GUIDELINE SERIES 84: MUTAGENICITY IN VIVO UDS

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DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: In vivo/in vitro unscheduled DNA synthesis assay in rat hepatocytes

EPA IDENTIFICATION Numbers:

Tox Chem. Number:

MRID Number: 415614-08

TEST MATERIAL: Dichlormid (R-25788)

SYNONYMS: None provided

SPONSOR: Imperial Chemical Industries, Macclesfield, Cheshire, UK

STUDY NUMBER: ICI Study No. SV0350; SRI Project No. LSC-7257

TESTING FACILITY: SRI International, Menlo Park, CA

TITLE OF REPORT: Evaluation of the Potential of Dichlormid to Induce Unscheduled DNA Synthesis in the In Vivo-In Vitro Hepatocyte DNA Repair Assay in the F-344 Rat

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REPORT ISSUED: August 18, 1989

CONCLUSIONS-EXECUTIVE SUMMARY: Two independent in vivo/in vitro unscheduled DNA synthesis (UDS) assays were conducted with dichlormid. Groups of male rats received single oral gavage doses of 350, 700, or 1400 mg/kg dichlormid and were sacrificed at 2 hours (2 males/dose in the first trial; 3 males/dose in the second trial) or 16 hours (3 males/dose in the first trial; 2 males/dose in the second trial) posttreatment, and hepatocytes were analyzed for UDS. Clinical signs of toxicity (e.g., weakness, loss of balance, and low body temperature), adverse effects on the target organ (i.e., mottled livers), and cytotoxic effects on the hepatocyte cultures were apparent in the high- and/or mid-dose groups. The evidence of overt toxicity in the test animals and cytotoxic effects on the target cells indicated that an appropriate range of test material levels was evaluated. The results further show that the selected doses did not increase the frequency of UDS in treated hepatocytes at

either sacrifice interval. Although guidelines do not currently exist for the in vivo/in vitro UDS assay, and the findings clearly demonstrated that dichlormid was not genotoxic in this study, the use of a single sex and less than the number of animals recommended for in vivo genetic toxicology assays (5 males, 5 females/dose/sacrifice time) precludes full acceptance of the results as valid evidence of a negative response. We conclude, therefore, that the study cannot be used to satisfy Guideline requirements for genetic effect Category III, Other Mutagenic Mechanisms.

STUDY CLASSIFICATION: The study is unacceptable.

A. MATERIALS

1. Test Material: Dichlormid (R-25788)
   Description: Amber liquid
   Identification Number: WRC 4921-35-11
   Purity: 97.2%
   Receipt date: 1/9/89
   Stability: Stable at <100°C
   Contaminants: None listed
   Solvent used: Corn oil
   Other provided information: The test material was stored at room temperature. Doses of the test material suspended in corn oil were prepared immediately prior to use.

2. Control Materials

   Negative/route of administration: None

   Vehicle/final concentration/route of administration: Corn oil at a 5 mL/kg was administered by oral gavage.

   Positive/final concentration/Route of administration:
   - Dimethylnitrosamine (DMN) was administered in water at 10 mg/kg by oral gavage.
   - Dinitrotoluene (DNT) was administered in corn oil at 125 or 150 mg/kg by oral gavage.
   - 2-Acetylaminofluorene (2-AAF) was administered in corn oil at 50 mg/kg by oral gavage.

3. Test Compound

   Route of administration: Oral gavage

   Dose levels used:

   (a) Range-finding test: 300, 600, 1200, 2500, 5000 mg/kg (3 males/group)

   (b) UDS assay: 350, 700, or 1400 mg/kg

- Trial 1--2 males/group (2-hour sacrifice)
  3 males/group (16-hour sacrifice)

- Trial 2--3 males/group (2 hour-sacrifice)
  2 males/group (16 hour-sacrifice)

4. Test Animals

a. Species rat ; Strain Fischer-344 ; Age >9 weeks (at dosing);
Sex males ; Weight range 155.3 to 282.9 g (at dosing)

Source: Harlan Sprague-Dawley Laboratories, Inc., Frederick, MD

b. Number of animals used per dose: See below

		Number of Male Rats/Trial/Sacrifice Interval			
		Trial 1		Trial 2	
Substance	Dose (mg/kg)	2 hours	16 hours	2 hours	16 hours
Vehicle Control					
Corn oil	--	1	1	1	1
Positive Control					
Dimethylnitrosamine	10	1	-	2	1
Dinitrotoluene	125	-	-	-	1
	150	-	1	-	-
2-Acetylaminofluorene	50	-	-	-	1
Test Material					
Dichlormid	350	2	3	3	2
	700	2	3	3	2
	1400	2	3	3	2

c. Properly maintained? Yes

B. TEST PERFORMANCE

1. UDS Assay

(a) Perfusion techniques/hepatocyte harvest: At 2 ani 16 hours post-treatment, animals in the appropriate groups were anesthesized

with sodium pentobarbital and livers were perfused with an undefined collagenase solution for an unspecified time. Hepatocytes were released with a comb and inoculated into six-well cultures dishes. Each well contained a coverslip and Williams' Medium E (WME), supplemented with 2 mM L-glutamine, 10% fetal bovine serum, and antibiotics. Cultures were placed in a humidified incubator at 37°C and 5% CO_2 for a 1.5- to 2-hour attachment period. Unattached cells were removed; viable cells were refed serum-free WME containing 10 µCi/mL [^{3}H]-thymidine for 4 hours, washed, and reincubated for 14-18 hours in WME containing 0.25 mM unlabeled thymidine.

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

(c) Preparation of autoradiographs/grain development: Slides were coated with Kodak NTB-2 emulsion, stored for 7 days at -20°C, stained with 1% methyl-green Pyronin Y, and coded.

(d) Grain counting: Hepatocytes harvested from animals that were sacrificed at 2 and 16 hours were used to determine UDS. The nuclear grains of at least 30 morphologically normal cells/slide/animal/dose group were counted with an automatic colony counter. Net nuclear grain counts were determined by subtracting the highest cytoplasmic grain count of two nuclear-sized areas adjacent to each nucleus from the nuclear grain counts of each cell. Mean net nuclear grain counts, standard error of the mean, and the percentage of cells in repair were calculated.

(e) Statistical methods: Data for the UDS were not analyzed statistically.

2. Evaluation Criteria

(a) Assay validity: The assay was considered acceptable if (1) the vehicle control data were within historical ranges and (2) if the positive controls showed an increase in net nuclear grain counts and the percentage of cells undergoing repair.

(b) Positive response: The test material was considered positive if the mean net nuclear grain count for any dose was >5 and the percentage of cells in repair was >20%. If mean net nuclear grain counts fell between 0 and 5, the data were assessed relative to dose response, increases in percentage of cells in repair, reproducibility of the results among animals, and the frequency of the cellular responses.

3. Protocol: A protocol was not provided.

C. REPORTED RESULTS

1. Preliminary Range-Finding Assay: Groups of 3 male rats were administered single oral gavage doses of 300, 600, 1200, 2500, or 5000 mg/kg of the test material and were sacrificed 7 days post-treatment. The report indicated that all rats in the high-dose group and 1 rat in the 2500-mg/kg dose group died following dosing on day 0. The remaining animals administered 2500 mg/kg dichlormid died on day 1; no further deaths occurred over the 7-day observation period. Clinical signs seen in the 1200-mg/kg group included uncoordinated motor control and rough fur. No treatment-related effects were reported from the gross examination of rats in the three lowest dose groups (300, 600, 1200 mg/kg). Based on these findings, the median lethal dose was calculated as 1734 mg/kg. The doses selected for the UDS assay, 350, 700, and 1400 mg/kg, therefore, represented 20%, 40%, and 80% of the $LD_{50/7}$, respectively.

2. UDS Assay

(a) Clinical observations: No deaths occurred in the treatment groups. Rats in the high-dose group showed signs of weakness, drowsiness, loss of balance, low body temperature, and rough fur at 2 and 16 hours posttreatment. Drowsiness and rough fur were also reported for the mid-dose animals at 16 hours. No clinical signs of compound toxicity were noted in animals receiving 350 mg/kg of the test material. At the time of perfusion, the livers of mid- and high-dose rats were reported to be mottled.

(b) UDS results: Two independent UDS assays were performed with the selected range of dichlormid doses. Pyknotic cells, indicative of cytotoxicity, were reported in monolayers established from all treatment groups; the effect was most pronounced in the high-dose cultures harvested at 16 hours. Hepatocytes recovered from a total of 5 male rats per test material group sacrificed at 2 hours (2 in the first trial; 3 in the second trial) or 16 hours (3 in the first trial; 2 in the second trial) were scored for UDS activity. Because of the small sample sizes used in both trials, the data were combined and are presented in Table 1. As shown, the total number of animals in the control groups was low; however, the net nuclear grain counts (NG) and the percentage of cells in repair (%IR) were within the historical range published by the reporting laboratory for male Fischer-344 rats administered corn oil (-5.1±0.5 NG; 1±0 %IR).[1] Similarly, the responses induced by the positive controls (10 mg/kg DMN, 125 mg/kg DNT, and 50 mg/kg 2-AAF) were comparable to the results published for these gentoxicants.[2] Our reviewers, therefore, used the historical data to evaluate the activity of the test material.

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[1]Mirsalis, J. C.; Tyson, C. K.; and Butterworth, B. E. Detection of genotoxic carcinogens in the in vivo-in vitro hepatocyte DNA repair assay. Environ. Mutagen. 4(1982):553-562.

[2]Ibid.

TABLE 1. Combined Representative Results of the In Vivo/In Vitro Unscheduled DNA Synthesis (UDS) Assays in Male Rats Dosed with Dichlormid

Substance	Dose (mg/kg)	Exposure Time[a] (hours)	No. of Animals per Group	No. of Hepatocytes Scored	Mean Nuclear Grain Count ± S.E.[b]	Mean Cytoplasmic Grain Count ± S.E.[b]	Net Nuclear Grain Counts ± S.E.[b]	Mean Percent Cells in Repair ± S.E.[b] (Cells with ≥5 Net Nuclear Grains)
Vehicle Control								
Corn oil	--	2	2	180	6.4	13.0	-6.6	0
		16	2	180	6.7	13.7	-7.0	2
Positive Control								
Dimethylnitrosamine[c]	10	2	3	270	37.2±4.7	10.7±0.4	26.5±4.4	93±2
Dinitrotoluene[d]	125	16	1	90	16.4	8.0	8.4	77
2-Acetylaminofluorene	50	16	1	90	29.2	14.7	14.4	88
Test Material								
Dichlormid	1400[e,f]	2	5	450	8.5±0.3	16.0±0.5	-7.5±0.5	2±1
		16	5	450	8.4±0.6	11.6±0.7	-3.2±0.4	3±(5±2)[g]

[a]Time after compound administration
[b]Means and standard error of the counts from 90 cells/animal (30 cells/slide)
[c]Two sacrifice times (2 and 16 hours) were conducted with dimethylnitrosamine; results from the 2-hour cell harvest were selected as representative.
[d]Dinitrotoluene was assayed at two concentrations (125 and 150 mg/kg); results from the lower level were selected as representative.
[e]Results for lower doses (350 and 700 mg/kg) did not suggest a genotoxic effect.
[f]Clinical signs of compound toxicity and mottling of the liver were reported for mid- and high-dose animals.
[g]Recalculated by our reviewers; presented mean was in error; S.E. value was not legible.

The combined results indicated that the single oral gavage administration of 350, 700, or 1400 mg/kg dichlormid did not increase the frequency of UDS in hepatocytes recovered from male rats 2 or 16 hours following treatment.

Based on the overall results, the study authors concluded that dichlormid was not genotoxic in this in vivo/in vitro UDS assay.

C. REVIEWER'S DISCUSSION AND INTERPRETATION OF STUDY RESULTS: We assess, in agreement with the study authors, that dichlormid did not increase the frequency of net nuclear grains in hepatocytes harvested from male rats 2 and 16 hours following dosing. The evidence of overt clinical signs (e.g., weakness, loss of balance, and low body temperature), adverse effects on the target organ (mottled livers), for the 700- or 1400-mg/kg treatment groups, and cytotoxic effects on the hepatocyte cultures (presence of pyknotic cells) indicated that an appropriate range of test material doses was evaluated.

Guidelines do not currently exist for the in vivo/in vitro hepatocyte UDS assay, and we concede that the historical ranges published by the reporting laboratory for negative and positive controls can be used in lieu of an adequate sample size in the concurrent controls. However, the use of male rats only and less than the number of animals recommended by the Guidelines for in vivo genetic toxicology studies (5/sex/dose/ sacrifice time) precludes full acceptance of the results as valid evidence of a negative response in the test system.

D. QUALITY ASSURANCE MEASURES: Was the test performed under GLPs? Yes. (A signed quality assurance statement, dated August 18, 1989, was present).

E. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 12-21.