azatin EC, Azatin-Plus EC, and Azatin Technical.

CONCLUSIONS: The studies are summarized below.

NO MRID #: Acute Oral Toxicity in Rats (§152-10)
In an acute oral toxicity study, Sprague-Dawley rats (5/sex) were give a limit dose of ATI-720 by gavage. Within the first 3 days post-dosing, 6/10 animals died (3/sex). Clinical signs included hypoactivity, labored breathing, hunched posture, and discoloration around the nose and mouth. Surviving rats appeared normal from day 4 post-dosing to the end of the 14-day study. The acute oral LD_{50} for ATI-720 in rats is 5 g/kg. This study is **acceptable**, and places the test material in **Toxicity Category III.**

MRID # 430148-01: Acute Inhalation Toxicity in Rats (§152-12)
In an acute inhalation toxicity study, Sprague-Dawley rats (5/sex) were exposed to an

aerosol of NPI-720-F for 4 hours. No deaths occurred during the study. Body weight loss for 4 males and 1 female on day 8 post-exposure was noted. All animals gained weight by the end of the observation period, but 1 male did not return to its pre-study level by day 14 of the study. Other clinical signs included redness around the eyes and nose, salivation, nasal congestion, rales, wheezing, and wet/discolored inguinal area. All clinical signs (except 1 rat with discolored inguinal fur) resolved by the end of the study. The LC_{50} (aerosol) in rats for NPI 720-F is > 2.41 mg/L. The study is **acceptable,** and places the test material in **toxicity category IV.**

MRID 431130-01 90-Day Feeding Study in Rats (§152-20)
Sprague-Dawley rats (10/sex/group) were fed diets which contained Azatin EC (7.74% azadirachtin, the active ingredient) at concentrations of 29.6, 145.2, and 584.6 mg/kg/day (equivalent to 2.3, 11.2, and 45.3 mg/kg/day a.i.) for males, and 34.5, 177.6 and 680.2 mg/kg/day (2.7, 13.8, and 52.7 mg/kg/day a.i.) for females for 90 days.

Both sexes exhibited decreased body weight gain at the highest dose from week 3 in males and week 4 in females. This persisted through the rest of the study, and was attributed to unpalatability of the diet. Females at the highest two doses exhibited elevated absolute and relative liver weights, compared with the control animals. Females in the 145.2 mg/kg dose group and both sexes at the highest dose group exhibited increased GGT activity, but this did not correspond to any histopathological changes in the liver. Significant hematological changes in the highest dose group animals included decreased mean cell volume (% of controls) of approximately 95% (both sexes); mean cell hemoglobin (92% in males, 95% in females); hemoglobin (95% in females only) and hematocrit (95% in females only).

Based on the liver effects, the LOEL value was 11.2 mg a.i./kg/day in males and 13.8 mg a.i. /kg/day in females. The NOEL value was 2.3 mg/kg/day a.i. in males and 2.7 mg/kg/day, in females. The study is coregraded **minimum.**

MRID 431627-01 Tier I Immunotoxicity Study in Mice (§152-18)
ATI-720 was fed to groups of 40 female mice at dietary levels of 500, 1250, or 5000 ppm (approximately 112, 295, or 1100 mg/kg body weight/day) and control diets (0 ppm) for 30 days. No compound-related effects were observed. No mortalities occurred and body weight gains, hematology, and clinical chemistry parameters were unaffected. Gross findings were unremarkable, and ATI-720 had no effect on spleen or thymic weights or bone marrow evaluations. The test compound had no effect on spleen or thymic cellularity.

No effects were observed in the anti-SRBC plaque-forming cell (PFC) assay; a significant (<0.05) decrease in tumor cell lysis was observed in the NK cell activity at the lowest dose tested (500 ppm), but only at the highest (100:1) effector:target cell ratio. The significance of this finding is unclear. In the cytotoxic T-lymphocyte (CTL) assay, lysis of target cells was markedly reduced at all dose levels; however, a positive control group (with a known immunosuppressant) was not included in the study. No effects on peritoneal macrophage numbers, viability or function were observed in the treated animals.

The study is **supplementary but upgradeable**, pending receipt of spleen cell viability data. The test substance appears to be **immunosuppressive to cytotoxic T lymphocytes**. Before a decision can be made as to whether Tier II testing is required for the test substance, the registrant must submit the spleen cell viability data. If a positive control group was tested in the CTL assay, or if a satellite recovery group in the present study was tested, that data must be submitted in order to determine if an appropriate host-resistance test would be required for the test substance.

MRID 429458-02 Genotoxicity: Unscheduled DNA Synthesis in Rats (§ 152-17).
ATI-720 was negative for the induction of unscheduled DNA synthesis in cultured rat hepatocytes at 1250, 2500, and 5000 mg/kg (139-555 mg/kg a.i.). The study is **acceptable**.

MRID 429458-01 Genotoxicity: In vivo Mammalian Cytogenetic Chromosome Aberration Assay in Mice (§ 152-17).
ATI-720 was negative for the induction of structural chromosome aberrations in bone marrow cells harvested from CD mice (both sexes) following oral administration at doses up to 5000 mg/kg (555 mg/kg a.i.). The study is **acceptable.**

The data evaluation reports are attached.

cc: J. Thomas McClintock
S. Reilly
P. Zubkoff
M. Locklear - Chronological file

S. Reilly/7501W/703-308-8265/agriaza.mem/azadirachtin (azatin technical)

EPA Reviewer: Sheryl Reilly, Ph.D. SKR 1/29/96
Secondary Reviewer: J. Thomas McClintock, Ph.D. JTMc 3/5/96
Biopesticides and Pollution Prevention Division

DATA EVALUATION REPORT

STUDY TYPE:	Guideline series 152-10; acute oral toxicity in rats
TOX CHEM NUMBER:	121701
MRID NUMBER:	Not assigned an MRID #
TEST MATERIAL:	ATI-720
SPONSOR:	AgriDyne Technologies, Inc. Salt Lake City, UT
STUDY NUMBER:	IITRI Project No. L08425
TESTING FACILITY:	IIT Research Institute, Life Sciences Research Chicago, IL
TITLE OF REPORT:	Acute Oral Toxicity Study of ATI-720 in Rats (Limit Test)
AUTHOR:	William D. Johnson
DATE OF REPORT:	March 11, 1993
QUALITY ASSURANCE:	The test was performed under Good Laboratory Practice Standards. A Quality Assurance Statement, signed March 11, 1993, was submitted.
EXECUTIVE SUMMARY:	In an acute oral toxicity study, Sprague-Dawley rats (5/sex) were give a limit dose of ATI-720 by gavage. Within the first 3 days post-dosing, 6/10 animals died (3/sex). Clinical signs included hypoactivity, labored breathing, hunched posture, and discoloration around the nose and mouth. Surviving rats appeared normal from day 4 post-dosing to the end of the 14-day study. The acute oral LD_{50} for ATI-720 in rats is 5 g/kg. This study is **acceptable**, and satisfies the guideline requirements for an acute oral toxicity study. Toxicity Category III.

A. MATERIALS

Test Compound

Test material:	ATI-720
Ident. number:	Lot no. 1111-37
Purity:	20% active ingredient (azadirachtin)
Physical description:	Yellow powder
Storage conditions:	Refrigeration (3°C)
Stability:	Not reported
Dose levels:	5 g/kg body weight (gavage)
Dosing volume:	10 ml/kg body weight of a 250 mg/ml suspension in 1% aqueous carboxymethylcellulose

Controls: There were no control animals.

Test Animals

Species:	Rat
Strain:	Sprague-Dawley (Crl:CD^{R}BR)
Source:	Charles River Laboratories, Portage, MI
Age at receipt:	6 weeks
Weight at receipt:	135-162 g
No. animals/dose:	10 (5/sex)
Acclimation:	7 days
Housing:	Individually
Food:	Purina Rodent Chow 5001, *ad libitum*
Water:	Purified water, *ad libitum*
Temperature:	22°C
Humidity:	31%
Photoperiod:	12 hours dark/12 hours light

B. TEST PERFORMANCE

Animals fasted:	18 hours before and 4 hours post-dosing
Dosing:	Gavaged twice (one hour apart) with a constant volume (10 ml/kg) of the test material
Observation period:	2 weeks
Observation frequency:	At least once per day
Body weight interval:	Prior to fasting, immediately before dosing, and at days 7 and 14 post-exposure
Gross pathology:	Yes
Histopathology:	No

C. RESULTS

Mortality

Mortality is summarized in Table 1. As shown, deaths occurred 1-3 days following dosing. Surviving rats appeared normal within 2-4 days post-dosing through the end of the observation period.

Table 1. Mortality

Dose (g/kg)	Number Dead/Number Tested Males	Females	Combined
5	3/5	3/5	6/10

Clinical observations

Treatment-related clinical signs of toxicity in both sexes included hypoactivity, hunched posture, labored breathing, coldness to the touch, ptosis, lacrimation, salivation, redness around the nose and eyes, wet and/or discolored inguinal fur, rough hair coat, and discolored paws.

Body weights: Body weight gain was normal.

Gross necropsy

Necropsy of the six rats that died on test revealed pale livers (6/6), mottled livers (1/6), red lungs (5/6), and redness of the brain (2/6). Necropsy of the four surviving rats was unremarkable.

LD_{50} determination

The estimated acute oral LD_{50} in rats for ATI-720 was 5 g/kg.

D. CONCLUSION

Acute oral exposure of rats to 5 g/kg ATI-720 resulted in 60% (6/10) mortality; therefore, the acute oral LD_{50} is approximately 5 g/kg; this places ATI-720 into Toxicity Category III. Significant treatment-related signs of toxicity included hypoactivity, hunched posture, labored breathing, and coldness to the touch.

This study is acceptable and satisfies the guideline requirements for an acute oral toxicity study.

EPA Reviewer: Sheryl Reilly, Ph.D. SR 1/29/96
Secondary Reviewer: J. Thomas McClintock, Ph.D. JTMc 3/6/96
Biopesticides and Pollution Prevention Division

DATA EVALUATION REPORT

STUDY TYPE: Subdivision M Guideline series 152-12; acute inhalation toxicity in rats

TOX CHEM NUMBER: 121701

MRID NUMBER: 430148-01

TEST MATERIAL: NPI 720-F (Azadirachtin)

SPONSOR: AgriDyne Technologies, Inc. (Native Plants, Inc.) Salt Lake City, UT

STUDY NUMBER: IITRI project no. L08270; study no. L06-1

TESTING FACILITY: IIT Research Institute
Life Sciences Research Department
Chicago, IL

TITLE OF REPORT: Acute Inhalation Toxicity Study of NPI 720-F in Rats. Final Report Amendment No. 2

AUTHOR OF AMENDMENT: Catherine Aranyi

STUDY COMPLETED: July 26, 1990

REPORT AMENDMENT DATE: July 21, 1993

QUALITY ASSURANCE: The test was performed under Good Laboratory Practice Standards. A Quality Assurance Statement, signed July 21, 1993, was submitted.

EXECUTIVE SUMMARY: In an acute inhalation toxicity study, Sprague-Dawley rats (5/sex) were exposed to an aerosol of NPI-720-F for 4 hours. No deaths occurred during the study. Body weight loss for 4 males and 1 female on day 8 post-exposure was noted. All animals gained weight by the end of the observation period, but 1 male did not return to its pre-study level by day 14 of the study. Other clinical signs included redness around the eyes and nose, salivation, nasal congestion, rales, wheezing, and wet/discolored inguinal area. All clinical signs (except 1 rat with discolored inguinal fur) resolved by the end of the study. The LC_{50} (aerosol) in rats for NPI 720-F is > 2.41 mg/L. **Acceptable. Toxicity Category IV.**

A. MATERIALS

Test Compound

Test material:	NPI 720-F (azadirachtin, active ingredient)
Source:	AgriDyne Technologies, Inc.
Identification no.:	Sponsor lot no. 13
Purity:	2.7% azadirachtin
Physical description:	Thick, brown viscous liquid
Storage:	Room temperature in closed glass containers
Stability:	Not reported
Exposure level:	Target: 5 mg/L aerosol; achieved: 2.41 mg/L
Exposure period:	Single 4-hour exposure, whole body
Observation period:	Two weeks

Controls

There were no control animals.

Test Animals

Species:	Rat
Strain:	Sprague-Dawley derived
Source:	Charles River Laboratories, Portage, MI
Age at exposure:	8 weeks
Mean body weight (at exposure):	258 g for males; 199 g for females
No. animals:	5/sex
Temperature:	20-26 °C (target range)
Relative humidity:	30-61% (target range)
Photoperiod:	12 hours dark/12 hour light cycle
Feeding:	Purina Rodent Chow 5001 pellets, *ad libitum*
Water:	Tap water, *ad libitum* (except during exposure)
Acclimation period:	1.5 weeks
Housing:	Individually
Selection:	Random

B. TEST PERFORMANCE

Inhalation chamber

A stainless steel, 0.5 m^3 inhalation exposure chamber was used.

Generation of test atmospheres

The aerosol atmosphere was generated by a gravity-fed Laskin Aerosol Generator, which was modified to accommodate the viscosity of the test material. The generator consisted of a 1/4 inch diameter stainless steel tube (generator body), about one foot long, which was placed in the center of the exposure chamber about ten inches above the rats. The generator body was connected to compressed air at one end and was sealed at the other end. Stainless steel capillary tubes were inserted into holes above the sealed end. Teflon tubes were attached to each capillary tube and were inserted into 500 mL Erlenmeyer flasks that were placed at the top of the exposure chamber to allow the test material to flow down the teflon tubes into the aerosol generator. Aerosolization of the test material was achieved by forcing compressed air through the generator body, which created a high velocity Venturi effect that drew the test material into the air stream and aerosolized it. However, the viscosity of the test material precluded the generation of an aerosol concentration > 2.41 mg/L.

Analytical determinations

Chamber total aerosol concentrations were determined gravimetrically by collecting 3-liter volumes of the test atmosphere from the rats' breathing zone on 25-mm Gelman glass fiber filters. Samples were

collected hourly, beginning one-half hour after initiation of exposure. The atmospheric concentration was determined by weighing the filter and calculating the difference between the pre-and post-sampling filter weights.

Particle size distribution was determined twice during the 4-hour exposure period using a Mercer Cascade Impactor. About 1.25 and 2.75 hours after initiation of exposure, the weight of each stage of the impactor was recorded before and after each sampling, and a cumulative weight by stage was calculated. Dust generation and exposure data are summarized in Table 1.

Table 1. Atmospheric Concentration (Aerosol) and Particle Size Distribution of the Test Material[a]

Gravity Filter (mg/L)	Nominal Conc. (mg/L)	Mass Median Aerodynamic Diameter (µm)	Geometric Standard Deviation
2.41 ± 0.15	NR	1.51	1.83

NR = Not reported
[a]Data were extracted from page 11 of the study report (IITRI Project No. L08270, MRID 430148-01).

Chamber monitoring

Temperature and total airflow were recorded nine times during the 4-hour exposure period. Results are presented in Table 2. Relative humidity was not measured due to the high aerosol concentration; air changes (10/hour) were sufficient to ensure an adequate oxygen level within the chamber.

Table 2. Chamber Environmental Conditions[a]

Mean Atmospheric Aerosol Conc. (mg/L)	Average Temp. (°C)	Relative Humidity (%)	Oxygen Concentration (%)	Average Airflow (L/min)
2.41 ± 0.15	22.3	NR	NR	69

NR = Not reported
[a]Data were extracted from page 11 of the study report.

Observations

Exposure period: Single 4-hour exposure, whole-body

Observation period: 14 days

Observation frequency: Immediately following exposure; 1.5 hours after removal from the chamber; and daily throughout the 14-day observation period.

Body weight interval: Prior to exposure, and at 8 and 15 days post-exposure

Gross pathology: Yes

Histopathology: No

C. RESULTS

Mortality

All rats survived to the end of the study period.

Clinical observations

The incidence of clinical signs in treated rats was as follows: rales (2/10), wheezing (6/10), mouth breathing (1/10), salivation (4/10), nasal congestion (5/10), redness around the eyes (7/10) and nose (10/10), and wet/discolored inguinal area (10/10). All signs cleared by the end of the observation period.

Body weights

Body weight gain was normal by the end of the study period; however, there was a slight body weight loss in 4/5 males and 1/5 females at day 8.

Organ weights

Organ weight data were not provided in the study report.

Gross necropsy

Gross necropsy was unremarkable.

D. CONCLUSIONS

The estimated 4-hour inhalation LC_{50} for rats exposed to NPI 720-F aerosol under these study conditions was > 2.41 mg/L. There was no mortality at this concentration.

Reviewer: Sheryl Reilly, Ph.D. SKR 11/30/94
Secondary Reviewer: Roy Sjoblad, Ph.D.
Biopesticides and Pollution Prevention Division

DATA EVALUATION REPORT

STUDY TYPE: Subchronic Oral Feeding - rat (82-1)

TOX CHEM NO.: 121701

MRID NO.: 431130-01

TEST MATERIAL: ATI-720 (Azadirachtin)

SYNONYM: Not Provided

PROJECT NUMBER: L08424

STUDY NUMBER: 4 (IITRI Project L08424)

SPONSOR: AgriDyne Technologies, Inc., Salt Lake City, UT

TESTING FACILITY: IIT Research Institute, Life Sciences Research, Chicago, IL

TITLE OF REPORT: 90-Day Oral (Diet) Toxicity Study of ATI-720 in Rats

AUTHOR: William D. Johnson

REPORT ISSUED: January 1994

EXECUTIVE SUMMARY: In a 90-day feeding study, ATI-720 (7.74% azadirachtin, the a.i.) was administered to male and female Sprague-Dawley rats (10/sex/group) at dietary levels of 0, 500, 2,500, and 10,000 ppm. The average daily intakes were 29.6, 145.2, and 584.6 mg/kg/day (2.3, 11.2, and 45.3 mg/kg/day azadirachtin) for males and 34.5, 177.6, and 680.2 mg/kg/day (2.7, 13.8, and 52.7 mg/kg/day azadirachtin) for females. A decrease in body weight gain in both sexes at the highest dose from week 3 in males, week 4 in females, which persisted through the rest of the study was attributed to decreased food consumption due to palatability of the diet.

Females at 2,500 and 10,000 ppm had elevated absolute and relative liver weights compared to the control group ($p \leq 0.05$). An increase in GGT activity was observed in females at 2,500-ppm and both sexes at 10,000-ppm, but the increase did not correspond to any histopathological changes in the liver. Significant hematological changes in the 10,000-ppm females included decreases in MCV (95% of control), hemoglobin (95%), and hematocrit (95%). The 10,000-ppm males exhibited decreases in MCV (94% of control), MCH (92%), and MCHC (97%). **The LOEL value of 2,500 ppm (11.2 mg/kg/day a.i./males; 13.8 mg/kg/day, females) is based on hepatic effects (increased liver weight and GGT level) in females. The NOEL value is 500 ppm (2.3 mg/kg/day a.i., males; 2.7 mg/kg/day, females).**

This study is Coregraded **MINIMUM** and satisfies the requirement for a subchronic oral toxicity study in rats.

A. MATERIALS:

1. Test Material: ATI-720
Description: Yellow-brown powder
Lot/Batch #: 21380
Purity: 7.74% a.i. (azadirachtin)
Stability of compound: Data not provided by sponsor
Chemical Formula: $C_{33}H_{44}O_{10}$ (active ingredient)
Structure: None provided

2. Vehicle: Certified Purina Rodent Chow 5002 (Purina Mills, Inc., St. Louis, MO)

3. Test animals: Species: Rat
Strain: Sprague-Dawley
Age and weight at study initiation: Approximately 8 weeks; 105-160 g
Source: Charles River Laboratories, Inc., Portage, MI
Housing: Animals housed individually in suspended stainless steel cages
Environmental conditions:
Temperature: 21-25°C
Humidity: 27-76%
Air changes: Not reported
Photoperiod: 12-hour light/dark
Acclimation period: 2 weeks

B. STUDY DESIGN:

1. Animal assignment

Animals were randomly assigned to the test groups shown in Table 1 so that no statistically significant differences in average weight existed between groups.

TABLE 1: STUDY DESIGN[a]

Test Group	Dietary Levels (ppm)	Males	Females
1 Control	0	10	10
2 Low	500	10	10
3 Medium	2,500	10	10
4 High	10,000	10	10

[a]Initiation of dosing 04/28/93; necropsies performed on 07/28/93 and 07/29/93.

Dose rationalization - Doses were chosen based on the results of a two-week range-finding study (this study was not provided for review).

2. Diet preparation and analysis

Test material was diluted with the diet (pulverized) (Certified Purina Rodent Chow 5002) to achieve the desired concentration. Test diets were prepared and analyzed every two weeks during the study and fresh diet was provided to the animals on a weekly basis. Diets were

stored frozen prior to and during use.

Results:

Homogeneity Analysis: Samples were homogeneous; samples collected at the right side, left side, or bottom of the blender (used to mix diet) ranged from 91.2% to 105.5%. The relative standard deviations were less than 5.3% for all diets; 4.6%, 5.1%, and 2.0% for diets at 500, 2500, or 10,000 ppm.

Stability Analysis: Concentration ranged from 93.5% to 103.1% of target after 14 days of storage in the freezer and 94.7% to 100.6% after 14 days at room temperature.

Concentration Analysis: Mean analyzed concentrations were 104.2%, 98.6%, and 100.1% of target for the 500, 2,500, and 10,000 ppm diets.

3. Diet

Certified Purina Rodent Chow 5002 (Purina Mills, Inc., St. Louis, MO) and reverse osmosis-purified water were provided ad libitum.

4. Statistics

All data were analyzed by one-way analysis of variance (ANOVA). When significant differences were seen in body weights, weight gains, food consumption, and hematology data, post hoc Dunnett's test was used to test for a significant difference compared with the control group. For significant differences in organ weights, food conversion ratios, and clinical chemistry data, Dunnett's test using SYSTAT software was used.

5. Quality Assurance

A signed Quality Assurance Statement (dated January 12, 1994) and a signed GLP Certification Statement (dated January 14, 1994) were provided.

C. METHODS AND RESULTS

1. Observations

All animals were inspected for mortality twice daily on weekdays and once daily on weekends and holidays. Observations for clinical signs of toxicity were made daily during the study.

Results: No deaths occurred in the study. Treatment-related clinical effects were not evident in animals during the study. Alopecia was observed in the 2,500-ppm (1 male and 2 females) and 10,000-ppm groups (1 male and 5 females), but the effect was not considered treatment-related.

2. Body weight

Body weights were recorded once weekly throughout the study.

Results: Mean weekly body weights were significantly decreased in the high-dose animals (week 3 for males and week 4 for females), but were not affected in the other treatment groups (Table 2). Mean weekly body weight gains were significantly decreased on weeks 3, 4, and 5 for the high-dose males. The mean cumulative body weight gains over the entire study period were significantly decreased in the 10,000-ppm males and females, but were increased in the 500-ppm females. The body weight losses were attributed to decreased palatability of the test diet.

TABLE 2. BODY WEIGHT GAIN DATA FOR RATS INGESTING ATI-720 IN THE DIET FOR 90 DAYS[a,b]

	Body Weight Gain (grams) Data by Dietary Level (ppm)			
	0	500	2,500	10,000
Males				
Week 0-4	168 ± 15	167 ± 23 (99)	171 ± 28 (102)	129 ± 26** (77)
Week 0-8	258 ± 25	256 ± 37 (99)	264 ± 36 (102)	188 ± 45** (73)
Week 0-13	315 ± 34	310 ± 43 (98)	305 ± 61 (97)	230 ± 55** (73)
Females				
Week 0-4	62 ± 10	71 ± 11 (115)	62 ± 7 (100)	47 ± 12* (76)
Week 0-8	90 ± 11	108 ± 19 (120)	94 ± 13 (104)	67 ± 15* (74)
Week 0-13	110 ± 13	130 ± 26 (118)	110 ± 15 (100)	78 ± 10** (71)

[a] Data extracted from Study No. L08424, Appendix 6.
[b] Numbers in parentheses indicate percent control.

* Significantly different from control, $p \leq 0.05$ using ANOVA and Scheffe's test performed by the reviewers
** Significantly different from control, $p \leq 0.01$ using ANOVA and Scheffe's test performed by the reviewers

3. Food consumption and compound intake

Food consumption for each animal was determined weekly during the study. Food conversion ratios were calculated weekly from body weight and food consumption data (food conversion = body weight gain [g]/food consumption [g]). Average compound intake (mg/kg/day) was calculated based on weekly food consumption and body weight data.

Results: Weekly food consumption was significantly reduced during most of the study period for high-dose males (weeks 3-6 and 8-11) and for high-dose females (weeks 2-11 and 13). A significant decrease in the food conversion ratios was seen in the 10,000-ppm males during weeks 3, 4, and 5. Decreased palatability of test diet was probably responsible for the decreased consumption in both sexes at the 10,000 ppm level.

Average compound intakes (mg/kg/day) for each group are shown in Table 3.

TABLE 3: AVERAGE DAILY COMPOUND INTAKE[a,b]

Test Group	Dietary Levels (ppm)	Males (mg/kg/day)	Females (mg/kg/day)
1 Control	0	0.0	0.0
2 Low	500	29.6 (2.3)	34.5 (2.7)
3 Mid	2,500	145.2 (11.2)	177.6 (13.8)
4 High	10,000	584.6 (45.3)	680.2 (52.7)

[a]From p. 13 of the study report.
[b]Numbers in parentheses indicate azadirachtin (a.i.) intake.

4. Ophthalmoscopic examination

Prior to and after 90 days of the study, examinations were conducted using an indirect ophthalmoscope following mydriasis with tropicamide. Ocular structures examined included the cornea, conjunctiva, anterior chamber, lens, anterior vitreous retina, retinal blood vessels, and optic nerve.

Results: No treatment-related effects were observed.

5. Clinical Pathology

After 18 hours of fasting, blood samples were collected from the abdominal aorta while animals were under anesthesia. Rats were then killed by exsanguination. Hematology and clinical chemistry parameters below were examined:

(a) Hematology

Hematocrit (HCT)*	Leukocyte differential count*
Hemoglobin (HGB)*	Mean corpuscular HGB (MCH)
Leukocyte count (WBC)*	Mean corpuscular HGB concentration (MCHC)
Erythrocyte count (RBC)*	Mean corpuscular volume (MCV)
Platelet count*	

*Required for subchronic studies

Results: Selected hematology data are summarized in Table 4. A significant decrease in MCV and MCH was seen in the 500 and 10,000 ppm males and a decrease in MCV in the 10,000 ppm females; however, no significant changes were seen in the 2,500 ppm group. In addition, the high-dose group exhibited significantly decreased MCHC and increased erythrocyte count in males and decreased hemoglobin and hematocrit in females. The changes observed in the high-dose group were considered to be treatment related and suggestive of a slight hypochromic, microcytic anemia.

(b) Blood (clinical) chemistry

Electrolytes

Calcium*
Chloride
Sodium*
Phosphorus*
Potassium

Enzymes

Creatinine phosphokinase*
Lactic acid dehydrogenase (LDH)*
Serum alanine
aminotransferase (ALT)*
Serum aspartate
aminotransferase (AST)*
Alkaline phosphatase (ALK)
Gamma glutamyl transpeptidase (GGT)

Other

Albumin*
Albumin/globulin ratio
Blood creatinine
Blood urea nitrogen (BUN)*
Cholesterol
Globulins (calculated)
Glucose*
Total bilirubin*
Total serum protein*

*Required for subchronic studies

Results: Selected clinical chemistry data are summarized in Table 5. The following statistically significant changes were observed in animals after study termination: increased activity of GGT in the 10,000-ppm males (600% of control) and females (650%) and the 2,500-ppm females (100%); increased levels of BUN (20%) and creatinine (18%) in 10,000-ppm females.

Statistically signficant ($p \leq 0.05$) decreases in AST (27%) and ALP (31%) in the 2,500-ppm females and a decrease in ALT (21%) in the 10,000-ppm females occurred, but were not considered treatment-related since no histopathological changes were observed in kidneys or livers of the affected animals which could account for the changes in these parameters. There was a slight but significant (2%) decrease in chloride in the high-dose females; however, the value (107.9 meq/L) was within the historical control range (range=105-111 meq/l; N-20).

6. Sacrifice and Pathology

All animals were sacrificed at study termination were subjected to gross pathological examination and the checked (X) tissues were collected for histological examination. The (XX) organs, in addition, were weighed.

Digestive System

- X Tongue
- X Salivary glands*
- X Esophagus*
- X Stomach*
- X Duodenum*
- X Jejunum*
- X Ileum*
- X Cecum*
- X Colon*
- X Rectum
- XX Liver*+
- X Pancreas*

Respiratory

- X Trachea*
- X Lung*

Cardiovascular/Hematologic

- X Aorta*
- X Heart*
- X Bone marrow*
- X Lymph nodes*
- X Spleen
- X Thymus*

Urogenital

- XX Kidneys*+
- X Urinary bladder*
- XX Testes*+
- X Epididymides
- X Prostate
- X Seminal vesicle
- XX Ovaries*+
- X Uterus*

Neurologic

- X Brain*+
- X Peripheral nerve (sciatic nerve)*
- X Spinal cord*
- X Pituitary*
- X Eyes (optic nerve)*

Glandular

- X Adrenals*
- Lacrimal gland
- X Mammary gland*
- X Thyroids*
- X Parathyroids*
- X Harderian

Other

- X Ear
- X Bone (femur)*
- X Skeletal muscle*
- X Skin*
- X All gross lesions and masses*

*Required for subchronic
+Organ weight required for subchronic and chronic studies.

Results:

(a) Organ weights and organ-to-body-weight ratios

Selected organ weight data are summarized in Table 6. The absolute and relative liver weights were significantly increased in mid- and high-dose females. The high-dose males also displayed a significant increase in relative liver weight. A decrease in absolute kidney weight and increase in relative testes weight were seen in the high-dose males, while the relative kidney weight was significantly elevated in the high-dose females. The reduced body weights in the high-dose animals probably account for all the organ weight changes, except for the increased liver weights in the mid- and high-dose females.

(b) Gross pathology

No treatment-related effects were observed, although the control and exposed animals exhibited red mandibular lymph nodes at 90 days.

(c) Microscopic pathology

No treatment-related lesions were observed in any organ or tissue. There were no histopathological changes in the liver corresponding to the decreases in liver weight in the mid- and high-dose groups.

D. DISCUSSION

This study is classified as Core Guideline and satisfies guideline requirements for a subchronic oral toxicity study in rats. No mortalities were reported.

The data presented in this study show that administration of ATI-720 in the diet of Sprague Dawley rats produced no major toxic effects. The increase in absolute and relative liver weight and the elevated GGT activity in 2,500- and 10,000-ppm females (13.8 and 52.7 mg/kg/day azadirachtin) suggest that these are early indicators of potential hepatotoxicity with ATI-720. These effects did not correspond to any histopathological changes in the liver of the affected animals; however, a subchronic oral study in rats (MRID No. 432291-01) indicated hepatic toxicity (bile duct proliferation) with 1000 mg/kg/day Neem Concentrate TGAI (46 mg/kg/day azadirachtin).

In addition, at the highest dose level (10,000 ppm), hematological changes (decreased MCV, MCH, hematocrit, and hemoglobin, increased erythrocyte count) were considered to be treatment-related and suggestive of a slight hypochromic, microcytic anemia.

Based on the evidence of hepatic toxicity reported in females, the LOEL for systemic toxicity is 2,500 ppm and the NOEL is 500 ppm.

E. DEFICIENCIES

There were no major deficiencies that affected the conclusions of the study.

TABLE 4. Selected Hematology Data for Rats Ingesting ATI-720 in the Diet for 90 Days[a,b]

	Hematology Data by Dietary Level (ppm)			
	0	500	2,500	10,000
Males				
Mean cell volume (fl)	50.8 ± 1.45	48.9 ± 1.05** (96)	49.6 ± 1.22 (98)	47.9 ± 1.45** (94)
Mean corpuscular hemoglobin (pg)	19.1 ± 0.84	18.2 ± 0.72* (95)	18.5 ± 0.86 (97)	17.5 ± 0.69** (92)
Mean corpuscular hemoglobin concentration (%)	37.6 ± 0.99	37.2 ± 1.14 (99)	37.3 ± 0.97 (99)	36.5 ± 0.78* (97)
Hemoglobin (g/dL)	15.4 ± 1.16	15.3 ± 1.17 (99)	15.4 ± 0.46 (100)	15.4 ± 0.82 (100)
Hematocrit (%)	41.0 ± 3.02	41.3 ± 3.80 (101)	41.2 ± 1.77 (100)	42.3 ± 2.31 (103)
Erythrocytes ($10^6/mm^3$)	8.08 ± 0.71	8.45 ± 0.78 (105)	8.30 ± 0.52 (103)	8.82 ± 0.40* (109)
Females				
Mean cell volume (fl)	52.4 ± 1.96	52.6 ± 1.57 (100)	53.6 ± 1.73 (102)	50.0 ± 1.84* (95)
Mean corpuscular hemoglobin (pg)	19.7 ± 1.14	19.5 ± 0.60 (99)	20.2 ± 0.71 (103)	18.7 ± 1.17 (95)
Mean corpuscular hemoglobin concentration (%)	37.5 ± 0.91	37.1 ± 0.53 (99)	37.7 ± 0.48 (101)	37.4 ± 1.04 (100)
Hemoglobin (g/dL)	15.2 ± 0.51	15.4 ± 0.45 (101)	14.9 ± 0.42 (98)	14.4 ± 0.61** (95)
Hematocrit (%)	40.6 ± 1.83	41.5 ± 1.51 (102)	39.6 ± 1.07 (98)	38.5 ± 1.84* (95)
Erythrocytes ($10^6/mm^3$)	7.78 ± 0.58	7.91 ± 0.32 (102)	7.39 ± 0.24 (95)	7.72 ± 0.50 (99)

[a] Data extracted from Study No. L08424, Tables 16 and 17.
[b] Numbers in parentheses indicate percent control.

* Significantly different from control, $p \leq 0.05$
** Significantly different from control, $p \leq 0.01$

TABLE 5. Selected Clinical Chemistry Data for Rats Ingesting ATI-720 in the Diet for 90 Days[a,b]

	Clinical Chemistry Data by Dietary Level (ppm)			
	0	500	2,500	10,000
		Males		
γ-glutamyl transpeptidase (U/L)	1 ± 0.9	2 ± 0.9 (200)	2 ± 0.9 (200)	7 ± 1.5* (700)
Creatinine (mg/dL)	0.47 ± 0.04	0.52 ± 0.07 (111)	0.52 ± 0.06 (111)	0.54 ± 0.09 (115)
Blood urea nitrogen (mg/dL)	13.9 ± 0.96	15.5 ± 2.76 (112)	15.2 ± 1.91 (109)	16.2 ± 2.89 (117)
		Females		
γ-glutamyl transpeptidase (U/L)	2 ± 0.7	2 ± 0.9 (100)	4 ± 1.4* (200)	15 ± 6.2** (750)
Creatinine (mg/dL)	0.50 ± 0.07	0.51 ± 0.03 (102)	0.51 ± 0.05 (102)	0.59 ± 0.06* (118)
Blood urea nitrogen (mg/dL)	17.1 ± 3.03	16.3 ± 2.67 (95)	16.2 ± 2.06 (95)	20.6 ± 2.69* (120)

[a] Data extracted from Study No. L08424, Tables 14 and 15.
[b] Numbers in parentheses indicate percent control.

* Significantly different from control, $p \leq 0.05$
** Significantly different from control, $p \leq 0.01$

TABLE 6. Organ Weight Data for Rats Ingesting ATI-720 in the Diet for 90 Days[a,b]

	Organ Weight Data by Dietary Level (ppm)			
	0	500	2,500	10,000
Males				
Kidneys				
–absolute weight (g)	3.37 ± 0.22	3.40 ± 0.39 (101)	3.31 ± 0.26 (98)	3.03 ± 0.33* (90)
–percent body weight	0.64 ± 0.05	0.66 ± 0.07 (103)	0.63 ± 0.05 (98)	0.69 ± 0.07 (108)
Liver				
–absolute weight (g)	14.86 ± 1.60	15.15 ± 2.20 (102)	15.59 ± 1.55 (105)	15.06 ± 2.30 (101)
–percent body weight	2.81 ± 0.25	2.91 ± 0.28 (104)	2.94 ± 0.15 (105)	3.41 ± 0.30* (121)
Testes				
–absolute weight (g)	3.49 ± 0.23	3.45 ± 0.34 (99)	3.49 ± 0.33 (100)	3.51 ± 0.27 (101)
–percent body weight	0.66 ± 0.06	0.67 ± 0.09 (102)	0.66 ± 0.06 (100)	0.81 ± 0.11* (123)
Females				
Kidney				
–absolute weight (g)	1.81 ± 0.10	1.87 ± 0.12 (103)	1.83 ± 0.11 (101)	1.73 ± 0.14 (96)
–percent body weight	0.69 ± 0.05	0.67 ± 0.07 (97)	0.70 ± 0.06 (101)	0.76 ± 0.04* (110)
Liver				
–absolute weight (g)	6.55 ± 0.46	7.19 ± 0.64 (110)	7.66 ± 0.83* (117)	9.52 ± 1.51* (145)
–percent body weight	2.50 ± 0.16	2.56 ± 0.09 (102)	2.91 ± 0.20* (116)	4.16 ± 0.57* (166)

[a] Data extracted from Study No. L08424, Tables 20-23.
[b] Numbers in parentheses indicate percent control.

* Significantly different from control, $p \leq 0.05$

Reviewer: Sheryl K. Reilly, Ph.D. SKR 1/31/96
Secondary Reviewer: J. Thomas McClintock, Ph.D. JTM 3/6/96
Biopesticides and Pollution Prevention Division

DATA EVALUATION REPORT

STUDY TYPE: 30-Day Oral (Dietary) Immunotoxicity Study - Mouse (152-18)

TOX. CHEM. NO.: 121701

MRID NOs.: 431627-01

TEST MATERIAL: ATI-720

SYNONYMS: Azadirachtin

STUDY NUMBER: Study No. 2 (Project No. L08434)

SPONSOR: AgriDyne Technologies Inc., Salt Lake City, UT 84109

TESTING FACILITY: IIT Research Institute, Life Sciences Research Dept., Chicago, IL 60616

TITLE OF REPORT: Tier I Immunotoxicity Study in Mice Using ATI-720

AUTHOR: Sherwood, R.L.

REPORT ISSUED: January 14, 1994

EXECUTIVE SUMMARY: ATI-720 was fed to groups of 40 female mice at dietary levels of 500, 1250, or 5000 ppm (approximately 112, 295, or 1100 mg/kg body weight/day) and control diets (0 ppm) for 30 days. No compound-related effects were observed. No mortalities occurred and body weight gains, hematology, and clinical chemistry parameters were unaffected. Gross findings were unremarkable, and ATI-720 had no effect on spleen or thymic weights or bone marrow evaluations. The test compound had no effect on spleen or thymic cellularity.

No effects were observed in the anti-SRBC plaque-forming cell (PFC) assay; a significant (<0.05) decrease in tumor cell lysis was observed in the NK cell activity at the lowest dose tested (500 ppm), but only at the highest (100:1) effector:target cell ratio. The significance of this finding is unclear. In the cytotoxic T-lymphocyte (CTL) assay, lysis of target cells was markedly reduced at all dose levels; however, a positive control group (with a known immunosuppressant) was not included in the study. No effects on peritoneal macrophage numbers, viability or function were observed in the treated animals.

The study is **supplementary but upgradeable**, pending receipt of spleen cell viability data. The test substance appears to be **immunosuppressive to cytotoxic T lymphocytes**. Before a decision can be made as to whether Tier II testing is required for the test substance, the registrant must submit the spleen cell viability data. If a positive control group was tested in the CTL assay, or if a satellite recovery group in the present study was tested, that

data must be submitted in order to determine if an appropriate host-resistance test would be required for the test substance.

A. MATERIALS

1. Test Material: ATI-720
Description: Yellow-brown powder
Lot/Batch #: 21380
Purity: 7.74% a.i. (azadirachtin)
Stability of compound: Stable at room temp. or freezer up to 14 days
Storage: Refrigerated

2. Positive controls/Vehicle:

a. Cyclophosphamide
Lot #: 86F0101
Source: Sigma, Chemical Co., St. Louis, MO

b. N-deacetyl-N-methylcolchicine (demecolcine)
Lot #.: 91H-4601
Source: Sigma Chemical Co., St. Louis, MO

c. Recombinant human interleukin-2 (rhIL-2)
Lot #: 4230
Source: Preprotech, Rocky Hill, NJ

Vehicles

For test compound: Diet - (Purina Rodent Chow #5002)
For cyclophosphamide: Dulbecco's phosphate buffered saline
For demecolcine: RPMI-1640 medium
For rhIL-2: Culture medium (Earle's salts/HEPES buffer-supplemented with L-glutamine, fetal bovine serum, and 2-mercaptoethanol)

3. Test animals

Species: Mice - female
Strain: $B6C3F_1$
Age and weight: 5-6 weeks-old on receipt; weight 13-26 g. At initiation the mice weighed 16-23 g
Source: Frederick Cancer Research Center from Charles River Laboratories
Housing: 4 mice/cage during acclimation; 1/cage during the study

Environmental conditions:
Temperature: 21-24.5°C
Humidity: 43-73%
Air changes: Not report
Photoperiod: 12-hour light/dark cycle

Acclimation period: At least one week.

B. STUDY DESIGN

1. Animal assignment

Animal were assigned to the following groups using a constrained randomization method:

Table 1. Study Design

Test Group	Dietary level (ppm)	Study Group			
		1[a]	2[b]	3[c]	4[d]
		Number of Mice			
Naive control	0	10	10	10	10
Low-dose (LDT)	500	10	10	10	10
Mid-dose (MDT)	1250	10	10	10	10
High-dose (HDT)	5000	10	10	10	10
Positive control[e]		--	10	--	--

[a] Hematology, clinical chemistry, gross necropsy, spleen and thymus weight, and histopathology.

[b] Antibody plaque forming cell response (antigen--SRBC on day 27).

[c] Cytotoxic T-lymphocyte (CTL) assay; lymphocyte cellularity and viability, spleen cellularity, and macrophage function.

[d] Natural killer cell response (NK), macrophage function, and spleen cellularity.

[e] Positive control -- Mice received cyclophosphamide (80 mg/kg) by intraperitoneal injection 24 hours prior to the terminal sacrifice.

2. Diet: Animals received food (Purina Certified Chow 5002) and water *ad libitum.*

a. Rationale for dose selection: A 14-day range finding study was conducted at ATI-720 dietary levels of 0, 1000, 5000, 10,000, or 20,000 ppm. At the two highest dietary levels, decreased body weights and food consumption and significant effects on the weight of the spleen and thymus were observed.

b. Diet Preparation: The test substance was mixed with basal diet in a 16-quart blender for 20 minutes to prepare the 5000-ppm diet. This mixture was appropriately diluted with untreated basal diet to prepare 1250 and 500 ppm diets. The diets were prepared 3 times at biweekly intervals, except the 500 ppm diet, which was prepared twice. Homogeneity of test compound in diets was verified for replicate samples at 6 locations in the mixer. Storage stability was determined over 14 days, and test compound concentration in the diets were analyzed twice. The concentration of ATI-720 was determined using an azadirachtin standard (98% pure) and calculating the amount based on the test material content (analyzed) of 7.74% azadirachtin. Samples were

extracted with acetonitrile and chromatographed on an HPLC column with detection by u.v. (214 nm).

c. Diet Analysis: Samples of diet were homogeneous; relative standard deviations for samples were 3.3, 3.6, and 2.7% for diets at 500, 1250, or 5000 ppm. Less than 1% loss was seen after 14 days storage at room temperature. Diets were within 95.8%, 100.2%, and 99.7% of the nominal concentrations, at increasing levels.

3. Statistics

Analysis of variance (ANOVA) was used to determine significant differences followed by Dunnett's test for pairwise comparison with controls. Means, standard deviations, standard errors and for appropriate data, ranges of values were reported in the summary tabulations.

4. Quality Assurance Measures

A signed and dated (1/14/94) quality assurance statement was present.
A signed and dated (1/14/94) GLP statement was present.

C. METHODS AND RESULTS

In-Life Findings

1. Observations

Animals were observed twice daily for mortality and once/day for morbidity and toxic signs. Clinical examinations were inclusive of all parameters indicating illness or toxicity and signs of recovery from these symptoms.

Results - There were no dose-related mortalities or clinical signs of toxicity. Alopecia was observed in 3 controls, 1 mid-dose and 3 high-dose mice; the earliest occurrence was at 17 days.

2. Body Weight

Mice were weighed prior to allocation on the first day of dosing, and weekly thereafter. Body weight gains were calculated weekly.

Results - Body weight and body weight gains were similar in negative control and dosed groups. Mean weights were slightly decreased in the positive control (nonsignificant); overall decrease was 1.0 g compared to 1.5 g in negative control.

3. Food Consumption

Food and water consumption/cage of two mice were determined weekly. For water consumption, a control value to correct for loss from drip-

ping of bottles was subtracted using two control bottles in an empty cage.

Results - Overall food consumption was similar in control and dosed groups. Based on body weight and food consumption, the mean compound intake was approximately 112, 295, and 1100 mg of ATI-720/kg body weight/day at the low-, mid-, and high-dietary levels.

4. Hematology

Blood was collected from the retroorbital plexus of 10 animals/group prior to the terminal sacrifice. The following parameters were examined

HCT	Leukocyte differential count
HGB	MCH
WBC	MCHC
RBC	MCV
Platelet count	

Results - No effects on red cell parameters, or total or differential leukocytes were observed. The hematocrit value was slightly (less than 5%) decreased in the high-dose group ($p<0.05$) but all individual values were within the normal range. An 11% increase in mean platelet counts at 5000 ppm (972×10^3) compared to control (872×10^3) may be compound related.

5. Clinical Chemistry

The following serum parameters were measured prior to sacrifice: glucose, glutamic-pyruvic transaminase, urea nitrogen, albumin and total protein.

Results - Total serum protein was slightly reduced ($p<0.05$) at the mid- and high-dose level and the albumin concentration in serum was slightly reduced in the high-dose group as compared to controls. However, these changes were within the normally expected range.

Sacrifice and Pathology

Mice were fasted for four hours prior to sacrifice (CO_2 asphyxiation) and necropsies conducted. The following tissues were saved in 10% buffered formalin for possible histologic examination:

Lungs	Pituitary
Kidney	Ovaries
Adrenals	All gross lesions
Liver	Lymph nodes (peripheral and mucosal associated)

The thymus and spleen were weighed wet and processed for cell counts and viability determinations with trypan blue. Bone marrow was removed from the femur and smears prepared on clean slides, fixed with methanol, and stained with Wright's stain.

Results - No gross lesions were seen in low- or mid-dose animals; one high-dose female was observed with a pale liver. The viability of the spleen cells was not reported in the study.

1. Organ Weights

Mean values for wet weights of spleen and thymus were calculated and organ-to-body weights were determined using the necropsy body weight.

Results - No differences were found in spleen or thymus weights (absolute or relative-to-body weight) in treated mice compared to controls. The positive control, cyclophosphamide, was associated with a reduction in absolute mean spleen weight (69 mg compared to 85 mg for controls) and relative spleen weight (0.313% compared to 0.382%).

2. Bone Marrow Evaluation

Myeloid, erythroid and megakaryocytic series and miscellaneous cells were counted for 500 total cells/animal. Total proliferating myeloid cells, total proliferating erythroid cells and the myeloid:erythroid (M:E) ratio were also calculated and tabulated.

Results - No changes were seen in any cell type comparing dosed groups with the control group. All individual values were within the normal limits.

3. Cellularity of the Spleen and Thymus

Results - Test compound had no effect on spleen or thymus cellularity. Viability data were not provided. The positive control had a marginal effect on spleen cellularity (14% decrease) compared to control.

c. **Immunotoxicology Tests**

1. Anti-Sheep Red Blood Cell (SRBC) Antibody-Forming Cell Assay

Mice were immunized by intraperitoneal (i.p.) injection of 2×10^8 SRBC at day 27 and sacrificed on day 31. Spleens were removed aseptically and weighed. Suspensions of splenic cells were prepared in supplemented RPMI-1640 medium. Duplicate tubes filled with optimal dilutions of splenocytes, SRBC, and an excess of guinea pig complement were prepared and duplicate Cunningham chambers filled with the mixture. The chambers were sealed with petrolatum and incubated at 37°C for 1 hour in a humidified chamber. Plaques were enumerated with a plaque counter. Positive control animals were dosed i.p. with 80 mg/kg body weight of cyclophosphamide 24 hours prior to sacrifice.

Results - No effect on the response of splenic cells to SRBCs was observed in groups fed ATI-720. Cyclophosphamide, the positive control, abolished the normal response as expected. Immunoglobulins IgM and IgG were not quantified.

2. Natural Killer (NK) Cell Assay

NK cell activity was quantified from spleens of individual mice using an *in vitro* ^{51}Cr-release assay, in which YAC-1 tumor cells were used as the target cell. Splenic cells were suspended at a concentration of 5×10^6 viable cells/mL and cultured for 24 hours at 37°C with or without an optimal concentration of rhIL-2. Following this induction, the effector cells were washed several times, adjusted to 5×16^6/mL and serial dilutions made to give different effector:target cell ratios (100:1; 33:1; 11:1). These cell suspensions (100 μL) were co-cultured in 96-well plates with ^{51}Cr-labeled YAC-lymphoma target cells. Following a 4-hour incubation, supernatant fluids were removed and chromium release was quantified.

Results - The basal activity was not significantly affected by the test compound. In the interleukin-augmented assay, low-dose mice exhibited a significant ($p<0.05$) reduction in response compared to controls at 100:1 ratio of effector cells:target cells, but not at ratios of 33:1 or 11:1 (Table 2).

Table 2. Effect of ATI-720 Feeding on IL-2 Augmented Splenic Natural Killer Cell Activity in Mice[a]

Dietary Level (ppm)	Percent of total cell lysis at an effector: target cell ratio of		
	100:1	33:1	11:1
0	13.71 ± 0.98	5.42 ± 0.68	2.62 ± 0.30
500	8.38 ± 1.59*	3.56 ± 0.88	2.72 ± 0.66
1,250	16.23 ± 1.33	6.77 ± 0.65	3.10 ± 0.68
5,000	11.55 ± 0.99	4.11 ± 0.60	2.85 ± 0.38

Source: Extracted from Table 13 of report (p.38). Values are mean percent of lysis ± standard error.

[a] Expressed as percent of total target cells
* Significantly different from control value, $p \leq 0.05$.

3. Cytotoxic T-lymphocyte (CTL) Induction

Single cell preparations of splenocytes (responder cells) were prepared and the erythrocytes lysed with ammonium chloride buffer. Cells were washed and resuspended to a concentration of 3×10^7 viable cells/T-25 flask in ultraculture medium (Bio Whittaker) supplemented with 2 mM glutamine and 5 mM antibiotic. The stimulator cells, log phase P815 tumor cells (mouse mastocytoma), were inactivated with mitomycin C and adjusted to 1.2×10^6 viable cells/mL. Stimulator cells (0.5 mL) were added to the T-flask with lymphocytes and the culture incubated at 37°C for five days. Contents of the flasks were decanted, cells were washed with phosphate buffered saline (PBS) and adjusted to 5×10^6 viable cells.

Three dilutions of cells in Eagles MEM were made so that for the killing assay the final effector:target cell ratios would be 15:1, 12.5:1 or 6.25:1. The dilutions (100 μL) were added to quadruplicate wells of a 96-well round bottom microtitre plate and then 100 μL of ^{51}Cr-labeled P815 cells were added to each well. The plates were incubated for four hours at 37°C and samples then removed to determine chromium release, correcting for spontaneous release in the absence of effector cells.

Results - Table 3 summarizes results. At all three dietary levels and at all three effector cell levels, the lysis of target cells was substantially reduced (50% or greater). The response was greatest in the mid-dose group and was statistically significant at all three dilutions. It was also significant at the 6.25:1 effector: target cell ratio in the 500-ppm group.

Table 3. Cytotoxic T-Lymphocyte Function in Mice Fed ATI-720

Dietary Level (ppm)	Percent total lysis at an effector/target cell ratio of		
	25:1	12.5:1	6.25:1
0	48.6 ± 6.9	37.4 ± 6.9	25.1 ± 5.0
500	26.9 ± 5.4 (-45)	19.9 ± 5.3 (-47)	12.4 ± 3.4* (-51)
1,250	20.1 ± 5.2* (-59)	13.5 ± 4.1* (-64)	8.21 ± 3.0* (-67)
5,000	28.6 ± 5.2 (-41)	19.9 ± 4.2 (-47)	12.3 ± 2.6 (-51)

Note: Percent decrease compared to controls found in parenthesis

Source: Extracted from Table 14 of report (p.39). Values are mean percent target cell lysis ± standard error.

* Significantly different from control value, $p \leq 0.05$.

4. Macrophage Numbers and Function

Peritoneal cells were obtained from test mice by lavage and pooled for 2-3 mice. Differential and total cell counts were performed, and viability of cells assessed by trypan blue exclusion. Macrophages were then isolated by adherence to plastic culture plates and the other cells were discarded.

To test for function, suspensions of macrophages (0.2 - 0.25 x 10^6 viable cells/mL) were incubated with latex beads while rotating in capped plastic tubes for one hour at 37°C. Phagocytic activity was determined in the presence or absence of bacterial lipopolysaccharide (LPS), an augmenting factor. A known immunosuppressive control (demecolcine) was also tested. Trypan blue was added (0.05%) and cells centrifuged onto clean glass microscope slides. One hundred viable cells/slide were counted and the number of beads ingested/cell determined.

Results - No significant effects on peritoneal macrophage phagocytosis were observed. LPS had a slight inductive effect on the number of beads ingested; demecolcine reduced the number of beads ingested and decreased the phagocytic index. However, ATI-720 did not affect the response in untreated or LPS-treated macrophages.

E. Discussion

In this study, the viability of the splenocytes was not reported and there is a possibility that the results seen in the cytotoxic-T lymphocyte function test are associated with decreased viability of splenocytes and are not related to dosing. The viability of splenocytes are required under Subdivision M, and should be reported to resolve the data interpretation.

The reviewer is aware of the difficulty of some of these assays and the variability from animal to animal. However, the test compound does appear to suppress cytotoxic T lymphocyte activity. The study report does not indicate that a satellite/recovery group was included with the CTL group, nor does it appear that a positive control group (with a known immunosuppressant) was tested. In order to determine if Tier II immunotoxicity testing should be undertaken, the spleen cell viability should be reported. In addition, if either a satellite/recover group was included, or tests with known immunosuppressants have been done to confirm the sensitivity of the assay, this data must be submitted by the registrant.

FINAL

DATA EVALUATION REPORT

AZADIRACHTIN

Study Type: Other Genotoxicity: *In Vivo* Unscheduled DNA Synthesis in Rat Hepatocytes

Prepared for:

Health Effects Division
Office of Pesticide Programs
Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Clement International Corporation
9300 Lee Highway
Fairfax, VA 22031-1207

Principal Reviewer [signature] Kristin Jacobson, MSPH Date 1/10/94

Independent Reviewer [signature] Nancy E. McCarroll, B.S. Date 1/10/94

QA Manager [signature] William L. McLellan, Ph.D. Date 1/10/94

Contract Number: 68D10075
Work Assignment Number: 3-29
Clement Number: 108
Project Officer: Caroline Gordon

Azadirachtin

GUIDELINE §84: MUTAGENICITY
UDS IN VIVO

EPA Reviewer: J. Thomas McClintock, Ph.D.
Biological Pesticide Section,
Science Analysis Branch, HED (7509C)
EPA Section Head: Roy Sjoblad, Ph.D.
Biological Pesticide Section,
Science Analysis Branch, HED (7509C)

Signature: [signature] for JTM
Date: 11/23/94

Signature: [signature]
Date: 3/6/96

DATA EVALUATION REPORT

STUDY TYPE: Other genotoxicity: *In vivo* unscheduled DNA synthesis in rat hepatocytes

TOX CHEM. NUMBER: Not provided

PC CODE: 121701

MRID NUMBER: 429458-02

TEST MATERIAL: ATI-720 (Azadirachtin) 11.1% a.i.

SYNONYM(S): None provided

STUDY NUMBER: HWA Study No. 15457-0-494

SPONSOR: Agridyne Technologies, Salt Lake City, UT

TESTING FACILITY: Hazleton Washington, Inc., Vienna, VA

TITLE OF REPORT: Genotoxicity Test on ATI-720 in the In Vivo/In Vitro Assay for Unscheduled DNA Synthesis in Rat Primary Hepatocytes with Two Timepoints

AUTHOR(S): M.E. McKeon

REPORT ISSUED: August 12, 1993

CONCLUSIONS-EXECUTIVE SUMMARY:

Negative for the induction of unscheduled DNA synthesis (UDS) in hepatocytes recovered from groups of male rats (three per dose, per sacrifice time) at 4 or 15.5 hours postexposure following oral gavage administration of 1250, 2500, or 5000 mg/kg ATI-720 (suspended in 0.5% carboxymethylcellulose; ≈139-555 mg/kg active ingredient).

Classification: ~~Core Guideline.~~ Acceptable. (SR)

The study satisfies the Guideline (§84-4) requirements for the evaluation of potential mammalian mutagenicity (*in vivo* UDS assay).

A. MATERIALS

1. Test Material: ATI-720

Description: Beige granules
Lot/batch number: 1111-71
Purity: 11.1% active ingredient (a.i.); the remaining 88.9% of the test material was not specified.
Stability of compound: Expiration date March 11, 1996
CAS Number: 11141-17-6
Vehicle used: 0.5% high-viscosity carboxymethylcellulose (CMC)
Other comments: Test material storage conditions were not reported. Dosing suspensions were prepared fresh for each treatment. Neither the achieved concentrations nor the homogeneity of the dosing suspensions were verified analytically. Purity adjustments were not made.

2. Control Materials:

Negative/route of administration: None

Vehicle/final concentration/route of administration: CMC was administered by oral gavage at a dosing volume ≤15 mL/kg.

Positive/final concentration/route of administration: Dimethylnitrosamine (DMN) was prepared in an unspecified solvent and administered by intraperitoneal (i.p.) injection at approximately 10 mg/kg for the 4-hour sacrifice interval, and 15 mg/kg for the 15.5-hour sacrifice interval.

3. Test Compound

Route of administration: Oral gavage

Dose levels used: 625, 1250, 2500, and 5000 mg/kg (≈69, 139, 278, and 555 mg/kg a.i.). Hepatocytes from animals exposed to 625 mg/kg were not scored for UDS.

Volume administered: ≤15.2 mL/kg

4. Test Animals

(a) Species: Rat; Strain: F344; Age: Young adult; Sex: Male; Weight range: 177.4-210.0 g; Source: Harlan Sprague Dawley Laboratories, Inc. (location not specified)

(b) Number of animals used per dose per sacrifice interval:

Treatment:	3 males;	0 females
Solvent control:	3 males;	0 females
Positive control:	3 males;	0 females

Note: Due to mortality and cytotoxicity, two additional animals were required in the positive control group (4-hour harvest); these animals were dosed separately from the rest of the study animals.

(c) All test animals were properly maintained.

B. TEST PERFORMANCE

1. UDS Assay

(a) Treatment: Groups of three male rats were administered selected doses of the test material or vehicle control by oral gavage; the positive control was administered by i.p. injection.

(b) Hepatocyte harvest/culture preparation: At ≈4 or 15.5 hours posttreatment, the livers from the appropriate experimental group were perfused *in situ* with Hanks' balanced salt solution (HBSS) containing 0.5 mM EGTA and HEPES buffer (pH 7.2), and with serum-free Williams' Medium E (WME), containing 2 mM L-glutamine, antibiotics and 50-100 units/mL collagenase. Livers were excised, cells were mechanically dispersed in WME with collagenase, allowed to settle to remove clumps and debris, centrifuged and resuspended. Cell viability was determined using trypan blue exclusion. Recovered cells were inoculated (0.5×10^6 viable cells/dish, 6 dishes/animal) into 35-mm culture dishes containing a plastic coverslip, and allowed to attach for 1.4-2.0 hours. Three dishes per animal were used for the UDS assay, and two dishes were used for the assessment of viability following attachment. Unattached cells were removed, and viability post-attachment was assessed using trypan blue exclusion. Viable cells were refed serum-free WME containing 10 μCi/mL [^{3}H]-thymidine for approximately 4 hours. Following the labeling period, cells were washed and reincubated for ≈18-19 hours in WME containing 0.25 mM unlabeled thymidine.

(b) Slide preparation: Hepatocytes attached to coverslips were washed, swollen with 1% sodium citrate, fixed in acetic acid:ethanol (1:3), dried and mounted on glass slides.

(c) Preparation of autoradiographs/grain development: Slides were coated with Kodak NTB2 emulsion, stored for 7 days at 4°C in light-tight boxes containing Drierite®, developed in D19 and fixed. Cells were stained with Williams' modified hematoxylin and eosin.

(d) Grain counting: Slides were coded, and the nuclear grains of at least 50 morphologically normal cells/slide (3 slides/animal) were counted. Net nuclear grain (NNG) counts were determined by subtracting the average grain count of three nuclear-sized cytoplasmic areas adjacent to each nucleus from the nuclear grain

counts of each cell. Mean NNG counts and standard deviations were calculated for each animal. The percentage of cells in repair (i.e., cells with ≥5 net nuclear grains) were also calculated. In addition, 1000-1500 cells/animal were scanned for heavily labeled nuclei in order to determine the incidence of cells in S-phase DNA synthesis.

3. Statistical Methods: The data were not analyzed for statistical significance.

4. Evaluation Criteria:

(a) Valid assay: The assay was considered acceptable if the following conditions were met: (1) hepatocyte viability prior to treatment was ≥70%; (2) the mean NNG in the vehicle control group was between -5.00 and 1.00; (3) the positive control produced positive responses; (4) the test material was evaluated over at least three dose levels; and (5) grain count data were obtained from at least 100 cells/animal and at least two of the three animals per treatment level.

(b) Positive response: The assay was considered positive if, for at least two of the three animals from any treatment group, (1) the average UDS response was increased ≥5 grains per nucleus above the concurrent vehicle control; and/or (2) the frequency of nuclei in repair (i.e., nuclei with ≥5 NNG) was 10% higher than the concurrent vehicle control value.

C. REPORTED RESULTS: The report indicated that selection of the high dose (5000 mg/kg) was based on information that was furnished by the sponsor from prior studies; no further details were provided. The test material was suspended in the vehicle (0.5% CMC) to yield concentrations ranging from 41.7 to 333.3 mg/mL. The study author stated that ATI-720 "appeared to form a light brown suspension in the vehicle."

No deaths or signs of clinical toxicity were reported for the males in the test material dose groups. There was also no indication that hepatocyte viability was affected by treatment with ATI-720.

Representative results from the UDS assay are presented in Table 1. The average NNG counts for all treatment levels were generally comparable to the vehicle controls, and the average frequencies of cells in repair (i.e., cells with ≥5 NNG) were within 4% of the vehicle control frequencies. By contrast, the positive control (10 or 15 mg/kg DMN via i.p. injection), induced a marked increase in both the average NNG and in the percentage of cells in repair at both sacrifice intervals.

Based on these findings, the study author concluded that ATI-720 was inactive in this *in vivo/in vitro* primary rat hepatocyte UDS assay.

Table 1

TABLE 1. Representative Results of the *In Vivo* Unscheduled DNA Synthesis (UDS) Assays in Male Rats Dosed with ATI-720

Substance	Dose (mg/kg)	Exposure Time[a] (hours)	No. of Animals Analyzed per Group	No. of Hepatocytes Scored	Average Net Nuclear Grain Counts per Group[b]	Average % Cells in Repair (Cells with ≥5 Net Nuclear Grains) per Group
Vehicle Control						
0.5% Carboxymethyl-cellulose	--	4	3	450	-0.22	1.3
	--	15.5	3	450	-0.71	2.2
Positive Control						
Dimethylnitrosamine	10	4	3	400	18.16	94.7
	15	15.5	3	450	9.02	68.9
Test Material						
ATI-720	5000 (555)[c]	4	3	400	-0.22	2.0
	5000 (555)[c]	15.5	3	450	0.16	6.2

[a]Time after test material or vehicle administration by oral gavage. The positive control was administered by intraperitoneal injection.

[b]Means and standard deviations of the counts from 2-3 slides per animal (50 cells/slide) were provided by the study author. Average group values were calculated by our reviewers.

[c]Results for lower doses (1250 or 2500 mg/kg; approx. 139 or 178 mg/kg active ingredient) at either 4 or 15.5 hours posttreatment did not suggest a genotoxic effect. Value in () is the nominal concentration of the active ingredient.

Note: Data were extracted from the study report, p. 23 and 25.

D. DISCUSSION/CONCLUSIONS: The study author's interpretation of the reported findings indicated that ATI-720 did not induce UDS in hepatocytes collected from male rats 4 or 15.5 hours postexposure to 1250-5000 mg/kg (69-555 mg/kg active ingredient). Although only male rats were evaluated, since ATI-720 was tested to a high insoluble level with no indication of a genotoxic effect, it is not likely that the inclusion of females would have altered the outcome of the study. Based on these considerations, the study provided acceptable evidence that ATI-720 was not genotoxic in this *in vivo* test system.

E. QUALITY ASSURANCE MEASURES: The test was performed under GLPs. A quality assurance statement, signed and dated August 12, 1993, indicated that the draft and final study reports were inspected.

FINAL

DATA EVALUATION REPORT

AZADIRACHTIN

Study Type: *In Vivo* Mammalian Cytogenetic Chromosome Aberration Assay in Mice

Prepared for:

Health Effects Division
Office of Pesticide Programs
Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by:

Clement International Corporation
9300 Lee Highway
Fairfax, VA 22031-1207

Principal Reviewer	Kristin Jacobson, MSPH	Date 1/10/94
Independent Reviewer	Nancy E. McCarroll, B.S.	Date 1/10/94
QA Manager	William L. McLellan, Ph.D.	Date 1/10/94

Contract Number: 68D10075
Work Assignment Number: 3-29
Clement Number: 107
Project Officer: Caroline Gordon

MUTAGENICITY STUDIES

EPA Reviewer: J. Thomas McClintock, Ph.D.
Biological Pesticide Section,
Science Analysis Branch, HED (7509C)
EPA Section Head: Roy Sjoblad, Ph.D.
Biological Pesticide Section,
Science Analysis Branch, HED (7509C)

Signature: [signature] for JTM
Date: 11/23/94

Signature: [signature]
Date: 3/6/96

DATA EVALUATION REPORT

STUDY TYPE: *In vivo* mammalian cytogenetic chromosome aberration assay in mice

TOX CHEM. NUMBER: Not provided

PC CODE: 121701

MRID NUMBER: 429458-01

TEST MATERIAL: ATI-720 (azadirachtin)

SYNONYMS: None provided

STUDY NUMBER(S): HWA Study No. 15457-0-451

SPONSOR: Agridyne Technologies, Inc., Salt Lake City, UT

TESTING FACILITY: Hazleton Washington, Inc., Vienna, VA

TITLE OF REPORT: Mutagenicity Test on ATI-720 Measuring Chromosomal Aberrations *in vivo* in Mouse Bone Marrow Cells

AUTHOR(S): H. Murli

REPORT ISSUED: June 28, 1993

CONCLUSION(S) -- Executive Summary:

Negative for the induction of structural chromosome aberrations in bone marrow cells harvested from male or female CD mice at 6, 18, or 30 hours postexposure following oral gavage administrations of ATI-720 (suspended in 0.5% carboxymethylcellulose) at doses up to 5000 mg/kg (555 mg/kg active ingredient).

Classification: ~~Core Guideline~~. Acceptable

The study satisfies the Guideline (§84-2) requirements for the evaluation of potential mammalian mutagenicity (*in vivo* cytogenetic assay).

A. MATERIALS

1. Test Material: ATI-720

Description: Tan powder
Lot/batch number: 1111-71
Purity: 11.1% active ingredient (a.i.); the remaining 88.9% of the test material was not specified.
Stability of compound: Expiration date March 11, 1996 (see Data Evaluation Record 3-29/108, MRID No. 429458-02)
CAS number: 11141-17-6
Vehicle used: 0.5% high-viscosity carboxymethylcellulose (CMC)
Other comments: Storage conditions for the test material were not reported. Immediately prior to treatment, samples of the test material were suspended in the vehicle (0.5% CMC) and homogenized for ≈5 minutes using a Tissue-mizer®. The dosing suspensions were mixed periodically to ensure homogeneity. The report did not indicate that dosing suspensions were analyzed for actual concentrations or homogeneity. Purity adjustments were not made.

2. Control Materials:

Negative/route of administration: None

Vehicle/final concentration/route of administration: CMC was administered once by oral gavage at a dosing volume of 20 mL/kg.

Positive/final dose(s)/route of administration: Cyclophosphamide (CP) was prepared in deionized water and administered by oral gavage at a dose of 40 mg/kg.

3. Test Compound:

Volume of test substance administered: 20 mL/kg, based on individual body weights

Route of administration: Oral gavage administration

Dose levels used: 1250, 2500, and 5000 mg/kg (approx. 139, 278, and 555 mg/kg a.i.)

4. Test Animals:

(a) Species: Mouse; Strain: Crl:CD®(ICR)BR; Age: ≈8 weeks (at dosing); Weight Range: 27.0-34.5 g (males); 20.6-26.5 g (females); Sex: Males and females; Source: Charles River Laboratories, Raleigh, NC

(b) Number of animals used per dose, per sacrifice interval:

- Treatment groups: 5 males 5 females
- Positive control: 5 males 5 females
- Vehicle control: 5 males 5 females

Note: A secondary high-dose group (7 males and 10 females exposed to 5000 mg/kg) was included in the study for use in case of mortality in the primary high-dose group; none of these secondary animals was needed in the study.

(c) All test animals were properly maintained.

B. TEST PERFORMANCE:

1. Treatment and Sampling Times:

(a) Test compound:
Dosing: x once ____ twice (24 hours apart)
____ other (describe):
Sampling (after last dose): x 6 hours ____ 12 hours
____ 24 hours ____ 48 hours ____ 72 hours
x other: 18 and 30 hours

(b) Vehicle control:
Dosing: x once ____ twice (24 hours apart)
____ other (describe):
Sampling (after last dose): x 6 hours ____ 12 hours
____ 24 hours ____ 48 hours ____ 72 hours
x other: 18 and 30 hours

(c) Positive control:
Dosing: x once ____ twice (24 hours apart)
____ other (describe):
Sampling (after last dose): ____ 24 hours ____ 48 hours
____ 72 hours
x other: 18 hours

(d) Administration of spindle inhibitor:

Inhibitor used/dose: Colchicine/2 mg/kg
Route of administration: Intraperitoneal injection
Administration time: 1.5-2.5 hours prior to sacrifice

2. Tissues and Cells Examined:

x bone marrow ____ other (list):
Number of cells examined per animal: 50

4. Details of Cell Harvest and Slide Preparation: Animals in the treatment or vehicle control groups were sacrificed by CO_2 asphyxiation at 6, 18, or 30 hours postexposure. Animals in the positive control group were sacrificed 18 hours posttreatment. Bone marrow cells were flushed from both femurs and transferred to Hank's balanced salt solution. Collected marrow cells were centrifuged, resuspended in 0.075 M KCl, recentrifuged, fixed twice in methanol:acetic acid (3:1) and held overnight at 4°C. Cells were dropped onto slides, air-dried, stained with 5% Giemsa (pH 6.8), coverslipped, and coded.

5. Metaphase Analysis: At least 50 metaphases per animal were scored for chromosome aberrations (excluding gaps). Means and the standard errors of the mean were calculated for the frequency of structural aberrations, percentage of cells with structural aberrations, and the percentage of cells with >1 aberration. Mitotic indices (MIs) were determined by scoring 500 cells per animal.

6. Statistical Methods: The percentage of cells with structural aberrations and the percentage of cells with ≥2 structural aberrations were evaluated for significance ($p=0.05$) using the Kruskal-Wallis test. Data from males and females were analyzed separately.

7. Evaluation Criteria: The assay was considered positive if a statistically significant dose-related increase in the number of structural aberrations was observed at one of the three dose levels.

C. REPORTED RESULTS:

1. Preliminary Range-finding/Solubility Determination: Selection of the vehicle and the test material doses for the cytogenetic assay were based on a previously conducted study (HWA Study No. 15457-0-459); no further details were provided. The stock suspension prepared for the cytogenetic assay contained 250 mg/mL of the test material and was reported to form an opaque tan suspension.

2. Cytogenetic Assay: No mortality or clinical signs of toxicity were observed in any treated animals. Representative results from the cytogenetic assay are presented in Table 1. There were no treatment-related effects on the MI or on the frequency of structural aberrations at any dose or sacrifice interval. By contrast, the positive control (40 mg/kg CP) induced significant ($p<0.05$) increases in the frequency of aberrant cells and the frequency of cells with more than one aberration.

Based on these findings, the study author concluded that ATI-720 was negative for inducing chromosomal aberrations in the bone marrow cells of male and female mice under the conditions of the study.

Table 1

TABLE 1. Representative Results of the Mouse Bone Marrow Cytogenetic Assay with ATI-720

Substance	Dose/kg	Exposure Time[a]	Number of Animals Analyzed	Mean % Mitotic Index ± S.E.	Number of Metaphases Analyzed	Mean % Cells with Aberrations[b] ± S.E.	Mean % Cells with >1 Aberration[b] ±S.E.	Biologically Significant Aberrations (No./Type)[c]
Vehicle Control								
0.5% Carboxymethyl-cellulose	20 mL	6	5M	3.3 ± 0.6	250	0.0 ± 0.0	0.0 ± 0.0	--
		18	5M	3.9 ± 0.7	250	0.4 ± 0.4	0.0 ± 0.0	1TB
		30	5M	3.2 ± 0.3	250	0.4 ± 0.4	0.0 ± 0.0	1TB
	20 mL	6	5F	4.0 ± 0.3	250	0.0 ± 0.0	0.0 ± 0.0	--
		18	5F	3.3 ± 0.8	250	0.0 ± 0.0	0.0 ± 0.0	--
		30	5F	4.9 ± 0.5	250	0.4 ± 0.4	0.0 ± 0.0	1SB
Positive Control								
Cyclophosphamide	40 mg	18	5M	1.4 ± 0.5*	250	31.2 ± 2.6*	20.8 ± 2.4*	143TB, 5SB, 9TR, 4QR, 1CR, 1D, 2CI, 7GT
	40 mg	18	5F	3.3 ± 0.8	250	25.2 ± 4.1*	10.8 ± 3.8*	99TB, 7SB, 11D, 5TR, 8QR, 2CR, 2GT
Test Material								
ATI-720	5000 mg (555 mg)[d]	6	5M	4.2 ± 0.2	250	0.4 ± 0.4	0.0 ± 0.0	1TB
		18	5M	5.3 ± 0.7	250	0.4 ± 0.4	0.0 ± 0.0	1TB
		30	5M	4.2 ± 0.4	250	0.4 ± 0.4	0.0 ± 0.0	1TB
	5000 mg (555 mg)[d]	6	5F	4.1 ± 0.3	250	0.0 ± 0.0	0.0 ± 0.0	--
		18	5F	6.7 ± 1.0	250	0.8 ± 0.8	0.0 ± 0.0	2TB
		30	5F	3.7 ± 0.7	250	0.0 ± 0.0	0.0 ± 0.0	--

[a]Time after compound exposure by oral gavage
[b]Excluding gaps
[c]Abbreviations used:

TB = Chromatid break SB = Chromosome break TR = Triradial
QR = Quadriradial CR = Complex rearrangement D = Dicentric
CI = Chromosome intrachange GT = Cell with >10 aberrations

[d]Results for lower levels (1250 and 2500 mg/kg; 139 and 278 mg/kg active ingredient, respectively) at all sacrifice times did not suggest a clastogenic effect. Value in () is the nominal concentration of the active ingredient.

*Significantly higher than the vehicle control group ($p<0.05$) by Kruskal-Wallis test

Note: Data were extracted from the study report, pp. 19-28.

D. DISCUSSION/CONCLUSIONS: The cytogenetic study was properly conducted and the study author's interpretation of the data was correct. ATI-720 was evaluated to 5000 mg/kg (555 mg/kg a.i.) but failed to induce either a cytotoxic or a clastogenic response in bone marrow cells of male or female mice. Although the actual concentration of the active ingredient was relatively low, it is doubtful whether higher levels could have been achieved since the stock concentration (250 mg/mL) used to deliver the high dose and prepare lower levels formed a suspension in the vehicle. In addition, the sensitivity of the test system was clearly demonstrated by the significant ($p<0.05$) increases in chromosome aberrations in both male and female mice exposed to the positive control (40 mg/kg CP). We conclude, therefore, that the study provided acceptable evidence that ATI-720 was not clastogenic in this *in vivo* cytogenetic assay.

E. QUALITY ASSURANCE MEASURES: The test was performed under GLPs. A quality assurance statement was signed and dated June 29, 1993.