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

009451

APR 20 1992

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Difenzoquat (Avenge, AC 84,777): Evaluation of the Following Studies: Acute Oral, Dermal and Inhalation; Dermal Sensitization, 21-Day Dermal and Three Mutagenicity Studies.

EPA ID No.: 106401
Case Number: 819395
Submission No.: S388447
HED Project No.: 1-0434
Tox. Chem. No.: 363A

FROM: Krystyna K. Locke, Toxicologist *Krystyna K. Locke 2/19/92*
Section I, Toxicology Branch I
Health Effects Division (H7509C)

TO: Lois Rossi/Terri Stowe, PM 74
Reregistration Branch
Special Review and Reregistration Division (H7508C)

THRU: Roger Gardner, Section Head *Roger Gardner 4-16-92* *KB 4/17/92*
Section I, Toxicology Branch I
Health Effects Division (H7509C)

Toxicology Branch I/HED has completed an evaluation of the following studies:

81-1. Oral LD_{50} Study in Albino Rats with AC 84,777; Joel E. Fischer; American Cyanamid Company; Study No.: A89-195; October 9, 1989. MRID No.: 413254-06

LD_{50} and 95% confidence limits:

Males: 617 (497-766) mg/kg
Females: 373 (266-524) mg/kg
Males and Females: 485 (372-631) mg/kg

Toxicity Category: II

Classification: Core-Guideline

82-2. Dermal LD_{50} Study in the Albino Rabbit with AC 84,777; Joel E. Fischer; American Cyanamid Company; Study No.: A89-191; October 9, 1989. MRID No.: 413254-07

LD_{50} = > 2000 mg/kg (Limit dose; males and females)

Toxicity Category: III

Classification: Core-Guideline

81-3. AC 84,777 Lot No.: AC 6027-118 Acute Inhalation Toxicity, LC_{50}, 4-Hour Exposure - Rats; Richard J. Hershman; Biosearch Incorporated, Philadelphia, PA; Study No.: 89-6760A; September 20, 1989. MRID No.: 413254-08

LC_{50} and 95% confidence limits (analytical values):

Males: 0.62 (0.47-0.83) mg/L
Females: 0.36 (0.27-0.48) mg/L
Males and Females: 0.50 (0.41-0.60) mg/L

LC_{50} values were calculated by the following procedure:

Litchfield, J.I. and Wilcoxon, F., "A Simplified Method of Evaluating Dose-effect Experiments." J. Pharmacol. Exp. Ther. 96:99-115 (1949).

Size of particles: respirable

Toxicity Category: II

Classification: Core-Guideline

81-6. Dermal Sensitization Study with AC 84,777, Lot Number: AC 6027-118 in Guinea Pigs; Jennifer Bielucke; American Cyanamid Company; Study No.: 89-6761A; September 14, 1989. MRID No.: 413254-09

Undiluted AC 84,777 (Difenzoquat; Avenge) Technical, 0.4 g, was not a skin sensitizer in this study (Beuhler test). Positive response was obtained with dinitrochlorobenzene, a known sensitizer. The animals used were male guinea pigs (Hartley strain).

Classification: Core-Guideline

82-2. AC 84,777 Lot # AC 6027-118: Twenty-One Day Dermal Toxicity Study - Rabbits; George E. Moore; Biosearch Incorporated, Philadelphia, PA; Study No.: 89-6762A; December 8, 1989.
MRID No.: 413254-10

<u>Dermal NOEL</u>: 250 mg/kg
<u>Dermal LEL</u>: 500 mg/kg (Eschar and focal epithelial hyperplasia)

<u>Classification</u>: Core-Minimum

84-Mutagenicity. Mutagenicity Testing of AC 84,777 in the <u>In Vitro</u> CHO/HGPRT Mutation Assay; E. Johnson; American Cyanamid Company; Study No.: 0488; December 17, 1984.
MRID No.: 413254-11

Difenzoquat was not mutagenic in this study, with and without metabolic activation. This study satisfies Guideline requirements for genetic effects Category I, Gene Mutations.

<u>Classification</u>: Acceptable

84-Mutagenicity. <u>In Vitro</u> Chromosomal Aberrations in Chinese Hamster Ovary Cells with AC 84,777; T. Cortina; Hazleton Biotechnologies Corporation; Study No.: HLA 362-172; January 30, 1985.
MRID No.: 414153-03

Difenzoquat was not mutagenic in this study, with and without metabolic activation. This study satisfies Guideline requirements for genetic effects Category II, Structural Aberrations.

<u>Classification</u>: Acceptable

84-Mutagenicity. Rat Hepatocyte Primary Culture/DNA Repair Test: AC 84,777; T. Barfknecht; Pharmakon Research International, Inc., Waverly, PA; Study No.: PH 311-AC-003-84; November 1, 1984.
MRID No.: 414153-04

Difenzoquat was not mutagenic in this study. This study satisfies Guideline requirements for genetics effects Category III, Other Mutagenic Mechanisms.

<u>Classification</u>: Acceptable

GUIDELINE: 81-1

Primary Review by: Krystyna K. Locke 4/19/92
Krystyna K. Locke, Review Section I, Toxicology Branch I/HED

Secondary Review by: Roger Gardner Roger Gardner 4-16-92
Section Head, Review Section I, Toxicology Branch I/HED

DATA EVALUATION RECORD

STUDY TYPE: Acute Oral Toxicity in the Rat

EPA IDENTIFICATION NOS: EPA MRID No.: 413254-06
Tox. Chem. No.: 363A

TEST MATERIAL: AC 84,777; white powder, soluble in water; purity: 99.4%.

SYNONYMS: Difenzoquat, AVENGE Technical

STUDY NUMBER(S): A89-195

SPONSOR: American Cyanamid Company (for Global Plant Industry Development Department)

TESTING FACILITY: American Cyanamid Company, Toxicology Department, Agricultural Research Division, Princeton, New Jersey

TITLE OF REPORT: Oral LD_{50} Study in Albino Rats with AC 84,777

AUTHOR: Joel E. Fischer, Senior Research Toxicologist

REPORT ISSUED: October 9, 1989

CONCLUSIONS:

LD_{50} and 95% confidence limits:

Males: 617 (497-766) mg/kg
Females: 373 (266-524) mg/kg
Males and Females: 485 (372-631) mg/kg

Toxicity Category: II

Classification: Core-Guideline

EXPERIMENTAL PROCEDURES

This study was started on August 9. 1989. Sprague-Dawley rats, Crl CD(SD)BR strain, 5/sex/dose, were given single doses of AC 84,777 and then were observed for toxic signs and mortality for 14 days. The test material (200, 400 or 800 mg/kg of body weight) was administered by gavage, using 10 ml of solution (in tap water)/kg of body weight. The selection of dose levels was based on previous studies done with the same test material. The animals were fasted for about 18 hours prior to dosing. The rats were:

1. About 5-6 weeks old when obtained from Charles River Laboratories, Wilmington, MA.

2. Acclimated for 7 days.

3. Housed by sex in groups of 5 at temperatures of 70-73° F, relativa humidity of 46-61% and 12-hour light/dark cycle.

4. Identified by ear notches.

5. Given unrestricted amounts of food (Purina Rodent chow) and water (automatic system).

6. Weighed individually just before dosing and during the observation days 7 and 14.

All animals were necropsied and the following organs were examined grossly: liver, kidneys, spleen, stomach, intestinal tract, lungs and urinary bladder.

The acute oral LD_{50} for each sex with 95% confidence limits was calculated by the method of moving averages as described by C.W. Weil, 1952, Tables for Convenient Calculation of Median Effective Dose and Instructions in their Use, Biometrics, volume 8, pages 249-263.

RESULTS

Mortality

Deaths occurred only in the high-dose males and females, and in the mid-dose females. Most animals died within 2-8 hours after dosing.

Toxic Signs

Toxic signs included salivation, decreased activity, prostration and diuresis. Salivation was observed in all groups; decreased activity and prostration, in the mid-dose and high-dose

groups; and diuresis, only in the high-dose group. Signs of toxicity generally occurred during the first 24 hours following dosing and disappeared completely, in the surviving animals, within 1-2 days.

Body Weight

All of the surviving animals gained weight during the 2-week observation period following treatment. The smallest weight gain occurred in the low-dose female group.

Gross Necropsy Findings

Nothing abnormal was observed in all of the survivors and a few nonsurvivors. Most of the nonsurvivors had congested livers and kidneys, and enlarged, fluid-filled, pale intestines. One high-dose nonsurvivor also had vascularized intestines and stomach.

COMMENTS

This study is well reported and meets the December 24, 1989 EPA acceptance criteria (Attachment). Statements of Good Laboratory Practice (GLP) and of Quality Assurance were included in the submission.

ATTACHMENT

MRID No.: 41520406
Study No.: A89-195
Study Date: 10/9/89

Subdivision F
Guideline Ref. No. 81-1
December 24, 1989

009451

81-1 Acute Oral Toxicity in the Rat

ACCEPTANCE CRITERIA

Does your study meet the following acceptance criteria?

1. ✓ Technical form of the active ingredient tested. (for reregistration only)
2.* ✓ At least 5 young adult rats/sex/group
3. ✓ Dosing, single oral may be administered over 24 hrs.
4.* — Vehicle control if other than water.
5. ✓ Doses tested, sufficient to determine a toxicity category or a limit dose (5000 mg/kg).
6. ✓ Individual observations at least once a day.
7. ✓ Observation period to last at least 14 days, or until all test animals appear normal whichever is longer.
8. ✓ Individual daily observations.
9.* ✓ Individual body weights.
10.* ✓ Gross necropsy on all animals.

Criteria marked with a * are supplemental and may not be required for every study.

009451

GUIDELINE: 81-2

Primary Review by: Krystyna K. Locke 2/19/92
Krystyna K. Locke, Review Section I, Toxicology Branch I/HED

Secondary Review by: Roger Gardner Roger Gardner 4-16-92
Section Head, Review Section I, Toxicology Branch I/HED

DATA EVALUATION RECORD

STUDY TYPE: Acute Dermal Toxicity in the Rabbit

EPA IDENTIFICATION NOS: EPA MRID No.: 413254-07
Tox. Chem. No.: 363A

TEST MATERIAL: AC 84,777; white powder, soluble in water; purity: 99.4%.

SYNONYMS: Difenzoquat, AVENGE Technical

STUDY NUMBER(S): A89-191

SPONSOR: American Cyanamid Company (for Global Plant Industry Development Department)

TESTING FACILITY: American Cyanamid Company, Toxicology Department, Agricultural Research Division, Princeton, New Jersey

TITLE OF REPORT: Dermal LD_{50} Study in the Albino Rabbit with AC 84,777

AUTHOR: Joel E. Fischer, Senior Research Toxicologist

REPORT ISSUED: October 9, 1989

CONCLUSIONS:

LD_{50} = > 2000 mg/kg (Limit dose; males and females)

Toxicity Category: III

Classification: Core-Guideline

EXPERIMENTAL PROCEDURES

This study was started on July 25, 1989. Five male and 5 female rabbits, New Zealand White strain, were treated once with a limit dose (2000 mg/kg of body weight) of AC 84,777 and then were observed for toxic signs and mortality for 14 days. The test material was applied by spreading it on a plastic sheet, moistening with tap water and then placing that sheet on the animal, covering about 10% of the (clipped) body surface area. The application site was occluded and, following a 24-hour exposure, wiped with the cloth wrap. The rabbits were:

1. About 12-16 weeks old when obtained from Skippack Farms, Skippack, Pennsylvania.

2. Acclimated for at least 3 days.

3. Housed individually at temperatures of 69-76° F, relative humidity of 49-86% and 12-hour light/dark cycle.

4. Identified by metal ear tags.

5. Given unrestricted amounts of food (Purina Rabbit Chow) and tap water.

6. Weighed just before treatment and during the observation days 7 and 14.

All animals were necropsied and the following organs/tissues were examined grossly: liver, kidneys, spleen, stomach, intestinal tract, lungs, urinary bladder and skin.

RESULTS

Mortality

One female died 24 hours after dosing. There were no deaths among males.

Toxic Signs

Toxic signs, observed during the course of the study, included severe skin irritation at the application site (erythema, edema, small sores, subdural hematoma, fissuring, dark red welts and necrosis), anorexia, diarrhea, emaciation and nasal discharge.

Body Weight

Males generally lost weight and females gained very little weight during the 14-day observation period. The mean weight

loss for the males was 99 g and the mean weight gain for the females was 16 g.

Gross Necropsy Findings

The nonsurviving female had pale kidneys, congested lungs and erythema and edema at the application site. The only findings observed in the survivors were small sores on abdomen (5 animals) and gas-filled intestinal tract (2 animals).

COMMENTS

This study is well reported and meets the December 24, 1989 EPA acceptance criteria (Attachment). Statements of Good Laboratory Practices (GLP) and of Quality Asuurance were included in the submission.

009452

ATTACHMENT

MRID No.: 41325407
Study No.: A89-191
Study Date: 10/9/89

009151

Subdivision F
Guideline Ref. No. 81-2
December 24, 1989

81-2 Acute Dermal Toxicity in the Rat, Rabbit or Guinea Pig

ACCEPTANCE CRITERIA

Does your study meet the following acceptance criteria?

1. ✓ Technical form of the active ingredient tested. (for reregistration only)
2.* ✓ At least 5 animals/sex/group
3.* ✓ Rats 200-300 gm, rabbits 2.0-3.0 kg or guinea pigs 350-450 gm.
4. ✓ Dosing, single dermal.
5. ✓ Dosing duration at least 24 hours.
6.* — Vehicle control, only if toxicity of vehicle is unknown.
7. ✓ Doses tested, sufficient to determine a toxicity category or a limit dose (2000 mg/kg).
8. ✓ Application site clipped or shaved at least 24 hours before dosing Not stated when clipped
9. ✓ Application site at least 10% of body surface area.
10. ✓ Application site covered with a porous nonirritating cover to retain test material and to prevent ingestion.
11. ✓ Individual observations at least once a day.
12. ✓ Observation period to last at least 14 days, or until all test animals appear normal whichever is longer.
13. ✓ Individual daily observations.
14.* ✓ Individual body weights.
15.* ✓ Gross necropsy on all animals.

Criteria marked with a * are supplemental and may not be required for every study.

009451

GUIDELINE: 81-3

Primary Review by: Krystyna K. Locke 2/19/92
Krystyna K. Locke, Review Section I, Toxicology Branch I/HED

Secondary Review by: Roger Gardner Roger Gardner 4-16-92
Section Head, Review Section I, Toxicology Branch I/HED

DATA EVALUATION REPORT

STUDY TYPE: Acute Inhalation Toxicity in the Rat

EPA IDENTIFICATION NOS: EPA MRID No.: 413254-08
Tox. Chem. No.: 363A

TEST MATERIAL: AC 84,777; white powder, soluble in water; purity: 99.4%.

SYNONYMS: Difenzoquat, AVENGE Technical

STUDY NUMBER(S): 89-6760A

SPONSOR: American Cyanamid Company, Agricultural Research Division, Princeton, NJ

TESTING FACILITY: Biosearch Incorporated, Philadelphia, PA

TITLE OF REPORT: AC 84,777 Lot No.: AC 6027-118
Acute Inhalation Toxicity, LC_{50},
4-Hour Exposure - Rats

AUTHOR(S): Richard J Hershman

STUDY COMPLETED ON: September 20, 1989

CONCLUSIONS:

LC_{50} and 95% confidence limits (analytical values):

Males: 0.62 (0.47-0.83) mg/L
Females: 0.36 (0.27-0.48) mg/L
Males and Females: 0.50 (0.41-0.60) mg/L

LC_{50} values were calculated by the following procedure: Litchfield, J.I. and Wilcoxon, F., "A Simplified Method of Evaluating Dose-effect Experiments." J. Pharmacol. Exp. Ther. 96:99-115 (1949).

Size of particles: respirable

Toxicity Category: II

Classification: Core-Guideline

EXPERIMENTAL PROCEDURES

This study was started on July 19, 1989. Outbred Sprague-Dawley rats, 10/sex/group, were exposed once to aerosolized AC 84,777 (whole body exposure in the individual restraining cages) and then were observed for toxic signs and mortality for 14 days. The test material was aerosolized as solutions in deionized water at the following mean concentrations (analytical values): 0 (air control group), 0.255, 0.438, 0.579, 1.14 and 1.72 mg/L. The controls and the first three treated groups were exposed for 4 hours, and the last two treated groups for 3 hours (all animals died during exposure). The capacity of the exposure chamber was 230 liters. There were at least 10 air changes per hour and the oxygen content of the air was at least 19%. The chamber temperature was 22±2° C. The concentration of the test material in the chamber and the size of the particles were measured for each group every hour during the exposure. The rats were:

1. Young adults weighing 204-316 g at the initiation of the study.

2. Acclimated for 7 days.

3. Housed individually at temperature of 70-80° F, relative humidity of 55±25% and 12-hour light/dark cycle.

4. Identified by ear punch.

5. Given unrestricted amounts of food (Purina Certified Rodent Chow 5002) and tap water.

6. Observed for toxic signs and mortality continually during the exposure, frequently immediately after exposure and twice daily starting with the observation day 2.

7. Weighed on the day before dosing, the day of dosing, during the observation days 7 and 14, and at sacrifice.

All animals on the study were necropsied and the following organs of all animals sacrificed at the termination of the study were weighed: lungs, liver, heart, kidneys and gonads. The following organs/tissues of the survivors were fixed in formalin and saved for possible future histopathological examination: lungs, trachea, bronchi, tissues from the nasal passages, liver, kidneys, heart, thyroid, adrenals, gonads, spleen, stomach, small and large intestine, bone marrow, brain and any tissue which appeared abnormal.

RESULTS

Mortality

There were no deaths in the control and the 0.255 mg/L groups, but all animals died in the 1.14 and 1.72 mg/L groups. In the remaining two groups, most of the females also died. All deaths occurred either during the exposure to AC 84,777 or within one day after the exposure. These data are summarized below.

AC 84,777 (mg/L)	Number of Rats Dying		Time of Death (Observation Days)	
	Males	Females	Males	Females
0	0	0		
0.255	0	0		
0.438	1	8	1	1[a]
0.579	4	9	0-1	0-1
1.14	10	10	E	E
1.72	10	10	E	E

This table is based on TABLES 1a-f, pages 12-17 of the submission. Observation day 0 = Day of exposure.
[a]One female in this group died during observation day 2.
E = These rats died during exposure which was terminated after 3 hours.

Toxic Signs

All rats in the control group appeared normal during and after exposure. Rats exposed to 0.255, 0.438 or 0.579 mg/L of AC 84,777 were inactive (no motion was visible), closed their eyes and had wet or stained noses during exposure. After exposure, these animals had ruffled appearance, brown/tan staining (ocular, mouth and nasal) and tremors. Most animals were also inactive and unsteady on their feet. Rats in the 0.255 mg/L group appeared normal by the observation day 4 and those in the 0.438 and 0.579 mg/L groups, by the observation days 5-6.

Most of the rats in the 1.14 and 1.72 mg/L groups could not be observed during the exposure because the chambers were too cloudy.

Body Weight

Comparing mean body weights on day 14 with those on day 0, the control males gained 64 g and females 12 g during the two weeks following exposure. The mean body weight gains for the treated males in the 0.255, 0.438 and 0.579 mg/L groups were 74, 85 and 67 g, respectively. The corresponding values for the females were 16, 17 (2 rats) and 42 g (1 rat), respectively. All rats in the 1.14 and 1.72 mg/L groups died during exposure.

Gross Necropsy Findings

Abnormalities were not observed in all survivors, both untreated and treated. Irrespective of the exposure level of AC 84,777, each nonsurviving rat had discolored lungs (bright red or dark red patches/areas). Mouth staining was also frequently observed in the nonsurvivors at each exposure level. In the 1.72 mg/L group, each rat had hark red lungs with free flowing dark red liquid on the cut surface of the lungs.

Organ Weights and Organ/Body Weight Ratios

These determinations were performed only for animals sacrificed at the termination of the study. The mean organ weights (heart, lungs/trachea, liver, kidneys, testes and ovaries) and organ/body weight ratios for the treated groups were similar to those obtained for the control group.

Particle Sizes

Rats were exposed to respirable particles of AC 84,777 in this study. The mass median aerodynamic diameters (MMAD) of the aerosolized particles ranged from 0.4 to > 9.0 µm. Over 60% (range: 45.17-80.56%) of the particles were 2.1 µm or smaller. Particles > 9.0 µm ranged from 0.2-2.0 %. The size of the particles to which each group was exposed is summarized below.

AC 84,777 (mg/L)	Average MMAD* ± SD (µm)	Range of MMAD ± SD (µm)
0.255	1.60 ± 0.08	1.53 ± 1.96 - 1.68 ± 1.88
0.438	1.37 ± 0.34	0.92 ± 3.04 - 1.71 ± 1.93
0.579	1.79 ± 0.60	0.93 ± 2.65 - 2.30 ± 1.93
1.14	1.88 ± 0.01	1.86 ± 1.74 - 1.89 ± 1.66
1.72	2.00 ± 0.14	1.91 ± 2.0 - 2.16 ± 1.89

*This table is based on TABLE 7 (a-e), pages 51-55 of the submission (Biosearch Project No. 89-6760A; MRID: 41325408).

Particle size was determined at 30, 90, 150 and 210 minutes during exposure for the first three groups and at 30, 90 and 150 minutes for the last two groups (all animals died during exposure).

COMMENTS

In general, this study is well planned, conducted and reported, and meets the December 24, 1989 EPA acceptance criteria (Attachment). Statements of Good Laboratory Practices (GLP) and Quality Assurance were included in the submission. The following omissions and inaccuracies, although not serious enough to downgrade the study, should be noted:

1. It was not reported how the exposure levels of AC 84,777 used in this study were selected. In the testing laboratory's protocol for an acute inhalation study, included in this submission, it was stated that dose levels for such a study would be based on the results of a pilot study. However, no data from a pilot study with AC 84,777 or reference to such data were included in the submission.

2. It was reported in the Results section on page 10 of the submission that all nonsurviving animals and one in the 0.579 mg/L group, sacrificed at the termination of the study, had discolored lungs. However, according to the individual gross pathology findings for that group, only the nonsurviving rats had discolored lungs (page 29 of the submission).

3. It was reported in the Results section on page 10 of the submission that the mean organ/body weight ratios for the five treatment groups were similar to the mean values obtained for the control group. However, organ weights were obtained only for animals sacrificed at the termination of the study (after the 14-day observation period). All rats in the 1.14 and 1.72 mg/L groups died during exposure and their organs were not weighed (pages 42, 43,48 and 49 of the submission).

ATTACHMENT

MRID No.: 41325408
Study No.: 89-6760A
Study Date: 9/20/89

009451

Subdivision F
Guideline Ref. No. 81-3
December 24, 1989

81-3 Acute Inhalation Toxicity in the Rat

ACCEPTANCE CRITERIA

Does your study meet the following acceptance criteria?

1. ✓ Technical form of the active ingredient tested. (for reregistration only)
2. — Product is a gas, a solid which may produce a significant vapor hazard based on toxicity and expected use or contains particles of inhalable size for man (aerodynamic diameter 15 um or less).
3.* ✓ At least 5 young adult rats/sex/group
4.* ✓ Dosing, at least 4 hours by inhalation.
5.* ✓ Chamber air flow dynamic, at least 10 air changes/hour, at least 19% oxygen content.
6. ✓ Chamber temperature, 22° C (±2°), relative humidity 40-60%.
7. ✓ Monitor rate of air flow
8. ✓ Monitor actual concentrations of test material in breathing zone.
9. ✓ Monitor aerodynamic particle size for aerosols.
10. ✓ Doses tested, sufficient to determine a toxicity catagory or a limit dose (5 mg/L actual concentration of respirable substance).
11. ✓ Individual observations at least once a day.
12. ✓ Observation period to last at least 14 days, or until all test animals appear normal whichever is longer.
13. ✓ Individual daily observations.
14.* ✓ Individual body weights.
15.* ✓ Gross necropsy on all animals.

Criteria marked with a * are supplemental and may not be required for every study.

GUIDELINE: 81-6

Primary Review by: Krystyna K. Locke 2/19/92
Krystyna K. Locke, Review Section I, Toxicology Branch I/HED

Secondary Review by: Roger Gardner Roger Gardner 4-16-92
Section Head, Review Section I, Toxicology Branch I/HED

DATA EVALUATION REPORT

STUDY TYPE: Dermal Sensitization in the Guinea Pig

EPA IDENTIFICATION NOS: EPA MRID No.: 413254-09
Tox. Chem. No.: 363A

TEST MATERIAL: AC 84,777; white powder, soluble in water; purity: 99.4%.

SYNONYMS: Difenzoquat, AVENGE Technical

STUDY NUMBER(S): 89-6761A

SPONSOR: American Cyanamid Company, Agricultural Research Division, Princeton, NJ

TESTING FACILITY: Biosearch Incorporated, Philadelphia, PA

TITLE OF REPORT: Dermal Sensitization Study with AC 84,777, Lot Number: AC 6027-118 in Guinea Pigs

AUTHOR(S): Jennifer Bielucke

STUDY COMPLETED ON: September 14, 1989

CONCLUSIONS:

Undiluted AC 84,777 (Difenzoquat; Avenge) Technical was not a skin sensitizer in this study (Beuhler test). Positive response was obtained with dinitrochlorobenzene, a known sensitizer. The animals used were male guinea pigs (Hartley strain).

Classification: Core-Guideline

EXPERIMENTAL PROCEDURES

This study was started on July 26, 1989, and the method used was similar to that described by Buehler[a]. Determination of sensitization was based on reactions to the challenge dose.

[a]Buehler, E.V., Delayed Contact Hypersensitivity in the Guinea Pig; Arch. Dermat. 92:171-175, 1965.

Young adult male guinea pigs (Hartley strain; weight on study day one: 407-565 g) were treated as follows:

Group	Number of Animals	Treatment
AC 84,777 (0.4 g)	10	9 induction applications (3/week) and, 2 weeks after the last application, one challenge dose.
Dinitrochlorobenzene (0.1 % w/v; positive control)	10	As above
AC 84,777 (0.4 g; naive control)	10	One challenge dose, but no induction applications.
Dinitrochlorobenzene (0.1% w/v; naive positive control)	5	As above

For each application, AC 84,777 was moistened with 0.4 ml of saline and applied on a one-inch gauze pad which was then placed on the intact (clipped) back of an animal and occluded. Dinitrochlorobenzene (1-chloro-2,4-dinitrobenzene) was applied in the same manner, but in 50% ethanol:0.9% saline solution. All induction applications were made on the left side and challenge applications on the right side of an animal. The control groups were treated with challenge doses when other groups were so treated. Application sites were scored for erythema and edema by Draize procedure[a] at 24 and 48 hours following each application. Each exposure lasted for 6 hours.

The concentration of AC 84,777 used in this study was based on the results of a pilot study, conducted to obtain a minimum irritation concentration of AC 84,777. In the pilot study, AC 84,777 was applied to 8 male guinea pigs, one each at 100% (0.4 g, as supplied, moistened with 0.4 ml of saline), 75%, 50%, 25%, 15%, 10%, 5% and 1% w/w concentration in saline for a 6-hour contact period. The volume of each application was 0.4 ml and each animal received 4 applications over an 8-day period. The guinea pigs weighed 309-439 g on study day one.

[a]Draize, J.H., Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics; in DERMAL TOXICITY, pp. 46-53. Topeka, Kansas: Association of Food and Drug Officials of the United States, 1959.

In both studies (pilot and main), the animals were:

1. Obtained from Backshire Corporation, Perkasie, PA.
2. Acclimated for 7 days.
3. Housed singly at temperature of 65-75° F, relative humidity of 55±25% and 12-hour light/dark cycle.
4. Identified by magic marker color coding. Animal's number, group and project number were displayed on the cage.
5. Allowed unrestricted amounts of food (Wayne Guinea Pig Diet) and water.

The animals were observed once daily for general behavior and appearance. Body weights were recorded one day before the induction application, at weekly intervals thereafter and at study termination. Gross necropsy was performed only on the nonsurvivors.

RESULTS

Pilot Study

No erythema or edema was observed at all concentrations of AC 84,777 tested. Based on these findings, AC 84,777 was used as supplied in the main study.

Dermal Sensitization Study

Guinea pigs treated with AC 84,777 had no erythema or edema during the induction phase and after the challenge. The Draize score was zero for each animal and at each reading interval. All animals appeared normal and all gained weight during the course of the study. The same results were obtained for the naive control and naive positive control groups.

Guinea pigs in the positive control group had erythema but no edema. During the induction phase, very slight to severe erythema and dry flaking skin were observed in all animals. During the challenge phase, 8 animals had well defined erythema and 2 animals had moderate to severe erythema at the 24-hour scoring interval. At the 48-hours scoring interval, 3 animals had very slight erythema and 7 animals had well defined erythema. With the exception of erythema, animals in this group appeared normal and all gained weight during the course of the study.

Based on the above findings, AC 84,777 did not appear to be a skin sensitizer, whereas dinitrochlorobenzene was a skin sensitizer in male guinea pigs.

009451

COMMENTS

This study is well planned, conducted and reported, and meets the December 24, 1989 EPA acceptance criteria (Attachment). Statements of Good Laboratory Practices (GLP) and Quality Assurance were included in the submission. However, the copy submitted to Toxicology Branch/HED for evaluation was of poor quality. Most of the submission was barely legible and, therefore, time-consuming to decipher and review.

ATTACHMENT

MRID No.: 41363404
Study No.: 89-6761A
Study Date: 9/14/89

81-6 Dermal Sensitization in the Guinea Pig

ACCEPTANCE CRITERIA

Does your study meet the following acceptance criteria?

1. ✓ Technical form of the active ingredient tested. (for reregistration only)
2. — Study not required if material is corrosive or has a pH of $\leq$ 2 or $\geq$ 11.5.
3. ✓ One of the following methods is utilized:
   - ___ Freund's complete adjuvant test
   - ___ Guinea pig maximization test
   - ___ Split adjuvant technique
   - ✓ Buehler test
   - ___ Open epicutaneous test
   - ___ Mauer optimization test
   - ___ Footpad technique in guinea pig
   - ___ Other test accepted by OECD (specify)________________
4. ✓ Complete description of test
5.* ✓ Reference for test.
6. ✓ Test followed essentially as described in reference document.
7.* ✓ Positive control included.

Criteria marked with a * are supplemental and may not be required for every study.

GUIDELINE: 82-2

Primary Review by: Krystyna K. Locke 2/19/92
Krystyna K. Locke, Review Section I, Toxicology Branch I/HED

Secondary Review by: Roger Gardner Roger Gardner 4-16-92
Section Head, Review Section I, Toxicology Branch I/HED

DATA EVALUATION REPORT

STUDY TYPE: Repeated Dose Dermal Toxicity (21-Day) in the Rabbit

EPA IDENTIFICATION NOS: EPA MRID No.: 413254-10
Tox. Chem. No.: 363A

TEST MATERIAL: AC 84,777; white powder, soluble in water, purity: 99.4%.

SYNONYMS: Difenzoquat, AVENGE Technical

STUDY NUMBER(S): 89-6762A

SPONSOR: American Cyanamid Company, Agricultural Research Division, Princeton, NJ

TESTING FACILITY: Biosearch Incorporated, Philadelphia, PA

TITLE OF REPORT: AC 84,777 Lot# AC 6027-118: Twenty-One Day Dermal Toxicity Study - Rabbits

AUTHOR(S): George E. Moore

STUDY COMPLETED ON: December 8, 1989

CONCLUSIONS:

Dermal NOEL: 250 mg/kg
Dermal LEL: 500 mg/kg (Eschar and focal epithelial hyperplasia)

Classification of Study: Core-Minimum

New Zealand rabbits, 5-7/sex/group, were exposed dermally to technical AC 84,777 for 3 weeks (5 days/week, 6 hours/day). The exposure of females was delayed by 21 days to allow for replacement of the animals due to the poor health of the initial shipment of females. The dose levels of AC 84,777 used were 0 (sham-treated), 250, 500 or 1000 mg/kg (limit dose). Eschar was the most frequent skin irritation observed in the mid-dose and high-dose animals.

EXPERIMENTAL PROCEDURES

New Zealand rabbits, used in this study, came from two different shipments. The first shipment of 36 males and 32 females arrived to the testing laboratory on 8/15/89. However, following a 27-day acclimation period, all females were rejected because of poor physical condition and only males were assigned to the study. The second or replacement shipment (36 females and 8 males) was received at the testing laboratory on 9/12/89 and these animals were acclimated for 20 days before they were assigned to the study. Dosing of males was, therefore, started on 9/11/89 and of females on 10/2/89.

It was originally intended to have 6 males and 6 females in each test group. However, following a moribund sacrifice of one male rabbit on study day 14 in Group III, another male rabbit (from the second shipment) was included in that group on 10/2/89. Data for this new rabbit were reported with the rest of the group, but were not used in statistical analyses. In the case of Group IV, it was noted at necropsy that one male rabbit was inadvertently placed on test with the females. This error resulted in Group IV consisting of 7 males and 5 females. The final assignment of animals to groups was as follows:

Group	AC 84,777 (mg/kg)*	Number of Animals in Group	
		Males	Females
I	0**	6	6
II	250	6	6
III	500	7	6
IV	1000***	7	5

*Nominal levels. Adjusted for 99.4% purity, these levels were 251.5, 503.0 and 1006.0 mg/kg for Groups II, III and IV, respectively.

**Sham-treated

***Limit dose

Selection of doses was based on results of the acute dermal toxicity studies. AC 84,777 was applied as a 50% w/v solution in saline using doses adjusted for purity. The applications were made on gauze pads which were then placed on clipped backs of the animals and occluded for 6 hours daily. After removal of the pads, the sites (test and control groups) were cleansed with 1% v/v dilution of ivory liquid soap. AC 84,777 was administered 5 days/week over a 3-week period. The test sites were examined for skin irritation before each application using the procedure of

Draize[a]. The rabbits were:

1. Purchased from Buckshire Corporation, Perkasie, PA.

2. Acclimated as indicated above. The rabbits were clipped and sham-wrapped for 3 hours/day during the last week of the acclimation period (days -7, -5, -4 and -3).

3. Assigned to groups using a computer-generated random number table.

4. Housed individually at temperatures of 68.5-81.9° F, relative humidity of 50-86% and 12-hour light/dark cycle.

5. Identified by ear tags.

6. Given unrestricted amounts of food (Wayne 15% Rabbit Ration) and tap water. Food consumption was determined weekly during the acclimation (days -10 and -3) and treatment (days 4, 11 and 18) periods, and before study termination (day 20).

7. Weighed on acclimation days -14 and -7, and on treatment days 0, 7, 14 and 21 (just before sacrifice). At the initiation of dosing, males weighed 2.43-3.32 kg and females 2.27-2.95 kg.

8. Observed daily for toxic signs and mortality.

Clinical tests were performed on all animals five days before the initiation of treatment and at the termination of the study. The rabbits were fasted at least 18 hours prior to taking blood samples. The following determinations were performed:

<u>Hematology</u>

Hematocrit
Hemoglobin
Erythrocyte count
Leucocyte count (total and differential)
Platelet and reticulocyte counts

---

[a]Draize, J.H., Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics; in DERMAL TOXICITY, pp. 46-53. Topeka, Kansas: Association of Food and Drug Officials of the United States, 1959.

Clinical Chemistry

Serum alanine aminotransferase (SGPT)
Serum aspartate aminotransferase (SGOT)
Gamma glutamyl transpeptidase
Total serum protein
Bilirubin (total and direct)
Urea nitrogen

Albumin	Calcium
Creatinine	Phosphorus
Glucose	Chloride
Potassium	Sodium

All animals were necropsied and the following organs were weighed: liver, kidneys, adrenals and gonads.

Histopathology was performed on all nonsurvivors (rabbits found dead or sacrificed moribund during the study) and on all rabbits from the control and high-dose groups sacrificed at the termination of the study. The following organs/tissues were stained with hematoxylin/eosin and examined:

Adrenals	Skin (treated area)
Kidneys	Skin (mammary area)
Liver	Testes/Epididymides
Ovaries	All tissues with gross lesions

Tissues with gross lesions and skin (treated area) were also examined histopathologically for all rabbits in the low-dose and mid-dose groups.

Statistical analyses were performed on body and organ weights, organ/body weight ratios, food consumption, and hematology and clinical chemistry data. The tests performed and procedures used are detailed in Attachment I.

RESULTS

Mortality

There were two deaths in this study. One male from the control group was found dead during the initial treatment (study day 0). Another male from Group III was sacrificed moribund on study day 14. Since there were no deaths in Group IV, the death in Group III did not appear to be treatment-related.

Toxic Signs

All of the male rabbits in the control group and most of the males in the treated groups appeared normal throughout the study. The male rabbit from Group III which was sacrificed moribund did not eat or drink for several days before sacrifice and was emaciated, dehydrated, bloated, lethargic and hypothermic. Three other males (1 in Groups II, III and IV) did not eat, had diarrhea and were bloated during some days of the study. One male in Group IV also had nasal discharge and wheezing.

Most of the female rabbits in Groups I, II and III, and all of the females in Group IV appeared normal throughout the study. Single incidences of ocular and nasal discharges, diarrhea, anogenital staining, wheezing and audible respiration were noted in Group I (controls). Diarrhea (single incidences) was also observed in Groups II and III. In Group II, two rabbits refused food during some days of the study.

Based on the incidence, toxic signs did not appear to be treatment-related. According to the testing laboratory, toxic signs seemed to be the result of coccidiosis and respiratory infections encountered in most animals. Toxicology Branch/HED agrees.

Body Weight

AC 84,777 had no effect on mean body weight gain of the surviving rabbits. Comparing mean body weights on study days 0 and 21, males and females in the controls and treated groups gained 0.2-0.3 kg. However, the moribund rabbit in Group III lost 1.06 lb by study day 14, when he was sacrificed.

Food Consumption

The control and treated male and female rabbits consumed similar amounts of food throughout the study. However, the food consumption of the moribund rabbit in Group III was decreased from 167 g at study initiation to 49 g during test week 1 and 22 g during test week 2, when he was sacrificed.

Skin Irritation

Skin reactions are summarized in the table below.

Group and /AC 84,777 (mg/kg)	Numbers of Animals Affected and, in Parentheses, Scoring Intervals When Skin Reactions Were Observed*		
MALES	Erythema	Edema	Eschar
I/0	0	0	0
II/250	0	0	0
III/500	0	0	1(15)
IV/1000	0	5(15)	7(10-15 11-15 12-15)
FEMALES	Erythema	Edema	Eschar
I/0	2(9-10 8, 9, 13)	0	0
II/250	0	0	0
III/500	1(5-11)	0	2(8-14 12-15)
IV/1000	0	0	4(8-10 8-15 14-15)

*This table is based on Discussion section, pages 14-15 and on Table 7(a-d), pages 39-42, of the submission.

Very slight erythema (score 1 on the Draize scale of 1-4) was observed in two female rabbits from the control group, in one prior to applications 9 and 10, and in another prior to applications 8, 9, and 13 (scoring was done before each application). A small area of well defined erythema on the center of the back (not a generalized irritation) was observed in one mid-dose female prior to applications 5, 6 and 7. This focal, well defined erythema became very slight erythema prior to applications 8-11.

Very slight edema (score 1) was observed only in five high-dose males prior to the last (15th) application.

Eschar was observed in eight males (one mid-dose and seven high-dose) and six females (two mid-dose and four high-dose). The scabs were located on the sides of animals or along the spine near taped areas and were attributed by the testing laboratory to mechanical damage "from shaving and dosing, exacerbated by contact with the test article." No generalized irritation was noted in either sex at the treatment site, at any time during the study.

Hematology

Relative to the control values, AC 84,777 had no effect on all of the parameters examined.

Clinical Chemistry

Relative to the control values, AC 84,777 had no effect on all of the parameters examined in the females and in the low-dose and mid-dose males. In the high-dose males, the mean blood glucose value was 14.2% lower than that of the controls at the termination of the study. This difference was statistically significant ($p \le 0.05$). However, blood glucose in one control male rabbit was much higher than in the remaining control male rabbits, resulting in a mean glucose value for this group being much higher than the historical control values, which were submitted. Also, one low blood glucose value in the high-dose male group lowered considerably the mean glucose value for that group. If these single outlier values are eliminated from the high-dose and control groups, the statistical difference between these groups is also eliminated. The lower blood glucose value in the high-dose males appears, therefore, to be treatment-unrelated.

Organ Weights and Organ/Body Weight Ratios

No statistically significant differences in mean absolute organ weights, in either sex, were noted between the control and AC 84,777-treated groups. With two exceptions, the same applied to organ/body weight ratios. A statistically significant difference ($p \le 0.05$) was noted in males for right adrenals/body weight ratio between Group I (ratio: 0.003) and Group II (ratio: 0.004) and IV (ratio: 0.005). Considering the difficulties encountered in obtaining accurate weights of very small organs and the lack of a dose-relationship, the difference noted above appears to be incidental rather than treatment-related.

Necropsy

Nothing remarkable was observed in most animals on the study, both treated and untreated. White/yellow spots and/or streaks were seen on the livers of 4 males and 2 females from the control group, and in 1-3 animals/group from the treated groups. Eschar/scabs on the treated skin was observed in 4 males and 1 female from the high-dose group. The control male which was found dead on study day 0 (1st sham treatment) had damaged GI tract and cloudy fluid in the peritoneal cavity and GI tract. The mid-dose male which was sacrificed moribund on study day 14 had partly atelectatic lungs and with wrinkled surface, very small spleen and testes/epididymides, liver covered with white spots, pale kidneys, gall bladder distended with bile, and mucoid fluid in GI tract.

Histopathology

The following effects, observed at the application sites, appeared to be treatment-related: 1) Epithelial hyperplasia in 5 high-dose males, 2 high-dose females and 1 mid-dose female; and 2) Necrosis and scab formation in 4 high-dose males. Treatment-unrelated effects, observed in the livers of most control males and females, included vacuolation of hepatocytes, cell infiltration and biliary hyperplasia and fibrosis.

COMMENTS

In general, this study is well reported and meets most of the December 24, 1989 EPA acceptance criteria (Attachment II). Statements of Good Laboratory Practices (GLP) and Quality Assurance were included in the submission. The study was inspected twelve times, first time on 9/12/89 and last time on 11/22/89.

The testing laboratory reported that possible secondary focal damage to the skin was observed in the high-dose animals and minimal focal epithelial hyperplasia was noted in one mid-dose female, but concluded that a dermal NOEL was 1000 mg/kg (HDT). The following explanation was given in support of that conclusion:

> "The focal changes observed both grossly and microscopically in the skin appear to be localized effects, probably the result of secondary infiltration of the test article into the skin mechanically damaged by shaving and dosing. The tissue adjacent to the focal changes was otherwise normal and no diffuse changes, or generalized damage, indicative of widespread or primary irritation were noted, which supports the conclusion that the test article does not appear to be a skin irritant to intact skin."

Toxicology Branch/HED disagrees with the above conclusion. Eschar was observed in 1 male and 2 females from the mid-dose group, and in 7 males and 4 females from the high-dose group. As long as skin reactions, macroscopic and/or microscopic, were observed at a dose level, that level cannot be regarded as a dermal NOEL. A dermal NOEL in this study is 250 mg/kg.

The following ambiguities should be noted:

1. It was stated in the experimental procedure section that the application sites were clipped. However, references were always made to shaved skin everywhere else in the report. Were the application sites then clipped or shaved?

2. Appendix A, page 154 of the submission, is a Draize Scale for Scoring Skin Reactions. Other entries on this page include: 1) Project Number: 89-6762A, which is this (21-Day Dermal) study and 2) Study Type: Acute Dermal Toxicity, Single Level - Rabbits, which is wrong. In fact, a Draize Scale is not even generally used in an acute dermal toxicity study (81-2).

Attachment I

Page 37 is not included in this copy.

Pages ____ through ____ are not included.

The material not included contains the following type of information:

____ Identity of product inert ingredients.

____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

____ Identity of the source of product ingredients.

____ Sales or other commercial/financial information.

____ A draft product label.

____ The product confidential statement of formula.

____ Information about a pending registration action.

X FIFRA registration data.

____ The document is a duplicate of page(s) ____.

____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

Attachment II

MRID No.: [illegible]
Study No.: 89-6762A
Study Date: 12/8/89

009451

Subdivision F
Guideline Ref. No. 82-2
December 24, 1989

82-2 Repeated Dose Dermal Toxicity (21-day) in the Rat, Rabbit or Guinea Pig

ACCEPTANCE CRITERIA

Does your study meet the following acceptance criteria?

1. ✓ Technical form of the active ingredient tested.
2. ✓ At least 5 animals/sex/group (3 test groups and control group).
3. ✓ Dosing duration at least 6 hour/day for 21 days or 5 days/week for 3 weeks.
4. ✓ Application site at least 10% of body surface area.
5. ✓ Doses tested include signs of toxicity at high dose, no or minimal dermal irritation, minimal lethality or a limit dose (1000mg/kg) if nontoxic.
6.* ✓ Doses tested include a NOEL.
7. ✓ Individual daily observations.
8. ✓ Individual body weights.
9. ✓ Individual or cage food consumption.
10. ✓ Clinical pathology data of 11 & 12 at termination.
11. ✓ Hematology.
    - ✓ Erythrocyte count
    - ✓ Hemoglobin
    - ✓ Hematocrit
    - ✓ Leucocyte count
    - *✓ Differential count
    - ✓ Platelet count (or clotting measure)
12. ✓ Clinical chemistry.
    - *___ Alkaline phosphatase
    - ✓ Aspartate aminotransferase
    - ✓ Alanine aminotransferase
    - *___ Creatinine kinase
    - *___ Lactic dehydrogenase
    - ✓ Glucose
    - ✓ Bilirubin
    - *___ Cholesterol
    - *✓ Creatinine
    - ✓ Total Protein
    - ✓ Albumin
    - ✓ Urea nitrogen
    - ✓ Inorganic phosphate
    - ✓ Calcium
    - *✓ Potassium
    - ✓ Sodium
    - *✓ Chloride
    - ✓ Gamma glutamyl transpeptidase
13.* No Urinalysis, only when indicated by expected or observed activity. As scheduled in 10.
    - ___ Blood
    - ___ Protein
    - ___ Ketone bodies
    - ___ Appearance
    - ___ Glucose
    - ___ Total bilirubin
    - *___ Urobilirubin
    - ___ Sediment
    - ___ Specific gravity (osmolality)
    - *___ Volume
14. ✓ Individual necropsy of all animals.
15. ✓ Histopathology performed on all control and high dose animals, all animals that died or were killed on study consisting of all gross lesions on all animals, target organs on all animals (to determine a NOEL), and skin (normal and treated) lungs, liver and kidneys.

Criteria marked with a * are supplemental and may not be required for every study.

CASWELL FILE

DOC920140
FINAL

003451

DATA EVALUATION REPORT

DIFENZOQUAT

Study Type: Mutagenicity: Gene Mutation in Cultured Chinese Hamster Ovary Cells (CHO/HGPRT)

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 Lynne T. Haber, Ph.D. Date 2/6/92

Independent Reviewer Nancy E. McCarroll, B.S. Date 2/6/92

QA/QC Manager Sharon Segal, Ph.D Date 2/6/92

Contract Number: 68D10075
Work Assignment Number: 1-39
Clement Number: 91-136
Project Officer: James Scott

GUIDELINE SERIES 84: MUTAGENICITY
MAMMALIAN CELLS IN CULTURE GENE MUTATION

MUTAGENICITY STUDIES

Reviewed by: Krystyna Locke, Toxicologist	Signature: Krystyna K. Locke
Section I, Tox. Branch I/HED	Date: 2/19/92
Secondary Reviewer:	
Roger Gardner, Section Head	Signature: Roger Gardner
Section I, Tox. Branch I/HED	Date: 4-16-92
Secondary Reviewer:	
Irving Mauer, Geneticist	Signature: [illegible]
Tox. Branch I/HED	Date: 04/15/92

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: Gene mutation in cultured Chinese hamster ovary cells (CHO/HGPRT)

EPA IDENTIFICATION Numbers:

Tox Chem. Number: 363A

MRID Number: 413254-11

TEST MATERIAL: Difenzoquat

SYNONYMS: AC 84,777; 1,2-dimethyl-3,5-diphenyl-1H-pyrazolium methyl sulfate

SPONSOR: American Cyanamid Company, Princeton, NJ

STUDY NUMBER: 0488

TESTING FACILITY: American Cyanamid Company, Princeton, NJ

TITLE OF REPORT: Mutagenicity Testing of AC 84,777 in the In Vitro CHO/HGPRT Mutation Assay

AUTHOR: Johnson, E.

REPORT ISSUED: December 17, 1984

CONCLUSIONS-EXECUTIVE SUMMARY: Under the conditions of the Chinese hamster ovary (CHO) cell HGPRT forward gene mutation assay, doses of nonactivated difenzoquat (500 to 1600 µg/mL), and doses of S9-activated difenzoquat (500 to 1250 µg/mL) did not induce a mutagenic response. Higher levels (2000 µg/mL -S9 and 1600-2000 µg/mL +S9) were severely cytotoxic. Based on these findings, it was concluded that difenzoquat was tested over an appropriate range of concentrations with no evidence of a mutagenic effect. The study, therefore, satisfies Guideline requirements for genetic effects Category I, Gene Mutations.

STUDY CLASSIFICATION: The study is acceptable.

A. MATERIALS:

1. Test Material: Difenzoquat

   Description: Not reported
   Identification No.: AC 84,777; Batch no. 233HA236
   Purity: 91%
   Receipt date: Not reported
   Stability: Not reported
   Contaminants: None listed
   Solvent used: Deionized sterile water (H_2O)
   Other provided information: Neither storage conditions nor frequency of dosing solution preparation were reported. However, based on data from the analytical determinations, it appears that dosing solutions were prepared the day they were used.

2. Control Materials:

   Negative: None

   Solvent/volume: H_2O/0.1 mL

   Positive: Nonactivation (concentrations, solvent): Ethyl methanesulfonate (EMS) was prepared in an unspecified solvent to yield a final concentration of 200 µg/mL.

   Activation (concentrations, solvent): 7,12-dimethylbenz(a)anthracene (DMBA) was prepared in an unspecified solvent and used at 7 µg/mL +/- S9 in the initial assay. In the confirmatory assay, DMBA was used at 3, 5, and 7 µg/mL +S9 and at 7 µg/mL -S9.

3. Activation: S9 derived from adult male Sprague-Dawley

x	Aroclor 1254	x	induced	x	rat	x	liver
	phenobarbital		noninduced		mouse		lung
	none				hamster		other
	other				other		

   The S9 was prepared by the testing laboratory.

   S9 mix composition: Not reported

4. Test Cells: Mammalian cells in culture

   _____ mouse lymphoma L5178Y cells
   __x__ Chinese hamster ovary (CHO) cells
   _____ V79 cells (Chinese hamster lung fibroblasts)
   _____ other (list):

   Properly maintained? Yes.

Properly maintained? Yes.
Periodically checked for mycoplasma contamination? Not reported.
Periodically checked for karyotype stability? Not reported.
Periodically "cleansed" against high spontaneous background? Yes.

5. Locus Examined:

_____ thymidine kinase (TK)
Selection agent: _____ bromodeoxyuridine (BrdU)
(give concentration) _____ fluorodeoxyuridine (FdU)

_x_ hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)
Selection agent: _____ 8-azaguanine (8-AG)
(give concentration) _≈10 µM_ 6-thioguanine (6-TG)

_____ Na^+/K^+ATPase
Selection agent: _____ ouabain
(give concentration)

_____ other (locus and/or selection agent; give details):

6. Test Compound Concentrations Used:

(a) Preliminary cytotoxicity assay: Six doses (10, 100, 500, 1000, 2500, and 5000 µg/mL) were evaluated with and without S9 activation. The nonactivated assay was repeated an additional two times, and the S9-activated assay was repeated once.

(b) Mutation assay:

Initial test: Four doses (500, 1250, 1600, and 2000 µg/mL) were evaluated in the presence and absence of S9 activation.

Confirmatory test: Five doses were evaluated in the presence and absence of S9 activation. The nonactivated doses were 400, 800, 1200, 1600, and 1800 µg/mL; the S9-activated doses were 400, 800, 1000, 1200, and 1400 µg/mL.

B. TEST PERFORMANCE:

1. Cell Treatments:

(a) Cells exposed to test compound for:
_5_ hours (nonactivated) _5_ hours (activated)

(b) Cells exposed to positive controls for:
_5_ hours (nonactivated) _5_ hours (activated)

(c) Cells exposed to negative and/or solvent controls for:
_5_ hours (nonactivated) _5_ hours (activated)

(d) After washing, cells cultured for _9_ days (expression period) before cell selection.

an unspecified number of days without selection medium to determine cloning efficiency.

2. Statistical Analysis: The mutation frequencies were transformed using a power transformation, and analyzed using the Duncan procedure at p = 0.05.

3. Protocol: Not provided.

C. REPORTED RESULTS:

1. Analytical Determination: Analytical determinations were performed on dosing solutions used in both the preliminary cytotoxicity and mutation assays; a total of 36 test samples were analyzed. Results indicated that all solutions contained ±12% of the target concentration.

2. Preliminary Cytotoxicity Assay: Three nonactivated preliminary cytotoxicity assays were conducted, all with the same range of difenzoquat doses (10 to 5000 µg/mL). Presumably, the assays were repeated because of the poor absolute survival (<50%) of the solvent control in the first two experiments. As shown in Table 1, doses ≥2500 µg/mL were too cytotoxic to be cloned. Results from the three trials at the 1000 µg/mL level were not in agreement; 94.1% relative survival (RS) was calculated for the third experiment, while 53.3 and 42.3% RS was calculated at this level for the first and second trials, respectively. Below 1000 µg/mL, RS was ≥87.1 for all trials.

   In the presence of S9 activation, severe cytotoxicity was seen at the two highest doses (2500 and 5000 µg/mL). RS for lower concentrations (10 to 1000 µg/mL) was ≥81.5% in the first assay and ≥79.4% in the repeat cytotoxicity test. We assume, as previously stated, that poor absolute survival of the solvent control prompted the performance of a second cytotoxicity test.

3. Mutation Assay: Doses for the mutation assays were chosen to give an estimated survival of 10%, 50%, and 100%; all levels were assayed in triplicate. In the first trial, difenzoquat was evaluated at four nonactivated and four S9-activated concentrations ranging from 500 to 2000 µg/mL. The 2000-µg/mL dose +/- S9 was severely cytotoxic. The average RS of cells exposed to 1600 µg/mL -S9 was 52.1% (Table 2); at the same level with S9 activation, difenzoquat was severely cytotoxic (RS <2%), and cultures were not cloned. Lower doses with and without S9 activation were not cytotoxic and the overall data did not suggest a mutagenic response.

   In the confirmatory assay, 400 to 1800 µg/mL -S9 and 400 to 1400 µg/mL +S9 were tested. RS at 1600 µg/mL -S9 was 0.7%; for lower nonactivated doses RS was ≥62.3%. In the presence of S9 activation, compound cytotoxicity was dose related; RS ranged from 0.5% at 1400 µg/mL to

TABLE 1. Representative Results of Preliminary Cytotoxicity Assays With Difenzoquat

Substance	Dose/mL	S9 Activation	Mean Relative Survival[a] Trial 1	Trial 2	Trial 3
Solvent Control					
Sterile Water	0.1 mL	-	100 (49.6%)	100 (49.6%)	100 (64.2%)
	0.1 mL	+	100 (44.4%)	100 (83.6%)	--
Test Material					
Difenzoquat	500 µg[b]	-	87.1	96.4	120.2
	1000 µg[c]	-	53.3	42.3	94.1
	500 µg[b]	+	108.1	93.8	--
	1000 µg[c]	+	81.5	79.4	--

[a]Values in parentheses represent the absolute survival of the solvent controls.

[b]Lower doses (10 and 100 µg/mL) showed no evidence of cytotoxicity.

[c]Higher doses (2500 and 5000 µg/mL) were too cytotoxic to clone.

>100% at 400 µg/mL. The results suggesting that the test material was more cytotoxic in the confirmatory trial may be related to the unacceptably low absolute survival of S9-activated solvent control cultures (40.4%) and the borderline acceptable nonactivated solvent control cultures (54%). Nevertheless, the data indicated, in agreement with earlier results, that difenzoquat was not mutagenic. Representative findings from the confirmatory trial are shown in Table 3.

From the overall results, the study author concluded that difenzoquat was not mutagenic in this test system.

D. REVIEWERS' DISCUSSION AND INTERPRETATION OF RESULTS: We assess that the study author's interpretation of the data was correct. Although the confirmatory assay was compromised because of low survival in the solvent control cultures, there was sufficient evidence from the initial assay to conclude that difenzoquat was adequately tested and was not mutagenic in this test system. The response of the test system to the positive controls (EMS at 200 µg/mL -S9 and DMBA at 7 µg/mL +S9) indicated that the assay was sufficiently sensitive to detect a mutagenic response. We, therefore, conclude that difenzoquat was negative in this test system.

E. QUALITY ASSURANCE MEASURES: Was the test performed under GLP? Yes. (A quality assurance statement was signed and dated December 7, 1984.)

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 9-14.

TABLE 2. Representative Results of the Initial Chinese Hamster Ovary (CHO) Cell Forward Gene Mutation Assay with Difenzoquat

Substance	Dose/mL	S9 Acti-vation	Relative % Survival (after treatment)[a]	Total Mutant Colonies/ 15 Dishes[b]	Average Number of Surviving Cells (at selection) $\times 10^5$ [a,c]	Average Mutation Frequency/ 10^6 cells[c,d]
Solvent Control						
Sterile water	0.1 mL	-	100 (84%)	1 (0.3)	7.09	0.47
	0.1 mL[e]	+	100 (85%)	1 (0.3)	5.49	0.61
Positive Controls						
Ethylmethane sulfonate	200 µg/mL[e]	-	62.6	258 (86.0)	6.54	131.50
7,12-Dimethylbenz-(a)anthracene	7 µg/mL	-	89.5	9 (3.0)	7.20	4.17
	7 µg/mL	+	2.6	212 (70.7)	2.79	253.41
Test Material						
Difenzoquat	1600 µg[f]	-	52.1	4 (1.3)	6.63	1.96
	1250 µg[e,g]	+	61.4	5 (1.7)	4.52	3.76

[a]Average of three cultures; values in parentheses represent the absolute survival of the solvent controls.
[b]Total of three cultures (five dishes per culture); calculated by our reviewers. Values in parentheses represent the average number of mutants per culture.

[c]Calculated by our reviewers.

[d]Average Mutation Frequency (MF) = $\dfrac{\text{Average Number of Mutant Colonies/Culture}}{\text{Average Number of Surviving Cells at Selection}} \times 10^6$.

[e]Includes at least one culture for which at least one selection plate was not counted due to contamination; the number of surviving cells was corrected accordingly.
[f]The highest assayed dose (2000 µg/mL) was severely cytotoxic; results for the lower doses (500 and 1250 µg/mL) did not suggest a mutagenic effect.
[g]Higher doses (1600 and 2000 µg/mL) were severely cytotoxic; results for the lowest assayed dose (500 µg/mL) did not suggest a mutagenic effect.

TABLE 3. Representative Results of the Confirmatory Chinese Hamster Ovary (CHO) Cell Forward Gene Mutation Assay with Difenzoquat

Substance	Dose/mL	S9 Activation	Relative % Survival (after treatment)[a]	Total Mutant Colonies/ 15 Dishes[b]	Average Number of Surviving Cells (at selection) x 10^5 [a,c]	Average Mutation Frequency/ 10^6 cells[c,d]
Solvent Control						
Sterile water	0.1 mL	-	100 (54.0%)	1 (0.3)	9.07	0.37
	0.1 mL	+	100 (40.4%)	4 (1.3)	6.10	2.19
Positive Controls						
Ethylmethane sulfonate	200 µg/mL[e]	-	33.0	238 (79.3)	6.60	120.15
7,12-Dimethylbenz-(a)anthracene	7 µg/mL	-	102	11 (3.7)	7.93	4.67
	3 µg/mL[f]	+	80.7	462 (154)	3.93	391.86
Test Material						
Difenzoquat	1200 µg[e,g]	-	62.3	0 (0.0)	4.80	<0.69
	1200 µg[h]	+	13.9	0 (0.0)	5.33	<0.63

[a]Average of three cultures; values in parentheses represent the absolute survival of the solvent controls.
[b]Total of three cultures (five dishes per culture); calculated by our reviewers. Values in parentheses represent the average number of mutants per culture.

[c]Calculated by our reviewers.

[d]Average Mutation Frequency (MF) = $\frac{\text{Average Number of Mutant Colonies/Culture}}{\text{Average Number of Surviving Cells at Selection}} \times 10^6$.

[e]Includes at least one culture for which at least one selection plate was not counted due to contamination; the number of surviving cells was corrected accordingly.
[f]DMBA was positive at 5 and 7 µg/mL.
[g]Higher doses (1600 and 1800 µg/mL) were severely cytotoxic; lower doses (400 and 800 µg/mL) did not suggest a mutagenic effect.
[h]The highest assayed dose (1400 µg/mL) was severely cytotoxic; lower doses (400, 800, and 1000 µg/mL) did not suggest a mutagenic effect.

APPENDIX A

MATERIALS AND METHODS
CBI pp. 9-14

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Pages 50 through 55 are not included.

The material not included contains the following type of information:

____ Identity of product inert ingredients.

____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

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____ Sales or other commercial/financial information.

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DATA EVALUATION REPORT

DIFENZOQUAT

Study Type: Mutagenicity: In Vitro Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells

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 ____________ Date 2-10-92
Nancy E. McCarroll, B.S.

Independent Reviewer ____________ Date 2/10/92
Lynne T. Haber, Ph.D.

QA/QC Manager ____________ Date 2/10/92
Sharon Segal, Ph.D.

Contract Number: 68D10075
Work Assignment Number: 1-39
Clement Number: 91-138
Project Officer: James Scott

I.

GUIDELINE SERIES 84: MUTAGENICITY
MAMMALIAN CELLS IN CULTURE CYTOGENETICS

EPA Reviewer: Krystyna Locke, Toxicologist — Signature: Krystyna K. Locke
Section I, Tox. Branch I/HED — Date: 4/14/92
Secondary reviewer: Roger Gardner, Section Head — Signature: [signature]
Section I, Tox. Branch I/HED — Date: 4-16-92
Secondary reviewer: Irving Mauer, Geneticist — Signature: [signature]
Tox. Branch I/HED — Date: [illegible]

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: In vitro chromosome aberration in Chinese hamster ovary (CHO) cells.

EPA IDENTIFICATION NUMBERS.:

Tox Chem. Number: 363A

MRID Number: 414153-03

TEST MATERIAL: Difenzoquat

SYNONYMS: AC 84,777; 1,2-dimethyl-3,5-diphenyl-1H-pyrazolium methyl sulfate

SPONSOR: American Cynamid Co., Princeton, NJ

STUDY NUMBER: HLA 362-172

TESTING FACILITY: Hazleton Biotechnologies Corp., Vienna, VA.

TITLE OF REPORT: In Vitro Chromosomal Aberrations in Chinese Hamster Ovary Cells with AC 84,777.

AUTHOR: T. Cortina.

REPORT ISSUED: January 30, 1985.

CONCLUSIONS-EXECUTIVE SUMMARY: AC 84,777 (difenzoquat) at five nonactivated doses of 100 to 10,000 µg/mL (actual concentrations based on analytical determinations indicate a range of ≥65 to ≥5700 µg/mL) and five S9-activated doses of 33 to 3330 µg/mL (actual concentrations: ≥24 to 1900 µg/mL) was assessed for the potential to induce structural chromosome aberrations in late, middle, and early stages of the Chinese hamster ovary (CHO) cell cycle. Nominal doses ≥3330 µg/mL +/-S9 (analytically, ≥1900 µg/mL) were cytotoxic. A significant increase in the percentage of cells with aberrations was seen at 3330 µl/mL-S9 (1900 µg/mL) 3 hours posttreatment. However, the significant effect was confined to one dose, was not reproduced in a repeat 3-hour treatment, and was not observed in cultures harvested 8 or 12 hours following treatment. There were no significantly increased aberration frequencies in cells recovered 3, 8, or 12 hours postexposure to the S9-activated test material. It was, there-

therefore, concluded that AC 84,877 was adequately tested and found to be nonclastogenic in this series of experiments. The study satisfies Guideline requirements for genetic effects Category II, Structural Aberrations.

STUDY CLASSIFICATION: The study is acceptable.

A. MATERIALS:

1. Test Material: AC 84,777

Description: White solid
Identification No.: Lot no. 233 HA 236
Purity: 99.1%; Dosing solutions were adjusted to 100% purity.
Receipt date: Not provided
Stability: Assumed to be stable under the conditions used.
Contaminants: None listed
Solvent used: Glass distilled, deionized water (DH_2O)
Other provide information: The test material was stored at room temperature. The frequency of dosing solution preparation was not reported. Solutions used in the 3-hour initial and repeat studies were analyzed for actual concentrations by the sponsor.

2. Control Materials:

Negative: Untreated cells grown in Ham's F-12 medium supplemented with 10% fetal calf serum (FCS).

Solvent/final concentration: DH_2O/0.05mL

Positive:
Nonactivation: (Concentrations, solvent): Mitomycin C (MMC) was prepared in culture medium at 80 µg/mL.

Activation: (Concentration, solvent): Cyclophosphamide (CP) was prepared in culture medium at 140 µg/mL.

3. Activation: S9 derived from male Sprague-Dawley

x Aroclor 1254	x induced	x rat	x liver
___ phenobarbital	___ noninduced	___ mouse	___ lung
___ none		___ hamster	___ other
___ other		___ other	

The S9 fraction was prepared by the performing laboratory and was standardized prior to use; the standardization methods were not reported.

The composition of the S9 mix per mL was as follows:

NADP	6.0 mg
Isocitric acid	11.25 mg
S9	0.25 mL
Fisher's medium	0.75 mL

4. Test Compound Concentration Used:

   (a) Preliminary cytotoxicity assay: Six doses (33, 100, 333, 1000, 3330, and 10,000 μg/mL) were evaluated with and without S9 activation.

   (b) Cytogenetic assay:

      (1) Nonactivated conditions: Five doses (100, 333, 1000, 3330, and 10,000 μg/mL) with a 3-, 8-, and 12-hour cell harvest. A repeat 3-hour cell harvest was conducted with a similar range of test material doses.

      (2) S9-Activated conditions: Five doses (33, 100, 333, 1000, and 3300 μg/mL) with a 3-, 8-, and 12-hour cell harvest. A repeat 3-hour cell harvest was conducted with a similar range of test material doses.

5. Test Cells: The Chinese hamster ovary cells (CHO/K-1) used in this assay were obtained from the American Type Culture Collection, Rockville, MD. CHO cells were initiated in Ham's F-12 medium supplemented with 10% FCS for 24 hours prior to use.

   Properly maintained? Yes.

   Cell line or strain periodically checked for mycoplasma contamination? Assumed to be mycoplasma-free.

   Cell line or strain periodically check for karyotype stability? Not reported.

B. TEST PERFORMANCE:

1. Cell Treatment:

   (a) Cells exposed to test compound for:
   3, 8, and 12 hours (nonactivated) 2 hours (activated)

   (b) Cells exposed to positive controls for:
   3, 8, and 12 hours (nonactivated) 2 hours (activated)

   (c) Cells exposed to negative and/or solvent controls for:
   3, 8, and 12 hours (nonactivated) 2 hours (activated)

2. Protocol:

   (a) Preliminary cytotoxicity assay: Cell cultures, seeded at $3x10^5$ cells/flask, were exposed to half-log dilutions of the test material ranging from 33 to 10,000 μg/mL, the negative control (culture medium) or the solvent (DH_2O) for 4 hours in both the presence and absence of S9 activation.

After exposure, cells were washed, refed with complete medium and reincubated for 24 hours. Colcemid (0.2 µg/mL) was added, and cultures were incubated for an additional 2 hours. Cultures were trypsinized and cells were stained; the mitotic index (MI) was determined from the count of 1000 cells per culture.

(b) Cytogenetic assay:

(1) Treatment: Three replicate cultures per treatment, per condition, per sampling time were seeded at $5x10^5$ cells and were exposed to the selected test material doses, the negative, solvent, or positive controls in both the presence and absence of S9 activation.

In the nonactivated system, cells were dosed for 3, 8, or 12 hours. Colcemid (0.2 µg/m/mL) was added 2 hours prior to each harvest time. Under S9-activated conditions, cells were exposed for 2 hours,washed and reincubated; colcemid was added 2 hours prior to cell harvest.

Metaphase cells were collected by mitotic shake-off, treated with a hypotonic solution of KCl (0.075 M), and fixed in methanol:glacial acetic acid (3:1). Slides were stained with 2% Giemsa and coded.

(2) Metaphase analysis: One hundred cells (50 cells/culture) from each dose level of the test material and the negative and solvent controls at the three harvest intervals were scored for chromosome aberrations. One hundred metaphases from the positive control groups (12-hour harvest only) were similarly scored. The percentage of growth and MIs were determined from the remaining culture.

(3) Statistical methods: The data were evaluated for statistical significance at $p<0.05$ by Chi-square, analysis of variance, and Dunnett's t-test.

3. Evaluation Criteria: No criteria were provided to establish assay validity or the biological significance of the results.

C. REPORTED RESULTS:

1. Cytotoxicity Assay: The study author indicated that the test material was soluble in DH_2O at 1000 mg/mL. Accordingly, the cytotoxicity assay was conducted with a concentration range of 33 to 10,000 µg/mL. No cells survived exposure to 10,000 µg/mL +/-S9 or 3330 µg/mL +S9. Percent growth for the remaining nonactivated concentrations (33, 100, 333, 1000 and 3330 µg/mL) was ≥66%. S9-activated doses ranging from 33 to 1000 µg/mL were not cytotoxic. Based on these findings, the cytogenetic assay was conducted with five nonactivated doses (100 to 10,000 µg/mL) and five S9-activated levels (33 to 3330 µg/mL).

2. Cytogenetic Assay: Under nonactivated conditions, no cells survived exposure to 10,000 µg/mL (actual concentration = 6100 µg/mL, see Section C.3) of the test material. Cytotoxicity, as indicated by reduced MIs, was also apparent at 3330 µg/mL (1900 µg/mL) following the 3- and 8-hour treatment; no cells were recovered at 12 hours. Although a significant ($p<0.05$) increase in the percentage of cells with aberrations but not the number of aberrations per cell was seen at 3330 µg/mL (1900 µg/mL) following the 3-hour treatment, the response was limited to this dose (Table 1). No significant effects were noted in cells harvested 8 and 12 hours posttreatment or in the 3-hour repeat study that was conducted with a comparable range of test material concentrations. The slight elevations in aberrations per cell, which occurred at several doses from each treatment period, were partially caused by scoring each exchange figure as two aberrations.

   In the presence of S9 activation, the selected doses of the test material did not induce a significant increase in the percentage of cells with aberrations or the number of cells with aberrations in cultures harvested 3, 8, or 12 hours posttreatment (Table 2). Similarly, the repeat 3-hour study showed no significant effects. With the exception of the 12-hour cell harvest, the high dose (3330 µg/mL; actual concentration = ≥1900 µg/mL) was cytotoxic. The "marked" increase in the aberration frequency at 3330 µg/mL (initial 3-hour harvest) resulted from scoring six cells at this level. As previously noted for the nonactivated experiments, counting exchanges as two aberrations and severely damaged cells (i.e., cells with ≥10 aberrations) as 10 aberrations was partially responsible for the elevated frequencies of aberrations per cell. Similarly, the occurrence of exchange figures in the negative and solvent control cultures with and without S9 activation diminishes the biological significance of this class of aberrations in the treatment groups. Significant clastogenic effects were, however, observed in cells exposed to the nonactivated (80 µg/mL MMC) and the S9-activated (140 µg/mL CP) positive controls.

3. Analytical Determination: Analytical determinations were performed on all dosing solutions used in the initial and repeated 3-hour harvest phase of testing. Results indicated that with the exception of the lowest concentration used in the initial study (3.3 mg/mL; 91.8% of target does), there were marked differences between actual and target levels. Percent of target was between 55.0 and 71.5% for the initial assay solutions and between 57.0 and 72.7% for the repeat assay solutions. The study author stated that the "large discrepancy" between stated and actual concentrations may have resulted from adding an equal number of grams to milliliters and making no adjustments for volume displacement. However, the example given (1.4 g dissolved in 1.4 mL to achieve 1000 mg/mL) conflicts with earlier information stating that the test material (99.1%) was adjusted to 100% purity for dosing purposes. We assess that even if the adjustment was made, it would not substantially alter the reported results. The study author also stated that "since the doses were tested to the limit of toxicity, the above discrepancy is not expected to affect the overall test results and conclusions."

TABLE 1. Representative Results of the Nonactivated *In Vitro* Chinese Hamster Ovary (CHO) Cell Cytogenetic Assays with AC 84,777

Substance	Dose	Harvest Time (Hours)	Mitotic Index (%)	Percent Growth[a]	No. of Cells Scored	No. of Aberrations per Cell[b]	% Cells with Aberrations[b]	Biologically Significant Aberrations No./Type[c]
Negative Control								
Culture medium	--	3[d]	1.8	--	100	0.04	2.0	2TB; 1E
	--	3[e]	3.8	--	100	0.00	0.0	--
	--	8	1.0	--	100	0.03	2.0	1TB; 1E
	--	12	2.8	--	100	0.04	2.0	2E
Solvent Control								
Glass distilled, deionized water	50 µL	3[d]	1.0	--	100	0.01	1.0	1SB
	50 µL	3[e]	1.8	--	100	0.00	0.0	--
	50 µL	8	1.2	--	100	0.00	0.0	--
	50 µL	12	3.8	--	100	0.04	1.0	2E
Positive Control								
Mitomycin C	80 µg/mL	12	0.2	7	100	1.99*	43.0*	37TB; 6SB; 18E; 12GT
Test Material								
AC 84,777	1000 (550)[f,g]	3[d]	1.8	180	100	0.15	5.0	1TB; 1SB; 7E
	3330 (1900)[h]	3	0.2	20	100	0.20	10.0*	6TB; 7E
	1000 (600)[g]	3[e]	1.2	67	100	0.06	3.0	3E
	3330 (2100)[h]	3	0.2	11	100	0.00	0.0	--
	1000 (550)[g]	8	0.6	50	100	0.02	1.0	1E
	3330 (1900)[h]	8	0.2	17	44	0.27	4.5	2TB; 1GT
	1000 (550)[h]	12	1.4	37	100	0.10	6.0	2TB; 4E

[a]Percent Growth = $\frac{\text{Mitotic Index of Test Group}}{\text{Mitotic Index of Solvent Group}} \times 100$.

[b]Gaps excluded.

[c]Abbreviations used:
TB = Chromatid Break
E = Exchange (counted as 2 aberrations)
SB = Chromosome break
GT = >10 aberrations/cell (counted as 10 aberrations)

[d]Results from the first 3-hour harvest.
[e]Results from the repeat 3-hour harvest.
[f]Values in () reflect actual concentrations used in the study based on analytical determinations.
[g]Findings for lower doses (333 and 100 µg/mL at all sampling times; actual concentrations ranged from 236 to 67 µg/mL) did not suggest a clastogenic response.
[h]Higher levels (10,000 µg/mL (≥5700 µg/mL) at all harvest times and 3330 µg/mL (1900 µg/mL)) at the 12-hour harvest] were severely cytotoxic.

*Significantly higher ($p<0.05$) than the solvent control value.

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TABLE 2. Representative Results of the S9-Activated In Vitro Chinese Hamster Ovary (CHO) Cell Cytogenetic Assays with AC 84,777

Substance	Dose	Harvest Time (Hours)	Mitotic Index (%)	Percent Growth[a]	No. of Cells Scored	No. of Aberrations per Cell[b]	% Cells with Aberrations[b]	Biologically Significant Aberrations No./Type[c]
Negative Control								
Culture medium	--	3[d]	0.2	--	100	0.10	4.0	1TB; 1SB; 4E
	--	3[e]	0.2	--	100	0.00	0.0	--
	--	8	1.4	--	10C	0.15	5.0	3TB; 6E
	--	12	5.2	--	100	0.06	5.0	3TB; 1SB; 1E
Solvent Control								
Glass distilled, deionized water	50 µL	3[d]	0.8	--	100	0.20	6.0	5E; 1GT
	50 µL	3[e]	0.2	--	100	0.03	2.0	1SB; 1E
	50 µL	8	2.4	--	100	0.02	1.0	1E
	50 µL	12	6.6	--	100	0.12	4.0	1TB; 3SB; 4E
Positive Control								
Cyclophosphamide	140 µg/mL	12	0.2	4	100	0.94*	35.0*	31TB; 1SB; 21E; 2GT
Test Material								
AC 84,777	1000 (550)[f,g]	3[d]	0.2	25	77	0.00	0.0	--
	3330 (1900)	3	0.2	25	6	0.33	17.0	1E
	1000 (600)[g]	3[e]	0.4	200	103	0.03	2.0	1SB; 1E
	3330 (2100)	3	0.2	100	90	0.18	5.6	2TB; 2E; 1GT
	3330 (1900)[g]	8	0.4	17	100	0.10	1.0	1GT
	3330 (1900)[f]	12	7.0	106	100	0.04	2.0	2E

[a]Percent Growth = $\frac{\text{Mitotic Index of Test Group}}{\text{Mitotic Index of Solvent Group}} \times 100.$

[b]Gaps excluded.
[c]Abbreviations used:
TB = Chromatid Break; SB = Chromosome break
E = Exchange (counted as 2 aberrations); GT = >10 aberrations/cell (counted as 10 aberrations)

[d]Results from the first 3-hour harvest.
[e]Results from the repeat 3-hour harvest.
[f]Results in () refelct concentrations used in the study based on analytical determinations.
[g]Findings for lower doses (333, 100, and 33 µg/mL at 3 hours posttreatment; actual concentrations ranged from 24 to 253 µg/mL) and 1000, 333, 100 and 33 µg/mL at 8 and 12 hours posttreatment; actual concentrations ranged from 30.3 to 550 µg/mL) did not suggest a clastogenic response.

*Significantly higher ($p<0.05$) than the solvent control value.

Our reviewers have adjusted the data to reflect actual concentrations used in the study. Therefore, in the initial assay, the evaluated concentrations of difenzoquat were 67, 236, 550, 1900, and 6100 µg/mL -S9 and 30.0, 67, 236, 550, and 1900 µg/mL +S9. For the repeat 3-hour treatment, actual concentrations were 65, 253, 600, 2100, and 5700 µg/mL -S9 and 24, 65, 253, 600, and 2100 µg/mL +S9.

Based on the overall results, the study author concluded that AC 84,777 was not clastogenic in this test system.

D. REVIEWERS' DISCUSSION/CONCLUSIONS: We assess that AC 84,777 was comprehensively tested in a series of experiments that evaluated potential adverse effects on chromosome morphology at sensitive stages in the cell cycle. The results show that the test material was assayed to cytotoxic levels in both the presence and absence of S9 activation but failed to induce a clastogenic response. We further assess, that the lack of agreement between actual and target concentrations did not adversely affect the outcome of the study. We have, however, adjusted the data to reflect actual concentrations used in the study. In addition, the sensitivity of the test system to detect a clastogenic response was adequately demonstrated by the significant results achieved with the nonactivated and S9-activated clastogens used in the assay. We conclude, therefore, that AC 84,777 was not clastogenic in a well-conducted study.

E. QUALITY ASSURANCE MEASURES: Was the test performed under GLPs? Yes. (A quality assurance statement was signed and dated December 11, 1984).

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 11-19.

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APPENDIX A

MATERIALS AND METHODS
CBI pp. 11-19

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Pages 66 through 74 are not included.

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FINAL

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DATA EVALUATION REPORT

DIFENZOQUAT

Study Type: Mutagenicity: Unscheduled DNA Synthesis Assay in Primary 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] Lynne T. Haber Ph.D. Date 2/6/92

Independent Reviewer [signature] Nancy E. McCarroll, B.S. Date 2/6/92

QA/QC Manager [signature] Sharon Segal Ph.D. Date 2/6/92

Contract Number: 68D10075
Work Assignment Number: 1-39
Clement Number: 91-137
Project Officer: James Scott

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GUIDELINE SERIES 84: MUTAGENICITY
UDS

MUTAGENICITY STUDIES

Reviewed by: Krystyna Locke, Toxicologist — Signature: Krystyna K. Locke
Section I, Tox. Branch I/HED — Date: 2/19/92
Secondary Reviewer:
Roger Gardner, Section Head — Signature: Roger Gardner
Section I, Tox. Branch I/HED — Date: 4-15-92
Secondary Reviewer:
Irving Mauer, Geneticist — Signature: I. Mauer
Tox. Branch I/HED — Date: [illegible]

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: *In vitro* unscheduled DNA synthesis assay in primary rat hepatocytes.

EPA IDENTIFICATION Numbers:

Tox Chem. Number: 363A

MRID Number: 414153-04

TEST MATERIAL: Difenzoquat

SYNONYMS: AC 84,777; 1,2-dimethyl-3,5-diphenyl-1H-pyrazolium methyl sulfate

SPONSOR: American Cyanamid Company, Princeton, NJ

STUDY NUMBER: PH 311-AC-003-84

TESTING FACILITY: Pharmakon Research International, Inc., Waverly, PA

TITLE OF REPORT: Rat Hepatocyte Primary Culture/DNA Repair Test: AC 84,777.

AUTHOR: Barfknecht, T.

REPORT ISSUED: November 1, 1984

CONCLUSIONS-EXECUTIVE SUMMARY: At concentrations ranging from 0.8 to 80 µg/well, difenzoquat did not induce unscheduled DNA synthesis (UDS) in primary rat hepatocytes. Higher levels (≥266 µg/mL) were cytotoxic. Based on these findings, it was concluded that difenzoquat was tested over an appropriate range of concentrations with appropriate controls and showed no evidence of UDS. Thus the study satisfies Guideline requirements for genetic effects Category III, Other Mutagenic Mechanisms.

STUDY CLASSIFICATION: The study is acceptable.

A. MATERIALS:

1. Test Material: Difenzoquat

Description: White powdery solid
Identification No: AC 84,777; Lot no. 233HA 236
Purity: Technical grade
Receipt date: July 7, 1984
Stability: Not reported
Contaminants: None listed
Solvent used: Dimethyl sulfoxide (DMSO)
Other provided information: The material was stored in a glass container; the temperature was not reported, but was probably room temperature. Test material solutions were used within four hours of preparation. Analytical determinations were performed on all dosing solutions.

2. Indicator Cells: Primary rat hepatocytes were obtained by the in situ perfusion of the liver of a 170 g, 8 to 10-week-old male Fischer 344 rat, purchased from Charles River Breeding Laboratories.

3. Control Substances: DMSO was used as the solvent control, untreated cells were the negative control, and 2-acetamidofluorene (2-AAF) at 0.1 µM was used as the positive control.

4. Medium: WME: Williams' Medium E; WME+: Williams' Medium E with 10% calf serum.

5. Test Compound Concentrations Used:

   (a) Concentrations assayed: 0.26, 0.8, 2.6, 8, 26, 80, 266, 800, 2666, and 8000 µg/well.

   (b) Concentrations scored: 0.8, 2.6, 8, 26, and 80 µg/well.

B. STUDY DESIGN:

1. Cell Preparation:

   (a) Perfusion technique: The animal was anesthetized with sodium pentobarbital and the liver was perfused with Hank's balanced salt solution containing 0.5 mM EGTA, Hepes buffer, pH 7.35, and gentamicin, for 4 minutes, and with WME containing 100 units/ml collagenase, Hepes buffer pH 7.35, and gentamicin, for 10 minutes. The liver was excised and placed in WME-collagenase medium; cells were detached by combing.

   (b) Hepatocyte harvest/culture preparation: Recovered cells were resuspended in WME+ and counted. Cell viability was measured by trypan blue exclusion and was found to be 93%.

2. UDS Assay:

   (a) Treatment: Cells were plated into multiwell culture dishes containing coverslips at a concentration of 1 x 10^5 viable cells/well and fed WME+. Hepatocytes were allowed to attach for ≈2 hours in a 37°C, CO_2 incubator. Unattached cells were removed; viable cells were fed WME containing 10 µCi/mL [^{3}H] thymidine.

   Triplicate cultures were exposed to each of the selected test material doses, the positive control (2-AAF), the solvent control (DMSO), or the negative control (medium only) for 18-20 hours. Treated hepatocytes, attached to coverslips, were washed, exposed to 1% sodium citrate for 10-15 minutes, fixed in acetic acid:ethanol (1:3), dried, and mounted.

   (b) Preparation of autoradiographs/grain development: Slides were coated with Kodak NTB-2 emulsion, dried overnight, stored for 7 days at 4°C in light-tight desiccated boxes, developed in Kodak D-19 developer (Eastman Kodak), fixed, stained with Harris Alum hematoxylin and eosin, coded, and counted.

   (c) Grain counting: Twenty cells on each coverslip were counted (60 cells/dose) for each test dose, as well as the solvent, untreated, and positive controls. Cytoplasmic background counts were determined by counting three nuclear-sized areas adjacent to the nucleus; the highest count was chosen as the cytoplasmic back-ground. Net nuclear grain counts were determined by subtracting the cytoplasmic background count from the nuclear grain count.

4. Evaluation Criteria:

   (a) Assay validity: For the assay to be considered valid, the following criteria must be satisfied: (1) solvent and untreated controls should have a net nuclear grain count of 1 or less; (2) the negative controls should fall within the 95% confidence limits of the reporting laboratory's mean historical data (DMSO: 0.21 ± 0.40, WME: 0.48 ± 1.1); and (3) the mean net nuclear count for the positive control should be within one standard deviation of the mean historical value for the reporting laboratory (mean = 29.6, S.D. = 14.5).

   (b) Positive response: The assay was considered positive if at least five net nuclear counts were consistently observed in triplicate wells.

5. Protocol: A protocol was not provided. However, a copy of the raw data accompanied the report.

C. REPORTED RESULTS:

1. Analytical Determinations: Analyses of all dosing solutions were performed by American Cyanamid Company, Princeton, NJ; analyses of the 0.4, 4, 40, 133, and 400 mg/mL solutions were performed in duplicate. The assayed concentrations of the 0.0133, 0.04, 0.133, 0.4, 1.33, 4.0, 13.3, 40, 133, and 400 mg/mL dosing solutions were 96%, 106%, 89%, 128%, 94%, 160%, 103%, 114%, 122%, and 80%, respectively, of their nominal concentrations.

2. UDS Assay: Ten concentrations of difenzoquat, ranging from 0.26 to 8000 µg/well in semi-log increments, were assayed on triplicate slides. A white precipitate formed at 2666 and 8000 µg/well. Doses above 80 µg/well were cytotoxic; grains were not counted for the lowest dose (0.26 µg/well). Accordingly, UDS was evaluated from cultures exposed to 0.8 to 80 µg/well. Representative data, shown in Table 1, indicated that there was no evidence of UDS at any of the noncytotoxic concentrations of difenzoquat that were tested. Although there was a slight increase in the mean net nuclear grain counts at the three highest noncytotoxic doses, the counts were still well below the reporting laboratory's criterion for a positive response. Our reviewers assume that the increased net counts at 80 µg/well was due to slight cytotoxicity, since the average cytoplasmic grain count calculated by our reviewers at this dose (10.0) was lower than the value for the solvent control (13.8). By contrast, the positive control, 2-AAF, induced a marked increase in UDS. Based on these findings, the study author concluded that difenzoquat was negative in the primary rat hepatocyte UDS assay.

D. REVIEWERS' DISCUSSION/CONCLUSIONS: We assess that the study author's interpretation of the data was correct. Difenzoquat was assayed to cytotoxic doses but failed to induce UDS. The response of the test system to the positive control indicated that the assay was sufficiently sensitive to detect a mutagenic response. We, therefore, conclude that difenzoquat did not induce UDS in this test system.

E. QUALITY ASSURANCE MEASURES: Was the test performed under GLPs? Yes. (A quality assurance statement was signed and dated September 10, 1984.)

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 9-12.

TABLE 1. Representative Results of the Unscheduled DNA Synthesis Rat Hepatocyte Assay with Difenzoquat

Treatment	Concentration	Average Cytoplasmic Grain Count[a]	Mean Net Nuclear Grain Count ±S.D.[b]	Percent Cells with ≥5 Net Nuclear Grains[a]
Untreated Control	--	13.8	0.2±0.2	1.7
Solvent Control				
Dimethyl sulfoxide	1%	13.8	0.2±0.2	0
Positive Control				
2-Acetamidofluorene	1×10^{-7} M	19.6	35.2±3.8[c]	100
Test Material				
Difenzoquat	26 µg/well[d]	13.8	0.4±0.2	3.3
	80 µg/well[e]	10.0	1.1±0.3	5

[a]Calculated by our reviewers.

[b]Means and standard deviations for triplicate slides.

[c]Fulfills reporting laboratory's criterion for a positive response (i.e., ≥5 net nuclear grains).

[d]Lower assayed levels (0.8, 2.6, and 8.0 µg/well) did not suggest a genotoxic effect.

[e]Higher assayed levels (266, 800, 2666, and 8000 µg/well) were cytotoxic.

APPENDIX A

MATERIALS AND METHODS
CBI pp. 9-12

Page_____ is not included in this copy.

Pages 82 through 85 are not included.

The material not included contains the following type of information:

- ____ Identity of product inert ingredients.
- ____ Identity of product impurities.
- ____ Description of the product manufacturing process.
- ____ Description of quality control procedures.
- ____ Identity of the source of product ingredients.
- ____ Sales or other commercial/financial information.
- ____ A draft product label.
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