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

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SEP 5 1991

OFFICE OF
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT: Molinate: 21-Day Dermal, Acute Delayed Neurotoxicity, and Four Mutagenicity Studies

TO: Ernestine Dobbins
Product Manager (50)
Generic Chemical Support Branch/
SRRD (H7508C)

FROM: Linda L. Taylor, Ph.D. 9/2/91
Toxicology Branch II, Section II,
Health Effects Division (H7509C)

THRU: K. Clark Swentzel for KCS 9/3/91
Section II Head, Toxicology Branch II
Health Effects Division (H7509C)

and

Marcia van Gemert, Ph.D. 9/3/91
Chief, Toxicology Branch II/HFAS/HED (H7509C)

Registrant: ICI Americas Inc.
Chemical: Molinate; S-ethyl hexahydro-1H-azepine-1-carbothioate
Synonym: Ordram
Project No.: 1-2163
Caswell No.: 444
Record No.: none
MRID No.: 409906-01, 133562, 409183-01, 409467-01, 163790, 410527-01,
Action Requested: TB II requested these studies, which were in-house, for review

Comment: The studies listed above have been reviewed, and the DER's are attached. A summary of each follows.

1) Molinate: 21-Day Dermal Toxicity to the Rat; AM Leah (dated 1/27/89). MRID No. 409906-01. There was no significant toxicity noted following dermal exposure to Molinate for 21 consecutive days at dose levels of 10, 25, and 50 mg/kg. The NOEL for skin irritation can be set at 10 mg/kg, the LEL at 25 mg/kg, based on skin irritation and acanthosis. Classification: Supplementary, but it can be upgraded.

1 OF

2) Acute Delayed Neurotoxicity Study with Ordram® Technical in Adult Hens; GL Sprague (dated 6/16/83). MRID No. 133562. Under the conditions of the study, oral doses of Ordram® (0.02, 0.063, 0.2, 0.63, and 2.0 g/kg) produced axonal degeneration in well-defined tracts of the brain and spinal cord and peripheral nerves at the two highest dose levels, as well as unsteadiness. The highest dose tested was greater than the LD_{50}. Before a NOEL for this study can be determined, a statistical analysis of the neurohistopathological data and the identification of the sensory areas affected by Ordram® should be requested. Classification: Supplementary, but it can be upgraded.

3) Molinate: An Evaluation in the Salmonella Mutation Assay; RD Callander (dated 9/28/88). MRID No. 409183-01. Under the conditions of the assay, Molinate was negative for mutagenic activity at exposure levels of 1.6, 8, 40, 200, 1000, and 5000 µg/plate, with and without metabolic activation in strains TA 1535, TA 1537, TA 1538, TA 98, and TA 100. Classification: Acceptable.

4. Mutagenicity Evaluation in Mouse Lymphoma Endpoint Test: Forward Mutation Assay; J. Majeska (dated 9/25/84). MRID No. 163790. Under the conditions of the study, Ordram® technical was mutagenic in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay with metabolic activation by both rat S9 activated and mouse S9 activated systems over the concentrations tested (0.01-0.1 µl/ml). Classification: Acceptable.

5. Molinate: An Evaluation in the In-Vitro Cytogenetic Assay in Human Lymphocytes; CA Howard and CR Richardson (dated 12/15/88). Molinate was not clastogenic in cultured human lymphocytes under the nonactivated and S9 activated system at the concentrations tested (24, 95, & 190 µg/ml). Classification: Acceptable.

6. Molinate: Assessment for the Induction of Unscheduled DNA Synthesis in Primary Rat Hepatocyte Cultures; RW Trueman (dated 3/22/89). MRID No. 410527-01. Two independent UDS assays were performed. Because of the deficiencies and problems noted, this study is not acceptable, and it is recommended that it be repeated. Classification; Unacceptable.

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Reviewed by: Linda L. Taylor, Ph.D. Linda Lee Taylor 7/29/91
Section II, Tox. Branch II (H-7509C)
Secondary reviewer: K. Clark Swentzel K. Clark Swentzel 7/30/91
Section II Head, Tox. Branch II (H7509C)

DATA EVALUATION REPORT

STUDY TYPE. 21-Day Dermal Toxicity - Rat TOX. CHEM. NO.: 444

MRID NO.. 409906-01

TEST MATERIAL. S-ethyl hexahydro-1H-azepine-1-carbothioate

SYNONYMS: Molinate, Ordram

STUDY NUMBER: LR0523

SPONSOR. ICI Americas Inc.

TESTING FACILITY: ICI Central Toxicology Laboratory

TITLE OF REPORT: Molinate: 21-Day Dermal Toxicity to the Rat

AUTHORS: AM Leah

QUALITY ASSURANCE: A quality assurance statement was provided.

REPORT ISSUED: January 27, 1989

CONCLUSIONS: There was no significant systemic toxicity noted following dermal exposure to Molinate for 21 consecutive days at dose levels of 10, 25, and 50 mg/kg. The NOEL for skin irritation can be set at 10 mg/kg, the LEL at 25 mg/kg, based on skin irritation and acanthosis.

Classification: Core Supplementary, pending submission of the raw individual data (body weights, organ weights, microscopic findings, etc.) and Batch #/ stability information for the Molinate used in the study. This study does not satisfy the guideline requirement (82-2) for a repeat dermal toxicity study but can be upgraded with the submission of the data/information described above.

A. MATERIALS

1. Test compound: S-ethyl hexahydro-1H-azepine-1-carbothioate; Description: amber liquid. Batch No.: not provided (reference. 10959-36), CTL reference # Y06367/001/001; Purity: 97.6%.

2. Test animal: Species. Rat; Strain: SPF Wistar-derived albino (Alpk:APfSD), Age: not provided, Weight: 192-225 grams (males), 185-227 grams (females); Source: Animal Breeding Unit, Pharmaceuticals Division, Alderley Park, UK.

3. Statistics

Body weight gain, final body weight, and food consumption: analysis of variance, separately for males and females; biochemical and hematological data: analysis of variance, male and female data analyzed together with the results examined to determine whether any difference between control and treated groups were consistent between the sexes; organ weights: analysis of variance and covariance on final body weight, separately for males and females. Each group mean was compared with the control group mean using a two-sided Student's t-test, based on the error mean square in the analysis.

B. STUDY DESIGN

Methodology

Rats were assigned randomly to one of four (three test and one control) groups of five males and 5 females. The dorsal-lumbar region (approximately 10 cm x 5 cm) of each animal was clipped 18 to 24 hours before application of the test material. The dose levels were: Control-occlusive bandage only, 10, 25, and 50 mg/kg. The undiluted test material was applied to the shorn backs with a glass syringe, with the differences in dose level being achieved by altering the dose volume applied (based on each animal's body weight at the time of dosing). The test material was kept in contact with the skin for approximately 6 hours using occlusive dressings, which consisted of a gauze patch of plastic film held in position with adhesive bandage secured by two pieces of PVC tape wrapped around the animal. At the end of the 6-hour period the dressing was removed, the site of application cleansed free of the test substance, and the test site was dried. Twenty-one 6-hour applications were made with an 18-hour "rest" period between each application. During the "rest" period, each animal was fitted with a plastic collar to prevent ingestion of the test material.

NOTE: Animals were acclimated to the collar prior to the study. Doses were based on results from a preliminary study in which doses of 10, 100, and 1000 mg/kg (undiluted test material) were applied (5 applications) as described above. Deaths occurred at 1000 mg/kg, and severe skin irritation was observed at 100 mg/kg. The high-dose for the main study was based on the skin irritation response. The undiluted test material was used, and no analysis of stability was provided in study report.

Observations

All rats were examined daily, prior to dosing and after decontamination, for signs of toxicity and dermal irritation at the site of application. Body weights were recorded daily to assess the amount of test substance to be applied, and food consumption (per cage over a 24-hour period) was measured once a week. Animals had free access to food (Porton Combined diet) and water.

RESULTS

Survival, Clinical Signs, and Dermal Irritation

There were no deaths, and no significant systemic effects were observed in any of the animals. Signs of skin irritation were observed at all dose levels. At the low dose, the irritation was minimal, consisting of slight desquamation, erythema, and thickening of the skin for several days in a few animals. These same observations were noted at the two higher dose levels, along with edema, but were slight to moderate in severity and more animals were affected. There were occasional observations of extreme desquamation and edema at the high dose.

Body Weight and Food Consumption

There were no treatment-related effects on body weight gain or food consumption.

Clinical Pathology

Blood was collected from each animal at necropsy for hematology and biochemical analyses. No information was provided as to whether the animals were fasted prior to sacrifice. The CHECKED (X) parameters were examined.

a. Hematology

X	Hematocrit (HCT)	X	Leukocyte differential count
X	Hemoglobin (HGB)	X	Mean corpuscular HGB (MCH)
X	Leukocyte count (WBC)	X	Mean corpuscular HGB conc.(MCHC)
X	Erythrocyte count (RBC)	X	Mean corpuscular volume (MCV)
X	Platelet count		Nucleated red blood cell count
X	Prothrombin time	X	Kaolin-cephalin time

b. Clinical Chemistry

	Electrolytes:		Other:
X	Calcium	X	Albumin
	Chloride	X	Blood creatinine
	Magnesium	X	Blood urea nitrogen
X	Phosphorous	X	Cholesterol
X	Potassium		Globulins
X	Sodium	X	Glucose
	Enzymes	X	Total Bilirubin
X	Alkaline phosphatase	X	Total Protein

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X	Cholinesterase (RBC & plasma)	X	Triglycerides
X	Creatine kinase		Serum protein electrophoresis
	Lactic acid dehydrogenase		
X	Plasma alanine transaminase		
X	Plasma aspartate transaminase		
	gamma glutamyl transferase		
	glutamate dehydrogenase		

RESULTS

No baseline data were provided. There were no treatment-related effects on clinical biochemistry or hematology. Although there were statistically significant differences in several parameters, none of the decreases was dose-related.

	MALES				FEMALES			
Dose Level (mg/kg)	0	10	25	50	0	10	25	50
PARAMETER								
Plasma total protein	6.2	5.9*	6.0	5.9*	6.4	6.2	6.1	6.2
Alkaline phosphatase	312	283	236*	251*	180	175	140	202
Plasma sodium	148	147	144**	145*	144	144	143	145
RBC cholinesterase	1962	1738**	782*	1652**	2166	1733**	1844**	1788**
Hemoglobin	17	16*	17	17	16	15	16	16
Hematocrit	.47	.44**	.46	.46	.45	.44	.44	.44
MCV	61	59*	59	58**	61	61	60	60
MCH	22	21*	22	21**	22	21*	22	22
MCHC	36	36	36	36	35	34*	36	36
Platelets	541	570	573	553	596	598	598	488**
WBC	8.9	6.7*	9.2	7.8	7.0	6.4	6.6	6.6
Neutrophil count	2.0	1.0**	1.5	1.6	1.4	1.4	1.1	1.0
Kaolin-cephalin time	18	9*	13	13	16	13	14	11

* $p<0.05$. ** $p<0.01$

Gross Pathology

At necropsy, each animal was subjected to a gross macroscopic examination of major abdominal and thoracic viscera, and the CHECKED (X) organs/tissues were collected. The liver, kidneys, brain, adrenals, and testes or ovaries were weighed at necropsy (all animals).

	Digestive System
	Tongue
X	Salivary glands
X	Esophagus
X	Stomach
X	Duodenum
X	Jejunum
X	Ileum
X	Cecum
X	Colon
X	Rectum
X	Liver
	Gall bladder
	Pancreas

	Cardiovasc./Hemat.
X	Aorta
X	Heart
	Bone marrow
X	Lymph nodes (cervical)
X	Spleen
X	Thymus
	Urogenital
X	Kidneys
X	Urinary bladder
X	Testes
X	Epididymides
X	Prostate
X	Seminal vesicle

	Neurologic
X	Brain
X	Periph. nerve (sciatic)
X	Spinal cord
X	Pituitary
X	Eyes (w/ Harderian gl.)
	Glandular
X	Adrenals
	Lacrimal gland
	Mammary gland
X	Parathyroids
X	Thyroids
	Other
X	Bone (femur)

Respiratory	|X| Ovaries	| | Skeletal muscle
| | Trachea	|X| Uterus	|X| Skin (test site)
|X| Lung	| | Cervix	|X| All gross lesions and masses
| | Nose	| | Vagina	|X| Skin (untreated)
| | Pharynx		
| | Larynx		

RESULTS

There were no treatment-related effects on gross observations or organ weight. Adrenal weight of the mid- and high-dose males was greater than their control value, but the increase was not dose-related, and there was no associated macroscopic or histopathologic effect. It was reported that the kidney weight of one high-dose male was approximately 50% lower than that of the other high-dose males, but there were no clinical or pathological findings to explain the occurrence. Analysis of the kidney weight data with and without this animal showed no statistically significant difference. The kidney weight of the mid- and high-dose females was higher than the control value, but the increase was not dose-related and statistical significance was found at the mid-dose level only.

Histopathology

Histological examination of the liver, kidneys, adrenals, brain, heart, sciatic nerves, spinal cord, spleen, untreated and treated skin, and abnormal tissues was performed on all animals.

RESULTS

Minimal to slight acanthosis was observed in treated skin from animals at all dose levels (both sexes) and in the male controls. The incidence and severity of the change showed a slight dose-related increase in animals at the two highest dose levels.

	MALES				FEMALES			
Dose Level (mg/kg)	0	10	25	50	0	10	25	50
Skin (untreated) n=5								
Acanthosis	0	0	1	0	-	-	-	-
minimal	0	0	1	0				
Skin (treated) n=5								
Acanthosis	4	3	4	5	0	1	2	3
minimal	3	2	1	1	0	1	1	2
slight	1	1	3	4	0	0	1	1

Unilateral hydronephrosis was observed only at the two highest dose levels in both sexes, but the author stated that this is a common spontaneous finding in the Alderley rat. No historical control data were provided.

	MALES				FEMALES			
Dose Level (mg/kg)	0	10	25	50	0	10	25	50
Kidney n=5								
Hydronephrosis	0	0	2	3	0	0	1	1

C. DISCUSSION

There was slight to moderate skin irritation observed grossly and minimal or slight acanthosis, unaccompanied by inflammatory cell infiltration, observed histologically at the 25 and 50 mg/kg dose levels in both sexes, which is consistent with low grade chronic irritation. At the low-dose level (10 mg/kg), there were some signs of slight skin irritation, but the incidence of acanthosis observed histologically was no greater than that observed in the controls and was considered to be due to the mechanical effect of daily application of an occlusive bandage.

There were no significant signs of systemic toxicity. The decrease in erythrocyte cholinesterase activity was considered to be within the normal range of variability for this measurement, it was not dose-related, and there was no evidence of the reduction in plasma cholinesterase activity.

D. CONCLUSION

There was no significant systemic toxicity noted following dermal exposure to Molinate for 21 consecutive days at dose levels of 10, 25, and 50 mg/kg. The NOEL for skin irritation can be set at 10 mg/kg, the LEL at 25 mg/kg, based on skin irritation and acanthosis.

This study is classified Core Supplementary, pending submission of the raw individual data (body weights, organ weights, microscopic findings, etc.) and batch #/stability information for the Molinate used in the study. This study does not satisfy the guideline requirement (82-2) for a repeat dermal toxicity study but can be upgraded with the submission of the data/information described above.

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Reviewed by: Linda L. Taylor, Ph.D. Linda L. Taylor 9/2/91
Section II, Tox. Branch II (H7509C)
Secondary Reviewer: K. Clark Swentzel [illegible] [for KCS] 9/3/91
Section II Head, Tox. Branch II (H7509C)

DATA EVALUATION REPORT

STUDY TYPE: Acute Delayed Neurotox. Hen TOX. CHEM. NO.: 444

MRID NO.: 133562/4330012-03

TEST MATERIAL: Ordram®

SYNONYMS: Molinate; S-ethyl he ahydro-1H-azepine-1-carbothioate

STUDY NUMBER: T-10510

SPONSOR: Stauffer Chemical Company

TESTING FACILITY: Richmond Toxicology Laboratory, Richmond, CA
(Histopathology: Napa Valley Histology Lab, CA)

TITLE OF REPORT: Acute Delayed Neurotoxicity Study with Ordram® Technical in Adult Hens.

AUTHOR: GL Sprague

REPORT ISSUED: June 16, 1983

QUALITY ASSURANCE: A quality assurance statement was provided.

CONCLUSIONS: Under the conditions of the study, oral doses of Ordram® (0.02, 0.063, 0.2, 0.63 and 2.0 g/kg) produced axonal degeneration in well-defined tracts of the brain and spinal cord and peripheral nerves at the two highest dose levels, as well as unsteadiness. The highest dose tested was greater than the oral LD_{50} for Ordram®. Before a NOEL for this study can be determined, a statistical analysis of the neurohistopathological data and the identification of the sensory areas affected by Ordram® should be requested. This study does not satisfy the guideline requirements (81-7) for an acute delayed neurotoxicity study in hens, but it may be upgraded with the submission of the above-requested data/ information as well as information regarding the identity of the test material used in the study.

Classification: Core supplementary, pending clarification of the difference between the Lot # identified as used in the study and the Lot # analyzed (Appendix I). This study does not satisfy the guideline requirement (81-7) for an acute delayed neurotoxicity study in hens, but it may be upgraded.

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A. MATERIALS

1. Test Material: Ordram Technical; Description: none provided; Batch/Lot #: CGB-1802; % Active Ingredient: % not provided*; Solvent: Mazola corn oil.

*NOTE: In Appendix I: Analysis and Stability of Ordram, it is stated that Lot # CGB-1802 was characterized twice (once on 3/10/80, the second on 5/7/81); the 2 characterizations were compared and a summary of the results was listed. It was stated that the composition of the 2 samples did not change substantially and that the weight % purity was 98.6%. However, the title of the table listing the results is: Major Components of ORDRAM® Technical Lot #CG13-1892. Additionally, the stability of ORDRAM® in corn oil was said to have been measured (21,000 ppm, stored at 4°C, ambient, and 60°C for 4 weeks); no evidence of instability was reported.

2. Control Material: Negative: corn oil, Lot #: 48001-05820; Positive: tri-o-tolyl phosphate (TOCP), Eastman Organic Chemicals Practical grade; Lot #: A7C.

3. Test Organism: Species: hen; Strain: White Leghorn; Age: adult in full egg production, 12-14 months at study initiation; Body Weight: 1.2-2.5 kg at study initiation; Source: Feather Hill Farms, Petaluma, CA; quarantined for unspecified period prior to treatment. Hens were treated with a mitocide (Ravap®) 3 days prior to arrival at laboratory and again during quarantine period (Ravap® or Sevin 4F®); more than 2 weeks elapsed between second mitocide treatment and test material administration.

B. STUDY DESIGN

Methodology

The study consisted of two phases: (A) acute toxicity, which was assessed by determining an oral LD_{50} and by measuring plasma cholinesterase activity; (B) acute delayed neurotoxicity test, which consisted of two parts: Part I was designed to test the acute delayed neurotoxic potential of Ordram® and Part II was designed to supplement Part I and determine whether the effects observed in Part I were reproducible, dose-related, or reversible. The data from the acute toxicity phase were used to select dose levels for the acute delayed neurotoxicity phase. Doses were administered by gavage to hens fasted for 17-20 hours; Ordram® was administered either neat or diluted in corn oil, depending on dose.

(A) An oral LD_{50} was determined for Ordram® using the up-and-down method of Dixon (1965). There were 6 groups, each consisting of 5 hens. One-half of the groups was given atropine sulfate (10 mg/kg, s.c.) prior to treatment with Ordram®. A reactivator (e.g., 2-PAM) was not used as a pretreatment because it can exacerbate cholinesterase inhibition by carbamates (such as Ordram®). All of

the hens were observed for 28-30 days post dose. An LD_{50} was determined for each group, and a mean group LD_{50} was calculated for the protected and unprotected groups. Single oral doses of Ordram® (0.00028 to 2.82 g/kg) were administered to hens to determine its effect on plasma cholinesterase activity (colorimetric method of Sprague, et al., 1981). Blood samples were obtained from the brachial wing vein 24 hours after treatment. Plasma protein concentrations were determined by the method of Lowry, et al., 1951. Activities of the treated hens were compared to the values of corn oil-treated hens.

(B) Hens were (1) randomly assigned an identification number upon arrival and (2) assigned sequentially to the groups outlined below.

Part I

Treatment	Dose (g/kg)	# of hens
Ordram	2.0; neat	26
Ordram	0.02; in corn oil, 2mL/kg	10
corn oil	9.8; neat	10
TOCP	0.5; in corn oil, 2 mL/kg	10

Part II

Treatment	Dose (g/kg)	# of hens
corn oil	10; neat	15
TOCP	0.5; in corn oil, 2 mL/kg	15
Ordram®	0.063; in corn oil, 2 mL/kg	10
Ordram®	0.2; in corn oil, 2 mL/kg	10
Ordram®	0.63; in corn oil, 2 mL/kg	15
Ordram®	2.0; in corn oil, 2 mL/kg	30

Hens were treated twice with 3 weeks between treatments. Three weeks after the second treatment, survivors were sacrificed, except those held for a recovery phase in Part II, which were observed for an additional 120 days before sacrifice.

All hens had access to feed (Layena ETS, Ralston Purina Co.) and water ad libitum.

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Observations

Cageside observations (see below) were performe once a week for the 2 weeks prior to dosing and then daily during the study until sacrifice. Those in the recovery phase were observed 5 times a week until sacrifice. Food consumption was recorded on 2 consecutive days once per week for 2 weeks prior to study initiation and during weeks 3 and 6 of the study and twice a week during all other weeks of the study. Body weight was recorded on the first of the 2 consecutive days on which feed consumption was recorded. Egg production was recorded once weekly, and walking behavior was scored once a week; all parameters for the hens of the recovery phase were checked once weekly.

Daily Observations Monitored

General Appearance
- Feather loss
- Curled toes
- Dry/atrophied comb
- Limp comb
- Blue-tipped comb
- Prostrate
- Moribund

Behavioral
- Listless
- Hyperactive
- Nervous when handled
- Non-vocal

Posture and Coordination
- Noticeably wobbly legs
- Leaning back
- Sitting on hocks
- Spreads wings for balance
- Loss of righting reflex
- Hunched posture

Physiological
- Difficult/labored breathing
- Exaggerated breathing
- Diarrhea
- Soft-shelled eggs
- Salivation
- Mydriasis or miosis
- Ptosis

Walking behavior was evaluated using a grading system (Sprague, et al., 1980). Hens were forced to walk on a horizontal surface in an enclosed area and the locomotion, posture, equilibrium, and leg strength were evaluated by a trained observer. A numerical score was assigned in each of the 4 categories; the sum comprised the hen's score (maximum score: 15).

Histopathology

In Part I, surviving or moribund hens were anesthetized, perfused systemically (cardiac infusion), and exsanguinated. Following perfusion, the intact brain, spinal cord and sciatic nerves were removed. Specimens examined (microscopically) included: (1) cross section through the cerebellum and attached medulla; (2) cross and longitudinal sections through the brain stem; (3) longitudinal and cross sections of (a) mid-cervical, (b) mid-thoracic, and (c) lumbosacral spinal cord; and (4) longitudinal and cross sections of sciatic nerves (from both legs) and their two major branches.

The tissues collected in Part I were collected in Part II also, along with 3 additional forebrain sections [cerebrum cut at 3 levels (anterior, mid at the optic chiasma, and posterior including the optic lobes)].

Statistics

Confidence limits for mean LD_{50} values: standard error of the mean and a two-tailed t-value (Sokal & Rohlf, 1960); Mean values for body weight, 24-hour feed consumption, and plasma cholinesterase activities: Dunnett's Test (Tallarida & Murray, 1981); Group scores for behavioral neurotoxicity: Mann-Whitney U-test (Sokal & Rohlf, 1969).

C. RESULTS

Acute Toxicity

1. Oral LD_{50} - Mortality was delayed, with most death occurring within 2-11 days after dosing. One death occurred at 22 and 26 days after dosing. Diarrhea, motor incoordination, and loss of body weight (10-30%) were the primary toxic signs observed. The oral LD_{50} for Ordram® (1) in the unprotected hens was calculated as 1.93 g/kg (95% confidence limits:0.56-3.30 g/kg) and (2) in protected hens was 2.30 g/kg (95% confidence limits: 0.86-3.74 g/kg). The two LD_{50}'s were not significantly different, and the toxic signs displayed were similar.

2. Cholinesterase Inhibition - There was a dose-related inhibition in plasma cholinesterase following a single oral dose of Ordram® at doses of 3.5 mg/kg or greater. No significant inhibition was reported following a dose of 2.8 mg/kg (or lower). NOTE: No individual data were provided; results were reported as a Figure (I, copy attached). Neither axis is labeled.

Acute Delayed Neurotoxicity

Survival: Part I - The highest dose (2 g/kg) tested was greater than the LD_{50}. Fourteen of the 25 hens treated at this dose level survived to Day 43 (sacrifice).

Part II - Twenty-three of the 30 high-dose (2.0 g/kg) animals died prior to study termination (2 were alive at the 43-day sacrifice and 5 for 163-day sacrifice). Eight of ten hens at 0.63 g/kg dose level survived to termination at day 43; all 5 scheduled for the recovery phase survived. The three lowest doses (0.2, 0.063, & 0.02 g/kg) produced no deaths.

Clinical Observations

Signs of toxicity displayed in Ordram®-treated hens (similar in both phases of the study) included mortality (67% at 2 g/kg;

average for Parts I & II), weight loss, decreased food consumption, diarrhea, motor incoordination (unsteadiness), and behavioral depression (non-vocal, listless). The unsteadiness reported for the Ordram® hens occurred immediately after dosing, but the severity did not increase with time, as was reported in the TOCP hens. Additionally, no clinical signs of delayed neurotoxicity were reported in the Ordram®-treated hens; leg weakness and incoordination were demonstrated in the TOCP-treated hens. NOTE: Although the report stated that incoordination (unsteadiness) was observed in the high-dose hens, it also stated that incoordination suggestive of delayed neurotoxicity was not.

PART I Clinical Observations (% incidence)

Parameter/Dose	0.02*	2.0*	0	TOCP
Appearance				
feather loss	50	71	20	10
Behavioral				
listless	0	29	0	0
non-vocal	0	100	0	0
Posture/Coordinat.				
unsteady	0	75	0	100
sitting on hocks	0	17	0	100
unable to stand	0	8	0	70
wide stance	0	21	0	30
wing/neck droop	0	75	0	0
Physiological				
ptosis	0	33	0	0
soft-shelled egg	20	42	0	0
diarrhea	90	92	0	90
labor.breathing	0	0	0	20

PART II Clinical Observations (% incidence)

Parameter/Dose	0.063*	0.2*	0.63*	2.0*	0	TOCP
Appearance						
feather loss	90	100	100	93	93	100
Behavioral						
listless	0	0	27	70	0	40
non-vocal	10	20	73	100	0	60
Posture/Coordin.						
unsteady stand.	0	20	93	90	0	100
sitting on hocks	0	0	20	80	0	93
unable to stand	0	0	20	20	0	67
wide stance	10	0	20	10	0	0
wing droop	0	0	13	33	0	0
neck droop	0	0	0	37	0	0

Physiological						
ptosis	0	0	33	50	0	40
soft-shelled eggs	10	20	0	17	0	0
diarrhea-mild	70	30	7	3	93	0
diarrhea-mod/sev	30	60	93	97	7	100
labor, breathing	0	0	7	3	0	0

* dose of Ordram®

Body Weight and Feed Consumption

Compared to the vehicle controls, the high-dose Ordram® hens and the TOCP hens had lower body weight following treatment. The data were presented as body weight on a particular day compared to the hen's own weight 7 days prior to study initiation. When viewed as a % of control, the positive control and the high-dose hens showed a decrease in body weight. NOTE: All hens lost weight during the course of the study, which may partially be accounted for by the fast prior to each dosing.

Part I Body Weight (% of control)

Day	-14	-7	1	5	22	42
TOCP	94	94	95	89	86	70
2.0*	102	98	98	80	96	91
0.2*	99	101	99	101	104	100

Part II Body Weight (% of control)

Day	-14	-7	1	5	22	42
TOCP	103	103	103	98	92	76
0.063*	102	103	101	101	98	100
0.2*	113	111	110	108	109	109
0.63*	106	103	103	97	95	95
2.0*	103	102	114	94	88	87

* dose levels of Ordram®

Food consumption was also decreased in the higher-dose and TOCP-treated hens following both treatments when compared to their own food intake measured 6 days prior to initial treatment.

Egg Production

In both parts of the study, the 2.0 g/kg dose of Ordram® resulted in a higher percentage of hens producing no eggs (within 2 weeks

after the first dose). The TOCP-treated hens showed a similar response at 4 weeks.

Part I Egg Production (% hens with no eggs)

Week	-2	-1	1	2	3	4	5	6
- Control	10	0	0	0	0	0	0	0
+ Control	10	10	10	20	10	88	88	100
0.02*	0	0	0	10	10	10	10	10
2.0*	12	8	8	100	69	50	100	100

Part II Egg Production (% hens with no eggs)

Week	-2	-1	1	2	3	4	5	6
- Control	47	40	33	47	57	50	50	50
+ Control	40	53	53	67	60	92	100	100
0.063*	70	90	60	40	60	30	50	40
0.2*	70	50	10	30	30	10	30	30
0.63*	80	60	0	20	50	0	23	23
2.0*	34	24	48	89	100	100	100	71

* dose levels of Ordram®

With regard to egg production, the high-dose (2.0 g/kg) hens in both parts of the study showed a decrease in the number produced per week compared to the negative controls. The TOCP-treated animals also showed decreased production, although in most cases the Ordram hens displayed a greater decrease than the TOCP hens compared with the corn oil controls.

PART I Egg Production (#/week)

Treatment/wk	-2	-1	1	2	3	4	5	6
- Control	5.4	4.5	5.4	5.6	5.3	5.0	5.3	4.0
+ Control	4.7	4.5	2.7	3.2	3.9	0.25	0.38	0
0.02 g/kg*	3.5	4.9	4.3	4.2	4.2	4.1	3.7	4.4
2.0 g/kg *	3.5	4.8	1.5	0	0.6	0.7	0	0

* dose levels of Ordram®

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PART II Egg Production (#/week)

Treatment/wk	-2	-1	1	2	3	4	5	6
- Control	1.1	1.9	1.7	2.0	1.4	1.9	1.5	1.7
+ Control	2.1	1.8	0.9	0.6	0.8	0.1	0	0
0.063 g/kg*	0.3	0.1	0.4	0.7	0.4	0.9	0.7	0.6
0.20 g/kg*	0.3	0.5	1.5	1.2	0.7	1.5	0.9	0.9
0.63 g/kg*	0.2	0.4	1.7	2.5	1.3	2.5	1.3	1.5
2.0 g/kg*	1.9	2.0	0.8	0.1	0	0	0	0.4

* dose levels of Ordram®

Motor Activity

Walking Behavior: In Part I, the mean score in the corn oil hens ranged from 0.2 to 0.6, with 2 being the highest score displayed by any hen. In the TOCP hens, the mean score ranged from 0.4 to 8.7 (increased with time after dose), with the highest score being 14. In the low-dose hens, the range was 0 to 0.7 (3 was the highest score). At the high dose, the range was 0.2 to 5.0, with the highest score for the group occurring on the day of dosing (highest score was 14). In Part II, the highest score for the corn oil controls was 2, with two exceptions (one moribund hen scored 12 and one hen scored 3 two weeks before dosing). The range was 0.1 to 0.8. Eight of the TOCP hens scored 14, with the range being 0.3 to 12.0. The mean score increased with time after dosing. At 0.063 g/kg, several hens scored 5 during the 2 weeks prior to dosing, and the mean score range prior to treatment (2.1 to 3.1) was greater than that following treatment (0.8 to 2.1). Similar findings occurred at 0.2 and 0.63 g/kg; i.e., the highest scores occurred prior to treatment and were higher than those displayed by the negative control. No comment was made regarding these findings. At 2.0 g/kg, the lowest scores occurred prior to treatment (0.4 to 0.6), and the scores increased with time after treatment (2.3 to 5.4).

PART I Mean Walking Behavior Scores

Treatment/day	-14	-7	1	8	15	22	29	36
- Control	0.2	0.3	0.5	0.4	0.2	0.3	0.6	0.4
+ Control	0.4	0.5	0.8	1.1	5.9	7.6	7.5	8.7
0.02 g/kg*	0	0.3	0.7	0.2	0.5	0.5	0.5	0.3
2.0 g/kg*	0.2	0.44	5.2	3.6	1.44	3.19	1.93	1.64

* dose levels of Ordram®

PART II Mean Walking Behavior Scores

Treatment/day	-14	-7	1	8	15	22	29	36
- Control	0.7	0.1	0.7	0.6	0.3	0.8	0.6	0.4
+ Control	0.4	0.4	0.3	2.1	7.4	8.4	8.8	12.0
0.063 g/kg*	2.1	3.1	1.5	1.5	2.1	0.8	1.7	1.7
0.20 g/kg*	3.6	3.0	0.6	0.7	1.8	0.4	0.6	1.5
0.63 g/kg*	1.5	1.0	0.5	0	1.1	0.1	0.1	0.8
2.0 g/kg*	0.6	0.4	2.3	3.9	3.5	3.9	5.4	3.4

* dose levels of Ordram®

In both parts of the study, the high-dose (2.0 g/kg) affected walking behavior. Although the scores at the lower dose levels in Part II were somewhat higher than corn oil control values on several occasions, these dose levels displayed higher scores prior to dosing than the controls. Therefore, these data on the 3 low dose levels do not clearly demonstrate either an effect or a no-effect level.

Neurohistopathology

Background histopathological alterations (lymphocytic perivascular cuffing, focal gliosis, perineural lymphocytic foci, lymphocytic foci, and focal Schwann cell hyperplasia) observed in the corn oil controls were said to be consistent with those associated with Marek's disease and/or other field and vaccine viruses common to commercial chickens. In the TOCP control hens, similar lesions as were seen in the negative controls were observed along with swelling and destruction of axon filaments and some associated fragmentation of the myelin sheaths in the brain and all 3 levels of the spinal cord, which were slight to extensive in severity and localized to specific white matter tracts (funiculi of Cavanaugh). Frequently, this axonal change was accompanied by reactive gliosis of varying intensity. The TOCP-induced axonal damage was distinct from the occasional swollen axons in the negative control in that the change was localized to specific tracts in the brain and spinal cord and generally more severe and more consistent in the positive control. In the sciatic nerve of all TOCP-treated hens, individual fiber degeneration was present, consisting of swelling, fragmentation or dissolution of the nerve fiber and vacuolar degeneration of the surrounding myelin sheath. Nerve fiber degeneration varied from minimal to extensive in severity and occurred bilaterally in all hens.

In the Ordram®-treated hens in Part I of the study, background lesions were also observed, but at the high dose (2.0 g/kg), degenerative changes were seen in the brain, spinal cord, and

nerves of all hens and were similar in nature and localization to those of the TOCP-treated hens. Axonal degeneration with accompanying vacuolation or fragmentation of myelin sheaths and reactive gliosis was particularly well developed in the brain stem, medulla, and cervical spinal cord and less so in the thoracic and sacrolumbar spinal cord segments. Nerve fiber degeneration, including formation of "digestive chambers" and focal Schwann cell hyperplasia was consistent and ranged from minimal to moderate severity. In 13 of 14 hens, nerve fiber degeneration was bilateral. At the low dose, lesions in the CNS were comparable to the negative control, and although 8 of 10 hens had detectable nerve fiber degeneration, the author stated that this lesion was bilateral in only 3. TB II notes that none of the lesions in the negative control hens were bilateral. The nerve fiber degeneration was said to have affected solitary or few fibers and generally was not more severe than that seen in 3 negative control hens. In Part II, hens at the 2 top dose levels (0.63 & 2.0 g/kg) had axonal degeneration and destruction in brain and cervical cord approximating that observed in the TOCP-treated hens, which affected identical white matter tracts and varied in severity from mild to extensive, the involvement of the lower levels of spinal cord was clearly less severe and less consistent than in the positive controls. Additionally, nerve fiber degeneration in sciatic nerves was less severe than in the + control. At the two lowest dose levels (0.2 and 0.063 g/kg), lesion patterns very similar to those observed in the negative controls were reported. It was concluded that Ordram® demonstrated morphological evidence of definite neurotoxicity, but the lesions caused by the compound do not conform exactly in distribution to the lesion pattern established for classical delayed neurotoxicants. The NOEL for this study was chosen at 0.2 g/kg, the LEL at 0.63 g/kg, based on axonal degeneration in the brain, spinal cord, and peripheral nerves.

NOTE: It was stated in the histopathological report that no remarkable involvement was observed in the forebrain, but no tabulation of the data were provided.

Recovery Phase - In the hens observed for recovery, both axonal degeneration in the CNS and nerve fiber degeneration in sciatic nerves were reduced in incidence and severity in both the TOCP- and Ordram®-treated hens. Additionally, reactive gliosis was minimal or absent. Swollen chromatolytic neurons were increased in both incidence and severity in the TOCP-treated and the high dose (2.0 g/kg) hens. Ordram®-treated hens at the 0.63 g/kg level were fully recovered (by day 163) from the lesions that were detected in hens sacrificed at the end of the study (day 43).

CONCLUSION

Acute Toxicity - Ordram® produced low acute toxicity (1.93 g/kg-LD_{50}) and plasma cholinesterase inhibition in adult White Leghorn hens. Atropine, an anticholinergic drug, had no effect on the

Ordram® LD_{50}.

Acute delayed neurotoxicity - No clinical signs of delayed neurotoxicity were observed in the Ordram®-treated hens, but histopathological lesions in the brain, spinal cord, and peripheral nerves were observed. In some cases the changes were similar to those produced by the positive control (TOCP), but the overall pattern of changes produced by Ordram® was distinctive. While the lesions produced by TOCP showed uniform distribution and severity in all nerve sections examined, the changes observed following Ordram® exposure were most severe and frequent in the brain stem and upper spinal cord, which the author indicated may have accounted for the absence of incoordination and leg weakness in these hens. Ascending tracts in the areas of the brain and cervical cord affected by Ordram® are primarily involved in sensory rather than motor function; therefore, motor pathways that control coordination appeared unaffected by toxic doses of Ordram®. Following recovery, the lesions observed in the Ordram® hens at termination on day 43 were no longer present in the hens terminated on day 163, while pronounced axonal degeneration in the brain and spinal cord of TOCP-treated hens was still evident, although there was mild recovery. It was concluded that Ordram® demonstrated morphological evidence of definite neurotoxicity, but the lesions caused by the compound do not conform exactly in distribution to the lesion pattern established for classical delayed neurotoxicants. NOTE: Since the dose of TOCP given is one that is meant to display a definite effect, the difference noted following recovery is difficult to assess; i.e., the more severely affected hens would be expected to "recover" less or more slowly than those less severely affected.

Before a NOEL for this study can be determined, a statistical analysis of the neurohistopathological data and the identification of the sensory areas affected by Ordram® should be requested. This study does not satisfy the guideline requirements (81-7) for an acute delayed neurotoxicity study in hens, but it may be upgraded with the submission of the above-requested data/information as well as information regarding the identity of the test material used in the study.

Discrepancies

1. On page 97, Egg Production. Hen #H0414-82 has no entry for either -2 or -1 week. In other places of the table, a 0 denotes no eggs and a blank indicates that the hen died. There are entries for this h en from week 1 on.
2. Figure 1. There are no units for either axis.
3. In Table 5, Part II, Scheduled termination date for 2.0 g/kg hens is listed as 1/7/81; the first treatment is listed as 7/29/82. NOTE: In Table 6, recovery animals terminated 1/7/83.
4. In Table 6, page 41, animal # listed as H3061-82 should be H0361 and animal #H3068-82 should be H0368-82 (as listed in Appendix II,

page 52).
5. On pages 68 and 69, Walking Behavior units are (g), as are the Egg Production units on pages 70-73; neither is accurate.
6. On pages 72 & 73, the heading across the top of the table indicates the numbers stand for study day; this should be study week.

Page 22 is not included in this copy.

Pages _____ through _____ are not included in this copy.

The material not included contains the following type of information:
Identity of product inert ingredients.

_____ Identity of product inert 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.

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Guideline Series 84: **MUTAGENICITY**

Reviewed by: Byron T. Backus, Ph.D. Byron T. Backus 9/3/91
Section 2, Tox Branch 2 (TS-769C)
Secondary reviewer: John H. Chen, D.V.M. John H. Chen 9/3/91
Section 1, Tox Branch 2 (TS-769C)
Tertiary reviewer: K. Clark Swentzel Jess Rowland (for KCS) 9/2/91
Section 2, Tox Branch 2 (TS-769C)

DATA EVALUATION REPORT II

CHEMICAL: Molinate Tox. Chem. No. 444

STUDY TYPE: Unscheduled DNA Synthesis in Primary Rat Hepatocytes

ACCESSION or MRID NUMBER: 410527-01

SYNONYMS/CAS No: S-Ethyl hexahydro-1H-azepine-1-carbothioate, CAS 2212-67-1, Ordram technical

SPONSOR: ICI Americas, Inc.

TESTING FACILITY: ICI Central Toxicology Laboratory
Alderley Park, Macclesfield
Cheshire, UK

TITLE OF REPORT: MOLINATE: Assessment for the induction of unscheduled DNA synthesis in primary rat hepatocytes

AUTHOR(S): Trueman, R. W.

STUDY NUMBER(S): Report No. CTL/P/2484
Study No. SV0332

REPORT ISSUED: 22 March, 1989

CONCLUSION(S):

1. A major deficiency of this study is the lack of quantitation of cytotoxicity, either in a preliminary cytotoxicity assay or concurrently with the UDS assays. In addition, we have no way of knowing what the viability of the cells was for any of the exposure level or how these values compared relative to those of the negative controls.

2. There are several additional difficulties in evaluating the reported findings of this study. One of these is attempting to recalculate the reported exposures in "molar" units into something like µg/mL. A reasonable approximation of the molecular

weight of molinate is 187, but does a 10^{-4} M treatment mean that cells were exposed to 1.87×10^{-2} g/mL concentration of the test substance within the medium, or was 1.87×10^{-2} g test substance added to the entire amount of medium? We should have the concentration of test material specified on a w/v basis.

3. The reporting does not specify actual numbers of cells that were analyzed at each exposure level (on p. 13 it is stated that: approximately 100 cells/treatment were analyzed," but this is insufficient).

4. Another area of concern relates to the selection of areas adjacent to the nucleus for cytoplasmic counts. According to the text on p. 28 this is defined as "the number of grains in an adjacent, nuclear-sized, most heavily labelled (underlining added by this reviewer) area of cytoplasm. Although the mean net nuclear grain counts presented in the summary data for molinate-exposed cells are well below zero, some cells are reported as exhibiting UDS, although percentages are well below the 20% criterion, suggesting the possibility of some induced UDS activity, perhaps in a relatively small proportion of cells. For this reason, grain counts for individual cells and their adjacent cytoplasmic areas should be reported.

5. Because of the deficiencies and problems indicated above, this study is not acceptable. It is recommended that this study be redone, and that the testing include both preliminary and concurrent cytotoxicity assays. This study, as reported, does not satisfy the data requirements for guideline [84-2(c)], other mutagenic effects.

A. MATERIALS

1. Test Material: Molinate, described as an amber liquid, which was stored at room temperature. "Under such conditions the test sample is stable for a period of 2 years."
Batch #: not reported Analyzed Purity: 97.6%
Contaminants: none reported
Solvent used: Dimethylsulphoxide (DMSO)
Other comments:

2. Control Materials:
Positive control: 6-p-dimethylaminophenylazobenzthiazole (6BT)
Negative (solvent) control: DMSO
From p. 24: Two negative control systems were utilized:

1) A solvent negative control, containing medium, ^{3}H-thymidine and 50 µl of DMSO.

2) A medium negative control containing medium and ^{3}H-thymidine only.

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From p. 11: "Dosing solutions of molinate and 6BT were prepared in DMSO. All dosing solutions were administered at a volume of 5 µl/ml of medium. A correction for the purity of the test article was not made when preparing the dosing solutions."

3. Indicator Cells: Primary rat hepatocytes were harvested from Alderley Park (Alpk:APfSD) specific pathogen free albino rats, supplied by the Animal Breeding Unit, ICI Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, UK.

4. Cell Preparation:

a. Hepatocyte Isolation:

The following buffers were used:

Buffer 1

150 mM NaCl, 3.73 mM $NaHCO_3$, 4.84 mM Na_2HPO_4, 4.97 mM KCl, 1.24 mM KH_2PO_4, 0.62 mM $MgSO_4.7H_2O$ and 0.62 mM $MgCl_2.6H_2O$, with 10 mg/L phenol red (Na salt) used as an indicator; this buffer was adjusted to pH 7.4.

Buffer 2

142 mM NaCl, 24 mM $NAHCO_3$, 4.37 mM KCl, 1.24 mM KH_2PO_4, 0.62 mM $MgSO_4.7H_2O$ and 0.62 mM $MgCl_2.6H_2O$. 10 mg/L phenol red (Na salt) was used as an indicator; this buffer was adjusted to pH 7.4.

Both Buffers 1 and 2 and were sterilized by filtration.

The rats were anesthetized by fluothane and the livers were perfused with approximately 450 ml of Buffer 1, followed by approximately 400 ml Buffer 2, until the reservoir volume fell to 200 ml. Then the collagenase solution containing calcium was added, and the waste line placed in the Buffer 2 reservoir so that the perfusate recirculated.

The perfusion was stopped when the reticular pattern of the liver began to break up and the liver became "spongy." The liver was then excised, placed in a glass beaker, and chopped with scissors. The crude homogenate was diluted in 75 ml Williams complete medium E, filtered, and then purified of cell debris and epithelial cells by centrifugation at 40xg for two minutes. The pellet was resuspended in Williams complete medium E, and the process repeated twice. After this the hepatocytes were resuspended in 25-30 ml William complete medium E and the viability of the suspension was assessed by the Trypan blue exclusion method. From p. 13

"Hepatocytes from the animal giving the highest cell viability were diluted in order to give a concentration of $1.5x10^5$ viable cells per ml and the cells allowed to attached to attach to a series of plastic coverslips."

b. Hepatocyte Harvest/Culture Preparation: From p. 23: "An appropriate volume of Williams complece medium 'E' containing 1.5×10^5 live hepatocytes/ml was then prepared. 3 ml of this suspension was plated out into each of 3, 3 cm petri dishes...containing a 25 mm round plastic coverslip...for each of the treatment regimes to be examined. The culture dishes were then placed in a 37°C incubator with a 5% carbon dioxide/air atmosphere for 1½-2½ hours to allow cell attachment."

B. STUDY DESIGN:

1. Preliminary Cytotoxicity Assay: There is no indication within the text of the report that there was a preliminary cytotoxicity assay.

2. UDS Assay: From p. 12: "The evaluation for UDS consisted of two separate studies designed to meet the following criteria:

a) To examine the effect of the test article at a range of dose levels, the highest of which is determined by cytotoxicity, or the limit of solubility. For freely soluble, non-toxic compounds, 10^{-2} molar is the highest dose examined.

b) To repeat the experiment at least once, thus establishing the reproducibility of the assay."

From p. 24: "In general, cells are exposed initially to a series of compound concentrations ranging from 10^{-2} molar to lower levels in decrements of 10, ie 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}M to assess for cytotoxicity. Only dose levels showing no major signs of overt toxicity (high incidence of pycnotic nuclei and damaged cells) are evaluated for UDS."

"For each dose level of each compound, 50 µl was added to 10 ml Williams incomplete containing 100 µl (3.7 MBq; 100uCi) of [methyl-^{3}H]-thymidine (37MBq/ml...) in a plastic screw capped container."

"The prepared media were thoroughly mixed to ensure even distribution of the compound and 3 ml plated out onto each culture, ie 3x3ml cultures for each dose level. The culture were incubated in a 5%/CO_2 air incubator overnight at 37°C (17-20h)."

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"The next day, each culture was washed three times with 2 ml Williams incomplete medium containing 0.25 MM thymidine. A further 3 ml of the thymidine-containing medium was then added to each dish and the culture incubated at 37° in a 5%/CO_2 air incubator until the next day (≈24h)."

3. Slide Preparation: "The medium was then removed by means of a vacuum pump and the coverslips washed with 2 ml of...WE-incomplete medium or saline. 2 ml of a freshly prepared mixture of 1:3 glacial acetic acid: absolute alcohol (v/v) was added to each plate for 10 minutes. This step was done 3 times. After fixation the cultures were washed 4 times with distilled water, and the coverslips dried in air. Coverslips were then mounted, cell side up on microscope slides with DPX... Each slide was marked with the study number and allocated a unique slide number. The slides were left flat for at least 17 hours for the DPX to set, in a dust-free environment."

   Photographic emulsion was then applied by dipping each slide vertically into molten emulsion. The slides were withdrawn from the molten emulsion, and their undersides were wiped with tissue. "After the emulsion had gelled, the slides were transferred to a light-proof cabinet and dried at room temperature for a minimum of 90 minutes. When dry the slides were placed into exposure boxes with a small amount of silica gel. The boxes were sealed and stored in a refrigerator at 4°C for 14 days." Following this, the slides were developed, and (from information on p. 13) were also stained.

4. Slide Assessment and Grain Counting: From p. 13: "Prior to evaluation for UDS the slides were examined to ensure that the culture as a whole was viable, that the radiolabelling procedure had worked and that there were sufficient numbers of cells to permit a meaningful evaluation for UDS. At this stage, a selection of the highest dose level to be examined was made. This was chosen to be that which extended into the cytotoxic range. Three addiitional lower dose levels which did not show signs of cytotoxicity were also read."

   "The slides were then analysed (approximately 100 cells/treatment), using an automated image analyser linked to a computer, to determine the mean net grain count (nuclear count - cytoplasmic count), the mean nuclear count and cytoplasmic counts and the percentage of cells in repair (mean net grain count = 5 or greater)."

5. Evaluation Criteria: From p. 28: "Criteria for a positive (ie genotoxic response): The criteria outlined in the ASTM Guideline

are adopted, the evaluation being based upon the mean net grain count and the percentage of cells in repair. Treated cultures showing mean net grain counts of 5 or greater (usually accompanied by 20% of greater of the cells in repair [a cell in repair is taken to be that exhibiting 5 or more net grains]) are considered unequivocally positive. Such a response must be reproduced in an independent experiment."

"Criteria for a negative (ie non-genotoxic) response: An unequivocal negative response is obtained where the mean net grain count of the treated cultures is less than 0 and the percentage of cells in repair is less than 20. This response must be reproduced in an independent experiment."

6. There is a signed and dated Statement of GLP Compliance on page 3 of the report, as well as a signed and dated Quality Assurance Statement on p. 5.

C. RESULTS

1. Cytotoxicity: From p. 14: "At the higher concentrations of molinate tested, 10^{-2}, and 10^{-3} M in experiment 1, and 10^{-2}, 10^{-3} and 10^{-4} M in experiment 2, a substantial cytotoxic response was observed. This was manifest as a reduction in the numbers of cells attached to the coverslips, together with the presence of many pyknotic nuclei amongst those cells present. This resulted in insufficient cells of normal morphology for an assessment of UDS at these concentrations. The highest dose levels of molinate selected for evaluation for UDS (10^{-4} M experiment 1 and 10^{-5} M experiment 2) showed some signs of cytotoxicity, however there were sufficient cells of normal morphology to permit an evaluation for UDS."

2. UDS Assay: Two independent UDS assays were performed. Refer to appended pages 1 and 2 for the reported results.

D. DISCUSSION

A major deficiency of this study is the lack of quantitation of cytotoxicity, either in a preliminary cytotoxicity assay or done concurrently with the UDS assay. In addition we have no way of knowing the viability of the cells for any exposure level, or, for that matter, the viabilities relative to those of the negative controls.

In addition, there are several difficulties in evaluating the reported findings of this study. The first of these is the reporting of treatment levels in "molar" units, and attempting to recalculate these values into µg/mL. A reasonable approximation of the molecular weight of molinate is 187, but does a 10^{-4} M treatment mean that cells were exposed to a 1.87×10^{-2} g/ml concentration of the test substance within the medium, or

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was 1.87×10^{-2} grams added to the entire amount of medium? The reporting should have the concentration of test material specified on a w/v basis. The reporting also does not specify numbers of cells that were analyzed at each exposure level (the text on p. 13 states: "approximately 100 cells/ treatment," but actual numbers should be reported) or for each slide; our preference would be to have 50 cells analyzed for each of the 3 slides at a dose level (150 cells/dose level). Another area of concern has to do with the selection of areas of cytoplasm adjacent to the nucleus for cytoplasmic count (on p. 28 this is defined as "the number of grains in an adjacent, nuclear-sized, most heavily labelled [underlining added by this reviewer] area of cytoplasm). Finally, although the mean net nuclear grain counts presented in the summary data are well below zero, the percentages reported for cells in repair, although well below the 20% criterion, indicate the possibility of some UDS activity, perhaps in a relatively small proportion of cells. For this reason grain counts for individual cells and their adjacent areas should be reported.

Because of the deficiencies and problems indicated above, this study is not acceptable. It is recommended that this study be redone, and that the testing include both preliminary and concurrent cytotoxicity assays. This study, as reported, does not satisfy the data requirements for guideline [84-2(c)] other mutagenic effects.

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Pages 30 through 31 are not included in this copy.

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Identity of product inert ingredients.

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_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

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008549

Guideline Series 84-2(a): **MUTAGENICITY**

Reviewed by: Byron T. Backus, Ph.D. Byron T. Backus 9/3/91
Section 2, Tox Branch 2 (TS-769C)
Secondary reviewer: K. Clark Swentzel [illegible] 9/3/91
Section 2, Tox Branch (TS-769C)

DATA EVALUATION REPORT I

CHEMICAL: Molinate Tox. Chem. No. 444

STUDY TYPE: Salmonella/mammalian activation gene mutation assay

ACCESSION or MRID NUMBER: 409183-01

SYNONYMS/CAS No: S-Ethyl hexahydro-1H-azepine-1-carbothioate, CAS 2212-67-1, Ordram technical

SPONSOR: ICI Americas, Inc.

TESTING FACILITY: ICI Central Toxicology Laboratory
Alderley Park, Macclesfield
Cheshire, UK

TITLE OF REPORT: MOLINATE: An Evaluation in the Salmonella Mutation Assay

AUTHOR(S): Callander, R. D.

STUDY NUMBER(S): Report No. CTL/P/2246

REPORT ISSUED: 28 September, 1988

CONCLUSION(S):

1. In two separate experimental assays technical molinate was assayed at exposure levels of 1.6, 8, 40, 200, 1000 and 5000 µg/plate. both with and without S9 activation, using Salmonella typhimurium strains TA 1535, TA 1537, TA 1538, TA 98, TA 100. Statistically significant increases in numbers of revertants were observed for strains TA 1535 and TA 1537 at 1.6 µg/plate in the presence of S9 in the second assay, resulting in a third assay with strains TA 1535 and TA 1537 only, with exposure levels of 0.32, 0.8, 1.6, 4, 8, and 20 µg/plate with S9. The findings indicate that technical molinate is negative for mutagenic activity under the conditions of this assay.

2. This study, with its negative findings, is acceptable. This study satisfies the guideline requirements [84-2(a)] for a gene mutation study.

A. MATERIALS

1. Test Material: Molinate technical material
Description: a liquid, "from the information supplied by the Sponsor, the sample was stable under normal storage conditions and under the test conditions used in this study."

Batch #: 4921-8-22 Analyzed Purity: 97.6% w/w
Contaminants: none reported
Solvent used: Dimethylsulphoxide (DMSO)
Other comments:

2. Control Materials:
Negative: DMSO
Solvent/final concentration:
Positive: Non-activation:

Sodium azide	not used	µg/plate	TA100, TA1535
2-Nitrofluorene	not used	µg/plate	TA98, TA1538
9-Aminoacridine	not used	µg/plate	TA97, TA1537
N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG)	1, 2, 5	µg/plate	TA100, TA1535
Daunorubicin	0.2,0.5,1	µg/plate	TA98
Acridine Mutagen	0.5, 1, 2	µg/plate	TA1537
4-Nitro-o-phenylenediamine	1, 2, 5	µg/plate	TA1538

Activation:
2-Aminoanthracene (2-anthramine) 0.2,0.5,1.2 µg/plate
TA1535, TA1537, TA1538, TA98, TA100

3. Activation: S9 derived from

X Aroclor 1254	X induced	X rat	___ liver
___ phenobarbital	___ non-induced	___ mouse	___ lung
___ none		___ hamster	___ other
___ other		___ other	

If other, describe below
Describe S9 mix composition (if purchased, give details):

S9 fraction	3 ml
Sucrose-Tris-EDTA Buffer	7 ml
Cofactor solution	20 ml

The S9-mix was incorporated into the top agar.

The S9 buffer was prepared to the following final concentrations:

Sucrose	250 mM
Tris Base	50 mM
EDTA tetrasodium salt (dihydrate)	1.0 mM

The cofactor solution was prepared to the following final concentrations:

	Final Concentration (mM) in Stock	S9-mix
Na_2HPO_4	150	100
KCl	49.5	33
Glucose-6-Phosphate	7.5	5
NADP (Na salt)	6	4
$MgCl_2$	12	8

Aliquots of both the S9 buffer and cofactor solution were sterilized by filtration.

4. Test organisms: S. typhimurium strains
_X_ TA97 _X_ TA98 _X_ TA100 ___ TA102 ___ TA104 _X_ TA1535 _X_ TA1537 _X_ TA1538 ; list any others: (none).
Properly maintained? Yes (From p. 27: prepared, then stored in liquid nitrogen; routine source of inocula for overnight cultures were one set of frozen cultures, used and discarded on a weekly to fortnightly basis; from p. 28: "When fresh frozen stocks were prepared (from the frozen permanent strains), the strains were tested for histidine requirement and for reversion properties using diagnostic mutagens...incorporated in the top agar layer as in a normal test...rather than spot tested as stated by Ames.").

Checked for appropriate genetic markers (rfa mutation, R factor)? Yes (From p. 27-28: Each new frozen culture was screened for the deep-rough characters, DNA repair deficiency and Ampicillin resistance as described by Maron and Ames (1983) with one modification: The presence of the uvrB deletion was confirmed by testing the sensitivity of each culture to mitomycin C (10 µl of a 10 µg/ml solution) in the same manner as sensitivity to crystal violet was tested. Damage to DNA caused by mitomycin C is repaired in normal bacteria by the uvr excision repair pathway, and is thus toxic to S. typhimurium which carry the uvrB deletion).

5. Test compound concentrations used:
Non-activated conditions: 1.6, 8, 40, 200, 1000, 5000 µg/plate (experiments 1 and 2).
Activated conditions: 1.6, 8, 40, 200, 1000, 5000 µg/plate for experiments 1 and 2; 0.32, 0.8, 1.6, 4, 8, and 20 µg/plate (experiment 3 only).

B. TEST PERFORMANCE

1. Type of Salmonella assay:
_x_ standard plate test
___ pre-incubation (___ minutes)
___ "Prival" modification (i.e. azo reduction method)
___ spot test
___ other (describe in a.)

a. Protocol (brief description, or attach copy to appendix, if appropriate; e.g. include mediums used, incubation times, assay evaluation):

From p. 29:

Agar Plates

"9cm diameter vented Petri-dishes pre-poured with Vogel Bonner minimal medium and containing 1.5% w/v agar and 2% w/v glucose were obtained from Gibco-Europe Ltd. The plates were stored at ambient temperature until needed to dry them and ensure sterility."

Growth Supplement Stock Solutions

"0.5mM Histidine/0.5mM Biotin: this was prepared in bulk on 19 May 1988. The solution was filter-sterilized on preparation, and stored refrigerated until required."

Top Agar

"This was prepared and sterilised at ICI Pharmaceuticals' media kitchen and consists of 0.6% w/v agar and 0.5% w/v NaCl in deionised water. Before use, sufficient aliquots were melted by brief autoclaving, then stored at 50-53°C in a water bath."

"For each test the molten top agar was prepared by adding sterile histidine/biotin stock solution (10ml solution:100ml agar)."

Method of Assay

From p. 30: "0.1ml aliquots of an overnight culture of each bacterial strain were dispensed by micropipette into the required number of sterile plastic bijou bottles fitted with screw caps and stored at room temperatue until required (1-2 hours)."

"0.5ml S9-mix (or Co-factor/Buffer) was then added by dispensing syringe to the number of bijou bottles of one strain required for one dose level, followed by 0.1 ml of the appropriate concentration of test compound solution added by micropipette. Finally, 2.0ml top agar was then added by syringe to each bijcu: the force of addition was sufficient to mix the contents. The resulting mixture was then poured rapidly onto the surface of a prepared Vogel Bonner plate and allowed to gel. Plates were then labelled before being incubated inverted at 37°C for 64-68 hours in the dark."

There were 3 plates for each culture at each dose level both with and without S9.

2. Preliminary cytotoxicity assay

There is no indication within the text of this report that any preliminary cytotoxicity assay was conducted. However, in the first two experimental runs of the mutagenicity assay the test material was assayed at concentrations of up to 5000 µg/plate.

3. Mutagenicity assay

For all strains, it is reported "background lawn absent/sparse on 5000 µg dose plates." This was the only concentration at which there was evidence of cytotoxicity (seen for all strains), along with concomitant reduction in numbers of revertants.

Refer to appended pages 1 and 2 (Table 1) for the plate counts from experiment 1, to appended pages 3 and 4 (Table 2) for plate counts from experiment 2, to appended p. 5 (Table 3) for experiment 3, to appended p. 6 through 8 (Table 4) for positve and negative control data for experiment 1, to appended p. 9 through 11 (Table 5) for positive and negative control data for experiment 2, and to appended p. 12 (Table 6) for positive and negative control data for experiment 3.

The only statistically significant increases in revertants in cultures exposed to the test material occurred with strains TA 1535 and TA 1537 in experiment 2 at the single dose level of 1.6 µg/plate in the presence of S9 (refer to appended page 3). In a repeat assay (experiment 3) for these two strains with dose levels of 0.32, 0.8, 1.6, 4, 8 and 20 µg/plate with S9 there was no indication of any increased number of revertants.

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The concurrent negative and positive controls elicited the appropriate responses.

4. Laboratory Criteria for Study Acceptability

From p. 11: "Test data from individual experiments are considered valid if:

a) the concurrent solvent control data are acceptable;

b) the positive control data show unequivocal ositive responses;

c) at least the lowest test compound dose shows no evidence of toxicity, and at least three test doses show no significant overt toxicity (ie significant loss of background growth and/or reductions in colony numbers)

Failure of one or more tester strain/S9 combinations does not invalidate the data for the remainder of a concurrent experiment."

5. There is a signed and dated statement of compliance with Good Laboratory Practice standards on page 3 of the report, and a signed and dated quality assurance statement on page 5.

C. DISCUSSION

Under the conditions of this assay the test material gave no indications of mutagenic activity in any of the five S. typhimurium strains (TA1535, TA1537, TA1538, TA98, TA100) used, either in the presence or absence of S9. While there is no indication within the report that a preliminary cytotoxicity assay was conducted, the technical molinate was tested at up to 5000 µg/plate, and at this highest-dose level there was evidence of substantial cytotoxicity, as demonstrated by a reduction in lawn and a corresponding substantial decrease in observed revertants. The test material was tested in two separate assays, with triplicate plates at each dose level and with or without S9.

It is concluded that the assay and its negative findings are acceptable. This study satisfies the guideline requirements [84-2(a)] for a gene mutation study.

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Pages 38 through 49 are not included in this copy.

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Reviewed by: Timothy F. McMahon, Ph.D.
Section I, Toxicology Branch II (H7509C)
Secondary Reviewer: John H.S. Chen, D.V.M.
Section I, Toxicology Branch II (H7509C)

Data Evaluation Report

Study type: Mutagenicity (84-1) Tox. Chem. No.: 444

TRID number: 470298-022 MRID No.: 163790

Test material: ORDRAM Technical (Molinate)

Study number: T-11840

Testing Facility: Stauffer Chemical Company
Environmental Health Center
Farmington, CT 06032

Sponsor: Europe Chemical Division

Title of report: Mutagenicity Evaluation in Mouse Lymphoma Endpoint Test: Forward Mutation Assay

Study Director: Jenness Majeska

Report issued: September 25, 1984

Conclusions:

Under the conditions of this study, ORDRAM technical was mutagenic in the L5178Y Mouse Lymphoma Mutagenesis Assay with metabolic activation by both rat S9 activat mouse S9 activated systems over the concentrations tested (0.01-0.1 µl/ml).

Core Classification: acceptable

This study satisfies the requirements (84-1) for a mutagenicity study.

I. MATERIALS AND METHODS

A. Test Material: ORDRAM technical; description: amber liquid; purity: 98.3%. lot no. W 4921-8-9. Stability: not specified; stated expiration date of 02/84

Solvent used: dimethylsulfoxide (DMSO), Lot # 144601, received 01

B. Control Materials: Vehicle- DMSO, plated for all tester strains with and without meta activation.

Positive (with and without metabolic activation):

Non-activation: Ethyl Methanesulfonate (Eastman Kodak lot # E8B)- 0.5 µl

Activation: N-nitrosodimethylamine (Aldrich Chemical Co. lot # 042397)- 0.05 0.1 µl/ml.

C. Activation

S-9 was prepared from Arochlor 1254-induced male Sprague-Dawley rat and mous (500 mg/kg i.p. x 1, five days prior to sacrifice)

S9 Mixture Composition (per 10ml treatment medium)

Isocitric acid	- 0.45 mg
NADP	- 0.24 mg
S-9 homogenate	- 1.0 ml

D. Test Cells

L5178Y TK+/- Mouse Lymphoma cells, derived from the 3.7.2 clone of Fischer L517 cells were obtained from Dr. Donald Clive. Properly maintained; periodically "clear with methotrexate to maintain a low background of TFT resistant cells (spontaneous mutants).

E. Locus Examined

x thymidine kinase (TK)
selection agent:
____ bromodeoxyuridine (BrdU)
____ fluorodeoxyuridine (FdU)
4 µg/ml trifluorothymidine (TFT)

___ hypoxanthine-guanine-phosphoribosyl transferase (HPRT)

___ Na^+/K^+ ATPase

selection agent: _____ ouabain

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

F. Test Compound Concentrations Used

Non-activated conditions: 0.0125, 0.025, 0.05, 0.1, 0.2, 0.22, 0.24, 0.26, and 0.28 µl

Activated conditions: 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, and 0.1 µl/m

G. Test Performance

1. Cell treatment:
   a. Cells exposed to test compound for:
      4 hours (non-activated) 4 hours (activated)

   b. Cells exposed to positive controls for:
      4 hours (non-activated) 4 hours (activated)

   c. Cells exposed to negative and/or solvent controls for:
      4 hours (non-activated) 4 hours (activated)

   d. After washing, cells cultured for 2 days (expression period) before cell selection

   e. After expression, cells cultured for 9-11 days in selection medium to determine numbers of mutants and for 9-11 days without selection medium to determine cloning efficiency

2. Protocol

The procedures used were based on the method of Clive and Spector (Mutation Re 17-29, 1975. Details of the test procedure (pp. 14-18) are attached.

H. Determination of Toxicity

Test article was diluted for testing at 3.0, 1.5, 0.75, 0.375, 0.188, 0.094, 0.047, 0.02 0.012, and 0.006 µl/ml. The highest concentration of test material resulted in > 90% cytotoxicity. Test article was added in amounts in which the final solvent concentratio 1%) was non-toxic to the cell suspension. Culture conditions are assumed to be simil those described for the mutagenicity assay (see below).

After 4 hours, cells were washed twice with culture medium and resuspended in 2 of growth medium. Approximately 20 hours later, cell numbers were determined by counting using a Coulter Counter. Test article toxicity was determined by comparing t cell population growth level with that of the solvent controls.

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Significant reduction in cell growth (< 10% survival) was observed at 0.375 µl.ml with S9 mix activation and at 0.094 µl/ml with activation. These results (Table 1) were used select the dose levels of Ordram for the mutation assay.

I. Mutagenicity Testing

It was not stated whether cells were cleansed to reduce the frequency of spontaneous TK -/- cells prior to mutagenicity testing. A cell suspension containing 6.0×10^6 cells/ 1 of treatment medium was exposed to one of six concentrations of test material or a solvent, medium, or positive control for 4 hours with or without S-9 activation. Control tubes received solvent only, while positive controls received ethyl methanesulfonate or nitrosodimethylamine. Culture conditions are assumed as 5% CO_2 in air at 37± 1 °C After four hours, cells were washed twice in medium , resuspended in 20ml of this same medium and incubated with agitation in the presence of 5% CO_2 in air at 37± 1 °C.

Following exposure to test article and washing, cells were incubated for two days with population adjustments at 24 and 48 hours. At the end of the expression period, cells were cloned in soft agar cloning medium for viable count and for mutant number in selective medium. The five highest dose levels that reached a cell density of 2.0×10^5 cells/ml were chosen for evaluation. Cloning medium consisted of RPMI 1640, heat inactivated horse serum (10%), glutamine (1.9mM), penicillin-streptomycin (95 µg/ml) sodium pyruvate (210 µg/ml), and noble agar (0.35% final concentration). Selection medium consisted of cloning medium with 4 µg/ml TFT added. Treatment medium was identical to cloning medium with serum reduced to 5%.

Guidelines for determining the validity of the study and positive response of test are listed on pages 11-12 of the registrant report.

A signed statement of compliance with Good Laboratory Practice was not provided.

A signed statement of Quality Assurance was provided.

II. RESULTS

Significant cytotoxicity was found in the preliminary toxicity study for test article concentrations above 0.188 µl/ml under non-activation conditions, and above 0.023 µ under activation conditions(Table 1, attached). In the mutagenesis assay, none of the non-activated cultures (Tables 2 and 3, attached) exhibited a statistically significant increase in mutant frequency (mutants/10^6 clonable cells) above the solvent control at concentrations tested (0.0125-0.28 µl/ml).

Under conditions of activation, significant increases (> 2.5x solvent control) in mutant frequency were found at test article concentrations of 0.04 and 0.05 µl/ml (Table 5). increase in mutant frequency was also evident in experiments in which one portion of treated cells were cloned at 48 hours (Table 6) and again at 96 hours (Table 7). In case where a significant increase in mutant frequency was observed, significant

cytotoxicity was also observed. While results with rat liver S-9 showed increases in mutant frequency slightly below that considered a positive response (Table 4, increa of 1.8x over solvent control at 0.05 µl/ml test article), a positive response was observe Table 5 using mouse liver S-9 , supporting the conclusion of a test article related effe Positive control compounds (EMS and DMN) demonstrated positive responses in mut frequency as expected.

III. CONCLUSIONS

The background mutant frequencies for the solvent controls under non-activation or activation conditions exceeded the normal range as recommended for the mammalian cell mutation assay (e.g., 23×10^{-6}) in Tables 2 and 4. The positive control compound (EMS and DMN) induced significant increases in the mutant frequency with respect to solvent controls by a factor of at least 5.1 in all experiments indicating that the test sys was sensitive to known mutagens. The highest concentration tested (1.0 µl/ml + S9 mi reduced survival to < 10% of that seen in the solvent control. Based upon the results o this study, the test article ORDRAM technical was m. agenic in the mouse lymphoma (L5178Y) cell mutation assay.

IV. CORE CLASSIFICATION: acceptable

This study satisfies the guideline requirements (84-1) for a mutagenicity study.

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Guideline Series 84: MUTAGENIC

Reviewed by: John H.S. Chen, D.V.M. John H S Chen 9/3/91
Section I, Toxicology Branch II (H7509C)
Secondary reviewer: Yiannakis M. Ioannou, Ph.D. Stephen C. Dapan for 9/3/91
Section I, Toxicology Branch II (H7509C)

DATA EVALUATION REPORT

CHEMICAL: Molinate **Tox. Chem. No.:** 4

~~**EPA File Symbol:**~~

STUDY TYPE: Mammalian cells in culture cytogenetics assay in human lymphocytes

ACCESSION NUMBER:
MRID Number: 409467-01
SYNONYMS/CAS No.:

SPONSOR: ICI Americas Inc., Agricultural Products. Wilmington, DL 19897

TESTING FACILITY: ICI Central Toxicology Laboratory. Alderley Park, Macclesfield, Cheshire, UK

TITLE OF REPORT: Molinate: An Evaluation in the In-Vitro Cyogenetic Assay in Human Lymphocytes

AUTHOR(S): C.A. Howard and C.R. Richardson

STUDY NUMBER(S): SV0328

REPORT ISSUED: December 15, 1988

CONCLUSION(S).- Executive Summary:

Molinate was not clastogenic in cultured human lymphocyt under the nonactivated and S9-activated system at the concentrations tested.

Concentrations tested: 24, 95, and 190 ug/ml

Classification: Acceptable

This study satisfies the requirements (84-3) for a mutagenicity study.

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A. MATERIALS

1. Test Material: Name: Molinate
Description (e.g. technical, nature, color, stability):

Batch #: SC11/88 Purity: 97.6%
Contaminants: if reported, list in CBI appendix
Solvent used: DMSO
Other comments:

2. Control Materials:
Negative: DMSO
Solvent/final concentration:
Positive: Non-activation (concentrations, solvent):
0.5 ug/ml/sterile physiological saline (85%)
Mitomycin C
Activation (concentrations, solvent):
50 & 100 ug/ml/sterile physiological saline (85%)
Cyclophosphamide

3. Activation: S9 derived from
_X_ Aroclor 1254 __X__ induced
___ phenobarbital ___ non-induced
___ none
___ other
If other, describe below

Sprague-Dawley albino
male rat __X__ liver
___ mouse ___ lung
___ hamster ___ other
___ other

Describe S9 mix composition (if purchased, give details):
Final in S9 Mix: Na_2HPO_4 100mM; KCL 33 mM; Glucose-6-phosphate 5 mM; NADP (Na salt) 4 mM and $MgCl_2$ 8 mM.

4. Test compound concentrations used:
Non-activated conditions: 24, 95, and 190 ug/ml

Activated conditions: 24, 95, and 190 ug/ml

5. **Test Cells**: mammalian cells in culture
Describe cell line, cell strain or primary cell culture (if human lymphocytes, describe conditions of subjects) used
Human blood was drawn aseptically from two healthy donors (1 male & 1 female). Cultures were initiated with 0.1 mg/ml phytohemagglutinin (PHA) and maintained in RPMI 1964 tissue culture medium with 10% FBS and 100 Units/ml Penicillin and Streptomycin.

Properly maintained? (Y) / N (circle one)

Cell line or strain periodically checked for Mycoplasma contamination? Y / N (circle one) Not Applicable

Cell line or strain periodically checked for karyotype stability? Y / N (circle one) Not Applicable

B. **TEST PERFORMANCE**

1. **Cell treatment:**
   a. **Cells exposed to test compound for:**
   3.25-3.75 **hours (non-activated)** 3.25-3.75 **hours (activated)**

   b. **Cells exposed to positive controls for:**
   3.25-3.75 **hours (non-activated)** 3.25-3.75 **hours (activated)**

   c. **Cells exposed to negative and/or solvent controls for:**
   3.25-3.75 **hours (non-activated)** 3.25-3.75 **hours (activated)**

2. **Protocol (brief description, or attach copy to appendix, if appropriate; include e.g. number of cell cultures; medium; incubation times; if lymphocytes, nature of mitogen and when added; cell density during treatment; harvest times; spindle inhibitor and when used; chromosome preparation and analysis number of cells/culture analyzed; statistics used):**

The protocol used in this study was based on the internationally accepte OECD Guidelines for conducting the in-vitro cytogenetic assay in human lymphocytes. The detailed procedures for culture initiation, treatments harvest of cultures, cell staining, and cell scoring are attached (Appen

3. Preliminary cytotoxicity assay (include concentration ranges, activation and nonactivation; reported results, e.g. cytotoxicity and solubility; rationale for determining harvest times (e.g. alterations in cell cycle) and concentration levels, if reported):

A cytotoxicity test with the test samples was performed using blood from both of the previously specified donors in the presence and absen of auxiliary metabolic activation using a range of dilutions (24, 48, 95, 190, 380, 475, 713 and 950 ug/ml). The top dose level selected for this study (950 ug/ml) was the limit of solubility of molinate in DMSO The highest dose level (190 ug/ml) of molinate chosen for chromosomal analysis was based on an observed reduction of mitosis within the range of 33-48%. Tables 1 and 2 show the results of mitotic index determination from both donors under the noactivated and S9 activated conditions.

IN VITRO MAMMALIAN CYTOGENETIC

4. Cytogenetics assay (reported results, e.g. induction of aberration frequency; types of aberrations, e.g. whether gaps are included in analysis or not, chromatid vs. chromosomal events, complex aberrations; positive and background aberration frequencies; number of cultures per concentration; levels of cytotoxicity obtained, e.g. effect on mitotic index or cell survival, if examined; include representative table, if appropriate):

Representative results from the cytogenetic assay conducted with molin are presented in Tables 1 and 2. At the high dose (190 ug/ml, +/- 39) MIs were reduced by 38-43% and 33-48% under nonactivated or S9-activat conditions, respectively. However, no statistically significant incre in the number of aberrant cells were observed in either donor, in eith the presence or absence of auxiliary metabolic activation. By contras both the nonactivated (0.5 ug/ml MMC) and S9-activated (50 & 100 ug/ml positive controls induced significant ($p<0.01$; Fisher's Exact Test) in in the number of chromosomally damaged cells.

Based on these findings, the authors concluded that "under the condit of this assay molinate is not clastogenic to cultured human lymphocyte in vitro."

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5. **Reviewer's discussion/conclusions** (include e.g. rationale for acceptability or not; necessity for repeat, if appropriate; address any discrepancies with author conclusions; remember, do not include gaps in final aberration frequency analysis):

(A) The positive control compounds, mitomycin C (0.5 ug/ml) and cyclophosphamide (50 & 100 ug/ml), adequately demonstrated the sensitivity of the cultured human lymphocytes with and without metabolic activation (S9 mix) to detect a clastogenic effect.

(B) The number of cells with chromosomal aberrations in the negative (solvent) control (0.5-1% metaphase in the presence or absence of rat S9 mix observed) was found within the acceptable range establis by the testing laboratory.

(C) The highest dose level of molinate (190 ug/ml) demonstrated cyto-toxicity to deviding lymphocytes resulting in reduction of mitotic index under the nonactivated and S9-activated conditions (38-43% c the solvent control, -S9; and 33-48% of the solvent control, +S9). In both the presence and absence of S9 activation, molinate was assayed to levels that induced cytotoxicity but failed to induce a clastogenic effect in cultured human lymphocytes.

(D) Although the preliminary assessment of cell cycle delay was not conducted in this study (Reference: EPA Test Guidelines for In-Vitr Mammalian Cytogenetics; Federal Reg./Vol 50, No. 188/Friday, Septe 27, 1985), the single harvest time (23 hrs post-treatment; 70 hrs total culture time) for cells exposed to the test material in the presence or absence of S9 activated system appeared adequate for t detection of chromosomal aberrations in cultured human lymphocytes

(E) Based on these findings, we assess that the study was properly con and that the authors interpreted the data correctly. The results this study provide no indication that molinate was clastogenic in human lymphocyte cytogenetic assay.

6. **Was test performed under GLPs (is a quality assurance statement present)?** (Y) / N (circle one)

7. **CBI appendix attached** (Y) / N (circle one)

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