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

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005368

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Review of 3 mutagenicity studies /MITC

FROM: James N. Rowe, Ph.D. James N. Rowe 8/1/85
Section V
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TO: Winnie Teeters, Ph. D.
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THRU: Laurence D. Chitlik, D.A.B.T.
Section Head
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8/6/85

8/19/86

ACTION REQUESTED: Review 3 mutagenicity studies(unscheduled DNA synthesis/ UDS test in rat hepatocytes , HGPRT locus test in V79 cells and chromosomal cell aberrations in V79 cells) for new use registration of methyl isothiocyanate(MITC) in utility pole fumigation. EPA no. 45639-OE; Caswell no. 573; Accession no . 257758 . Received on 5/1/85.

RECOMMENDATIONS: 1)UDS: Although the test for UDS appears negative, it is classified as unacceptable due to the lack of raw data to enable independent confirmation of the study's findings. Upon submission of this data and acceptance of its adequacy, this test should be redesignated as acceptable. 2)HGPRT locus assay: MITC, under the conditions of the assay, was adjudged to be negative for the induction of mutagenicity on the HGPRT locus. However, the test is considered to be an inconclusive study due to a lack of a comprehensive cytotoxicity /cytogenicity dose-response curve. Additional testing of doses at and above the highest dose used(2.5 ug/ml) or submission of data on hand clarifying this issue is requested. 3) Chromosomal cell aberration: Although there is some suggestion of a solvent control effect on background cell aberrations, this study clearly demonstrated the production of chromosomal aberrations including an excess of chromatid exchanges at the two highest doses of MITC utilized. However, the study is designated inconclusive due to the lack of a complete dose-response curve for cytotoxicity. Additional concerns are listed in the methods section. A repeat study is recommended unless there is additional data available for submission which will clarify our concerns.

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DATA EVALUATION RECORD 005368

A.

1. Study Type: Unscheduled DNA synthesis(UDS) assay in the rat primary hepatocyte

2. Chemical: Methylisothiocyanate(MITC); SN 584.

3. Test Material: technical grade; batch no. 340178; dissolved in dimethylsuloxide(DMSO) and diluted in Williams' Medium E containing 1% fetal bovine

4. Study I.D.:

a. Rat primary hepatocyte UDS assay using fresh hepatocyes harvested from an adult male Fischer 344 rat(Charles River Breeding Laboratories, Inc.)

b. Laboratory: Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895

c. Study #: PF-84.818/LBI Project no. 20991

d. Date of report: February, 1985

e. Study director: Dr. M.A. Cifone

f. Caswell # 573; Accession # 257758 ; EPA # 45639-OE

5. Conclusions: This test for UDS in the rat hepatocyte appears negative for methyl isothiocyanate but is judged to be unacceptable due to the lack of the raw data to allow independent confirmation of the authors findings. Additional information regarding the positive control and reasons for the requirement of three trials is also recommended for submission.

6. Methods: A copy of the methods(photocopied) is attached. The following comments are presented:

a. On page 5 of the report the authors note that the positive control, 2-acetyl aminofluorene, did not have the same activity as observed in previous studies, therefore the concentration was increased in order to maintain the UDS activity in the range of the historical data. Explanation for this comment should be included since this statement could be interpretated as indicating either degradation of the stock chemical used in making the positive control or some change in the test procedure which could possibly diminish the sensitivity of the assay to detect UDS. Protocol no. 447 of Litton Bionetics states that typical responses to treatments with 0.05 ug/ml of 2-AAF produces: 16+ 7 mean net nuclear grain counts, 87 + 17% of the nuclei having 6 or more net grains, and 31+ 23% of the nuclei having 20 or more net grains--an effect not produced at twice the dose in this assay(see table below).

b. The author also reports on page 1 that three trials of the UDS assay were performed , but Trials 1 and 2 were terminated because of technical difficulties. No mention of the reasons for the additional trials were stated, this could possibly be due to problems in harvesting viable rat hepatocytes. Clarification of the need for repeating of the assay should be included.

c. No individual data were presented.

7. Results: A summary table is presented below:

Treatment	Concentration	UDS*grains/ nucleus	Avg.¶ % nuclei (> 6grains)	Avg.¶% nuclei (>20grains)	Survival† at 20.5 hrs (%)
Solvent Control: DMSO	1 %	0.72	0.7	0.0	100.0
Positive Control: 2-AAF	0.1ug/ml	7.06	48.7	4.7	109.5
Test Material					
	30.3 ug/ml	Excessive toxicity; grain analysis not possible			0.0
	15.2 ug/ml	0.95	1.3	0.0	39.7
	10.1 ug/ml	1.82	8.0	0.0	66.7
	5.05 ug/ml	1.27	1.3	0.0	79.6
	2.53 ug/ml	1.43	4.0	0.0	88.1
	1.01 ug/ml	1.66	2.7	0.0	97.1
	0.505 ug/ml	1.42	1.3	0.0	101.0
	0.253 ug/ml	1.32	1.3	0.0	ND

*UDS= Average on net nuclear grain counts on triplicate coverslips(150 total cells)
¶ Average values for triplicate coverslips
†Survival= number of viable cells per unit area relative to the solvent control x 100%
ND= not determined
Mean cytoplasm grain count for the negative control= 2.28

The authors reported the metabolic condition of the harvested hepatocytes to be good with 87% viability(as determined by trypan blue exclusion) and about 95% viable cells attaching to the culture dishes during the 1.5 hour settling period. The treatments were initiated approximately 2.5 hours later with cell monolayers having about 93% viability. After an additional 20.5 hours in culture (encompassing the 18-hour treatment period), the average viable cell count was reported as 99% of the viable count at the beginning of the treatments.

The summary data table indicated a dose-related toxicity with 0.0% survival at the highest dose used(30.3 ug/ml) and 40, 67, 80, 88, 97 and 101% survival of hepatocytes at concentrations of 15.2, 10.1, 5.05, 2.53, 1.01, and 0.505 respectively. Examination of the UDS grains/nucleus, average % nuclei with >= 6

grains and average % nuclei with >= 20 grains using the assay evaluation criteria cited in the methods section(p.11) showed an active response for the positive control (7.06, 48.7, and 4.7, respectively) but was not active for concentrations of 5.05 or less(based on a criteria of >=70% survival rate for the monolayer cell culture; p.10 of methods). No dose-response effect was noted either.

8. Discussion: This assay for unscheduled DNA synthesis in rat hepatocytes appears to be negative for methyl isothiocyanate but it is not possible to confirm the authors' findings since no raw data were submitted, including standard deviations, to allow independent analysis. Inclusion of SDs would allow assessment of the response to be judged by an additional criteria. Probst et al., 1981(Envir. Mut. 3: 11-32) judges a compound to have induced a positive response for UDS when at least two successive concentrations produced nuclear grain counts which exceeded those of the control by at least two standard deviations of the control value. Further, some discrepencies were noted in regards to the positive control and its potential effect on the sensitivity of the hepatocyte assay, and explanation of the reasons for the assay having to be repeated three times(see methods section).

B.

1. Study Type: Hypoxanthine-Guanine Phosphoribosyl Transferase Locus in V79 Cells(HGPRT-test)

2. Chemical: Methylisothiocyanate(MITC); SN-584.

3. Test Material: technical grade; batch no. 340178; ZK 3.318; analysis no. X 7047 Schering AG, D-1000 65 ; stability of the test substance in solution reported as unknown; dissolved in dimethy sulfoxide(DMSO) and diluted in MEM-medium supplemented with 10% fetal calf serum= FCS(Gibco Europe, D-7500 Karlsruhe, F.R.G.)

4. Study I.D.:

a. HGPRT-test using cell cultures of Chinese hamster cell line V79

b. Laboratory: Laboratorium für Mutagenitatsprufung Technische Hochschule Darmstad- LMP DARMSTADT-; Schnittspahnstr. 3, D-6100 Darmstadt,F.R.G.

c. Study #: LMP 075A

d. Date of report: November 30, 1984

e. Study director: Prof. Dr. H.G. Miltenburger

f. Caswell # 573; Accession # 257758 ; EPA # 45639-OE

5. Conclusions: Under the conditions of this test system, methyl isothiocyanate was negative for the production of mutants at the HGPRT-locus in the V79 Chinese hamster lung cell line as judged by the criteria of either a three fold increase in the mutation rate over the control values or in terms of a dose-response effect. However, this is considered to be an inconclusive test for gene mutation since the cytotoxicity/cytogenicity dose-response curve was not adequately delineated nor was the rationale for excluding a high dose(2.5 ug/ml) treatment group, without the microsomal fraction, presented. Thus, this is not a comprehensive assay.

6. Methods: A copy(photocopied) of the experimental procedures is attached. In general, the methods appear to be acceptable. However, some comment is appropriate:

1. No reason is given for the use of only - S9 mix in the low dose(0.1 ug) or only + S9 mix in the high dose(2.5 ug).

2. The treatment doses should have been extended "up" to around 90-95% cytotoxicity in order to adequately delineate the cytotoxic /cytogenetic effects.

7. Results: A summary of test results is presented below:

Treatment	Conc./ml	S9 mix	PE%(absolute)* Expt 1/Expt 2	PE%(relative)† Expt 1/Expt 2	Mutant colonies¶ per 10^6 cells Expt 1/ Expt 2
negative control	0.00 ug	-	90.2/104.1	100.0/100.0	15.7/27.4
	0.00 ug	+	84.1/ 88.7	100.0/100.0	7.5/19.9
neg.con./ solvent	0.00 ug	-	83.7/ 82.6	100.0/100.0	6.4/20.0
neg. con./ solvent	0.00 ug	+	70.6/ 88.0	100.0/100.0	7.4/25.1
positive con./EMS	1.00 ug	-	58.4/ 69.9	64.7/ 67.2	527.9/538.6
positive con./DMBA	15.40 ug	+	1.0/ 7.9	1.5/ 9.0	265.6/298.5
test cmpd.	0.10 ug	-	83.6/ 85.4	99.9/103.4	10.6/39.9
"	0.25 ug	+	65.4/ 83.2	92.6/ 94.6	6.5/43.4
"	0.25 ug	-	81.1/ 81.7	96.9/ 98.9	13.1/18.5
"	0.50 ug	+	74.9/ 82.5	106.1/ 93.8	6.1/42.2
"	0.50 ug	-	50.0/ 60.9	59.8/ 73.8	10.5/16.5
"	1.00 ug	+	45.9/ 55.2	65.0/ 62.7	4.1/13.6
"	1.00 ug	-	22.4/ 36.1	26.7/ 43.8	14.7/30.9
"	2.50 ug	+	17.7/ 33.1	25.0/ 37.6	5.6/27.0

* Plating effiency(mean # of cells/flask divided by # of cells seeded)
† Plating effiency(PE% absolute divided by corresponding control)
¶ Only colonies with more than 50 cells 7 days after seeding in thioguanine(TG) medium were scored; number of mutant colonies divided by the total number of cells surviving after plating in TG medium
EMS= ethylmethanesulfonate, DMBA=9,10-dimethyl-1,2-benzanthracene

A dose-related toxicity(decrease in plating efficiency) was established in two experiments with concentrations of 0.1, 0.25, 0.5, 1.0 and 2.5 ug/ml of MIC with the PE% relative ranging from 99.0 down to 25% and 103 down to 38% for experiments 1 and 2 , respectively(+/- S9 mix).

The positive controls (EMS, DMBA) produced significant cell toxicity at the doses used(1.0 and 15.4 ug,resp) as well as a significant mutation rate in the V79 cells(528,539/10^6 cells for EMS in experiments 1 and 2, resp.; 266, 299/10^6 cells in experiments 1 and 2, resp.) indicating the responsiveness of the assay system to known mutagens. No significant increase in the mutation rate was evident in any treatment group as judged by the criteria of a three-fold increase over the control values(negative control with of without solvent) or in terms of a dose-related increase in mutants for either experiment.

8. Discussion: Treatment of V79 Chinese hamster cell cultures with MITC at concentrations eliciting from approximately 75-62% reduction in plating efficiency (2.5 ug/ml) to no effect, 0% reduction in plating efficiency (0.1 ug/ml) as compared to the negative control(solvent), produced no increase in the mutation rate as assayed by the HGPRT locus test. No rationale was given by the authors for not testing the high dose (2.5 ug/ml) without the microsomal fraction. In addition, and more importantly, the relationship between cytotoxicity and cytogenicity was not adequately explored. Thus, the assay does not comprehensively evaluate the potential for the production of chromosomal aberrations.

C.

1. Study Type: In vitro chromosome aberration test in Chinese hamster V79 lung cells

2. Chemical: Methylisothiocyanate(MITC); SN-584.

3. Test Material: technical grade; batch no. 340178; ZK 3.318; analysis no. X 7047 Schering AG, D-1000 Berlin 65 ; dissolved in dimethy sulfoxide(DMSO) and diluted in MEM medium supplemented with 10% fetal calf serum= FCS (Gibco Europe, D-7500 Karlsruh F.R.G.)

4. Study I.D.:

a. Chromosomal aberrations in Chinese hamster lung cell line V79.

b. Laboratory: Laboratorium für Mutagenitatsprufung Technische Hochschule Darmstad- LMP DARMSTADT-; Schnittspahnstr. 3, D-6100 Darmstadt,F.R.G.

c. Study #: LMP 075B

d. Date of report: November 30, 1984

e. Study director: Prof. Dr. H.G. Miltenburger

f. Caswell # 573; Accession # 257758 ; EPA # 45639-OE

5. Conclusions: Although there is some suggestion of a solvent effect on background cell aberrations, this study clearly demonstrates the production of chromosomal aberrations including chromatid exchanges at the two highest doses of MITC utilized(1.00, 2.50 ug/ml). However, the study is designated as inconclusive due to lack of a comprehensive dose-response curve for cytotoxicity. Additional concerns are listed in the Methods section. A repeat study is recommended unless data can be submitted to resolve these issues.

6. Methods: A copy of the methods(photocopied) is attached. The following comments are noted:

1. No statistical analysis of the data was performed.

2. The raw data for plating efficiency for the doses tested for cell toxicity in the cytotoxicity range-finding experiment were not included. Additional doses should be used to completely delineate the dose-response curve in terms of cell toxicity and genotoxicity. Data on the solvent control should also be included.

3. Some explanation should be provided for the rationale why ± S9 mix was not used for all doses tested as well as why all doses were not examined at all observation time periods of 6, 12 and 28 hours.

4. The number of aberrations per cell can be considered significant in this type of mutagenicity assay(Hayes, A.W.,Principles and Methods of Toxicology; 1982, p.251). If available, this data should be submitted.

5. It is difficult to understand how the mitotic indices could not be

determined at 1.00 and 2.5 ug/ml doses due to nuclear disintegrations but the chromosomal aberrations can still be scored. Some further explanation would seem to be appropriate.

7. Results: A summary table of the studys' findings abstracted by the reviewer from tables I-XX, and XXIV is presented below:

Treatment per ml	S9 mix	6 hrs. after treatmt initd*		12 hrs. after treatmt initd*		28 hrs. after treatmt initd*		Mitotic Index(%)
		incl. gaps	excl. gaps	incl. gaps	excl. gaps	incl. gaps	excl. gaps	
Neg.cont. (0.0ug)	-	-	-	11	4(0)	-	-	5.5
Neg.cont. (0.0ug)	+	-	-	13	3(0)	-	-	4.3
Solv.cont. (0.0ug)	-	9	2(0)†	-	-	-	-	6.2
	-	-	-	7	1(0)	-	-	9.2
	-	-	-	-	-	4	0(0)	10.6
Solv.cont. (0.0ug)	+	20	6(0)	-	-	-	-	4.8
	+	-	-	15	3(0)	-	-	4.2
	+	-	-	-	-	11	6(1)	12.4
Pos.cont. EMS/3.72mg	-	-	-	40	20(5)	-	-	2.4
Pos.cont. CPA/2.62mg	+	-	-	84	54(11)	-	-	0.7
Test substance								
0.10 ug	-	-	-	7	3(1)	-	-	7.5
0.25 ug	+	-	-	15	6(0)	-	-	6.2
0.50 ug	-	-	-	10	2(0)	-	-	6.7
0.75 ug	+	-	-	15	6(0)	-	-	5.2
1.00 ug	-	14	3(0)	-	-	-	-	2.3
1.00 ug	-	-	-	17	5(0)	-	-	7.2
1.00 ug	-	-	-	-	-	30	21(8)	-**
2.50 ug	+	18	12(0)	-	-	-	-	2.1
2.50 ug	+	-	-	20	8(1)	-	-	3.2
2.50 ug	+	-	-	-	-	26	16(9)	-**

* Total of 4 flasks(100 cells/flask) examined = 400 cells; incl. gaps= all aberrations, excl. gaps= minus chromosomal gaps

** Reported not scorable because of nuclear disintegrations induced by the test substance

† Number of exchanges per treatment

EMS= ethyl methanesulfonate, CPA= cyclophosphamide

In a range-finding test(not presented in table) a dose-dependent cytotoxicity was reported by the authors with media concentrations of 0.25, 0.50, 1.00, 5.00 and 10.00 ug/ml producing 104, 78, 35, 0 and 0 %(without S9 mix) and 80, 86, 63, 0, and 0%(with S9 mix) of control cell survival(1% DMSO). Based on these results the authors selected the doses presented in the table. The doses selected appear to be acceptable for demonstrating both cytotoxicity and potential chromosomal aberrations. It is unclear to the reviewer why only the two highest doses were examined at all incubation time periods or the rationale for testing the concentrations, 0.1, 0.50, and 1.00 ug/ml, without the S9 mix or 0.25, 0.75, and 2.5 ug/ml with the S9 mix only.

The positive controls (EMS, CPA) demonstrated the responsiveness of the test system to the induction of chromosomal aberrations producing 40(5 chromatid exchanges) and 84(11 chromatid exchanges) changes, respectively, as well as a significant effect on the mitotic index(2.4 and 0.7% respectively versus 5.5 and 4.3 % in negative control. The mitotic index was also decreased at 1.00 and 2.5 ug/ml(2.3, 7.2 and 2.1, 3.2%) at 6 and 12 hours as compared to the solvent controls (6.2, 9.2 , 10.6/-S9 mix and 4.8, 4.2, 12.4/+S9 mix) at 6, 12, 28 hours. No effects were reported at the 28 hour period due to nuclear disintegrations of the cells as a result of exposure to the test substance.

There is a strong suggestion that the solvent controls (+ S9 mix) may be different in their effect on chromosomal aberrations in the V79 cell line as indicated by the total number of chromosomal aberrations produced during the test assay ,i.e., 20, 15, 11 (+) vs 9, 7, 4(-) at 6, 12, and 28 hours of treatment , respectively. The report does not indicate whether or not the solvent control affects the cytotoxicity values. No statistical analysis was performed. The impact of this effect on the study findings is unclear.

The authors report the negative control for cell with aberrations to range from 0 to 2.5%. The negative controls in the assay were 2.75%(- S9 mix) and 3.25% (+ S9 mix), and the solvent controls (DMSO) were 1-2.25%(- S9 mix) and 2.75-5%(+ S9 mix) at various times during the assay.

At 6 hours after treatment is initiated there is a elevation in total aberrations in the 1.00 ug/ml treatment group as compared to the solvent control (-S9 mix)(14 vs 9, resp.), while the higher dosage (2.50 ug/ml) does not differ significantly in terms of total aberrations with the solvent control(+ S9 mix) (18 vs 20, resp.), but has a higher number of effects if the chromosomal gaps are excluded(12 vs 6, resp.).

At 12 hours after treatment initiation, doses above 0.25 ug/ml appear to be increased over the solvent controls(+ S9 mix)--see table.

By 28 hours after treatment is initiated, there is a clear increase over control values in the number of cell aberrations and particularly chromatid exchanges with incidences higher or similar(for exchanges) than the 12 hour positive controls , i.e., 4 total aberrations /0 exchanges in solvent controls (-S9 mix) versus 30 total aberrations /8 exchanges in the 1.00 ug/ml dose and 11 total aberrations/1 exchange in solvent control(+S9 mix) versus 26 total aberrations /9 chromatid exchanges in the 2.50 ug/ml dose.

On a percentage basis(solvent control divided by the treatment group aberrations), the solvent control(- S9 mix) was consistently less for total chromosomal aberrations than the 1.00 ug/ml dose(64, 41, 13%) at the consecutive

time periods as opposed to the solvent control(+ S9 mix) which was 111, 75, and 42% of the high dose group for the consecutive time periods. Again, this suggests that the DMSO is having some effect on cell chromosomal aberration incidences when incubated in the presence of S9 mix.

8. Discussion: Although there is is a strong suggestion that the solvent control containing DMSO increased the number of aberrations in the V79 cell line when incubated in the presence of rat liver homogenate, there is clear evidence, at the two highest doses utilized, that MITC is producing a significant increase in the total number of chromosomal aberrations, including and excluding cell gaps, and in the number of chromatid exchanges as compared to the solvent controls(± S9 mix) and in reference to the positive controls(EMS, CPA). This study is classified as inconclusive based on the need for submission of additional data(outlined in the methods section) to clarify justification for the doses utilized in the study and the rationale for the treatment groups(± S9 mix). A repeat study is recommended if the requested data is not available

Methyl isothiocyanate science review

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Pages 12 through 36 are not included in this copy.

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