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DATA EVALUATION RECORD

PYRIDATE

Mutagenicity--*In vitro* Cytogenetic Study with Chinese Hamster Ovary Cells

STUDY IDENTIFICATION: Taalman, R. D. F. M., and Hoorn, A. J. W. *In vitro* chromosome assay with Pyridate technical. (Unpublished study No. E-9550 prepared by Hazleton Biotechnologies, Veenendaal, The Netherlands, for Chemie Linz, A.G., Linz, Austria; dated January 7, 1987.) MRID No. 401865-02.

APPROVED BY:

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Date: 8-14-87

1. CHEMICAL: Pyridate.

2. TEST MATERIAL: Pyridate technical, from batch No. 2556520, was described as a brown, oily liquid; the purity was not reported.

3. STUDY/ACTION TYPE: Mutagenicity--In vitro cytogenetic study with Chinese hamster ovary cells.

4. STUDY IDENTIFICATION: Taalman, R. D. F. M., and Hoorn, A. J. W. In vitro chromosome assay with Pyridate technical. (Unpublished study No. E-9550 prepared by Hazleton Biotechnologies, Veenendaal, The Netherlands, for Chemie Linz, A.G., Linz, Austria; dated January 7, 1987.) MRID No. 401865-02.

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7. CONCLUSIONS:

A. Pyridate technical, assayed without metabolic activation over a dose range of 5 to 100 µg/mL and with S9 activation over a dose range of 10 to 250 µg/mL induced cytotoxic effects in Chinese hamster ovary (CHO) cells, but was not clastogenic.

B. The study is acceptable; pyridate is not clastogenic.

Items 8 through 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

A. Materials and Methods: (See Appendix A for details.)

1. Test Material: Pyridate technical, from batch No. 2556520, was described as a brown, oily liquid; the purity was not reported. The test material was stored at room temperature in the dark and was dissolved in dimethylsulfoxide (DMSO).

2. Cell Line: The Chinese hamster ovary cells (CHO-WBL) used in this assay were originally obtained from Dr. Sheldon Wolff, University of California, San Francisco, CA. The CHO cells were grown in McCoy's 5a, supplemented with 10% fetal calf serum (FCS), L-glutamine, and antibiotics, for 24 hours prior to use.

3. S9 Fraction: The S9 fraction was obtained from an unspecified commercial source, and was derived from the livers of male rats (strain not reported) induced with Aroclor 1254. The S9-reaction mixture contained 15 µL/mL rat liver S9.

4. Preliminary Cytotoxicity Assay: Prepared cell cultures, seeded at 0.5×10^6 cells/flask, were exposed to half-log dilutions of the test material at doses ranging from 0.03 to 1000 µg/mL, the solvent control (DMSO), or the positive controls [mitomycin C (MMC) at 0.4 and 0.5 µg/mL/-S9 and cyclophosphamide (CP) at 20 and 25 µg/mL/+S9] with or without S9 activation.

In the nonactivated system, cells were exposed for 2 hours to the test material; BrdU (10 µM) was added to the cultures, and incubation was continued for 22.5 hours. Cell monolayers were washed, refed with fresh, complete medium containing BrdU, and reincubated in the presence of 0.1 µg/mL colcemid for 2.5 hours. In the S9-activated system, cultures were exposed for 2 hours without FCS. After exposure, cells were washed twice, refed with complete medium containing BrdU

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[1]Only items appropriate to this DER have been included.

(10 µM), and reincubated for 22.5 hours. Colcemid was added, and cultures were incubated for an additional 2.5 hours.

After incubation, monolayers were visually evaluated for confluency, and metaphase cells were collected by mitotic shake-off. Cells were swollen in a hypotonic 0.075 M solution of KCl and washed three times in fixative (methanol: acetic acid, 3:1), and slides were prepared. Estimation of cell-cycle delay was accomplished by staining the cells with the modified fluorescent-plus-Giemsa techniques of Perry and Wolff[2] and Goto et al.[3] One hundred metaphase cells per culture were examined for the percentage of first (M_1), between first and second (M_1+), and second (M_2) division metaphase cells.

5. Cytogenetic Assay:

   a. Treatment: Prepared cultures (in duplicate), seeded at 0.8×10^6 cells, were exposed to the selected test material doses, the solvent (DMSO), or the positive controls, MMC (0.5 and 1.0 µg/mL) without S9 activation or CP (25 and 50 µg/mL) with S9 activation.

      In the nonactivated system, cells were dosed for 9 hours. Cultures were washed, refed medium containing colcemid, and reincubated for approximately 2.75 hours. Under S9-activated conditions, cells were exposed for 2 hours, washed, refed culture medium, and either incubated for 10 hours (normal harvest time) or 17.5 hours (delayed harvest time). Colcemid was added 2.5 to 2.75 hours before the cultures were harvested.

      Metaphase cells were collected and fixed. Slides were stained and coded.

   b. Metaphase Analysis: One hundred morphologically normal cells (containing 19 to 23 centromeres) per culture from at least four levels of the test material and the negative and solvent control were scored for chromosome aberrations. One hundred cells were scored for each level of the positive controls.

6. Statistical Methods: The data were evaluated for statistical significance by chi-square analysis. The results for negative (culture medium) and solvent controls were pooled.

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[2]Perry, P. and Wolff, S. New Giemsa method for the differential staining of sister chromatids. Nature (1974) 251: 156-158.

[3]Goto, K., Maeda, S., Kano, Y., and Sugiyama, T. Factors involved in differential Giemsa-staining of sister chromatids. Chromosoma (1978) 66: 351-359.

7. Evaluation Criteria: The biological significance of the results was evaluated relative to the overall chromosome aberration frequencies, percentage of cells with aberrations, percentage of cells with >1 aberration, dose response, and the types of aberrations observed. The results were considered significant ($p < 0.05$) when a 1 to 5 percent increase in aberrant cells, as compared to the pooled negative and solvent control values, was observed.

B. Protocol: A protocol was not present; however, a protocol number (E-437, Ed 1) and a set of deviations were provided (CBI p. 19).

12. REPORTED RESULTS:

A. Preliminary Cytotoxicity Assay:

The cytotoxicity assay was conducted with test doses ranging from 0.03 to 1000 µg/mL, separated by half-log dilutions, in the presence or absence of S9 activation.

1. Without S9 Activation: The report stated that no cells survived treatment at doses ≥100 µg/mL. Results presented for 1.0, 3.3, 10.0, and 33.3 µg/mL indicated monolayer confluency was ≥70%; however, a slight delay in cell-cycle progression was apparent at 33.3 µg/mL (Table 1). No interference of cell-cycle kinetics was seen at the lower doses. Based on these results, the nonactivated assay was performed with doses ranging from 0.5 to 100 µg/mL with a normal 10-hour cell harvest.

2. With S9 Activation: The test material caused a total loss of monolayer confluency, i.e., 100% cytotoxicity, at 1000 and 333.3 µg/mL. A 90% reduction in monolayer confluency and severe cell-cycle delay were noted at 100 µg/mL. Below this level, monolayer confluency and progression through the cell cycle were essentially normal (Table 1). Based on these data, 100 and 250 µg/mL were evaluated with a delayed cell harvest at 17.5 hours; lower doses (1-50 µg/mL) were assayed using a normal 10-hour harvest interval.

B. Cytogenetic Assay:

1. Nonactivated Test Material: No cells survived the highest dose (100 µg/mL) of test material. At 50 µg/mL, cell cycle delay and reduced monolayer confluency were also observed. Based on these results, metaphase cells from the 5-, 10-, 25-, and 50-µg/mL dose groups were analyzed for chromosome aberrations. No increase in the number of cells with aberrations, percent cells with aberrations, or percent cells with >1 aberration was seen at the four analyzed doses. By contrast, both concentrations of the positive control (MMS, 0.5 and 1 µg/mL) caused marked increases in all parameters. Representative results are presented in Table 2.

TABLE 1. Representative Results from the Preliminary Test for Delay of Cell-Cycle Progression with Pyridate

Substance	Dose/mL	S9 Activation	% Cells[a] M_1	% Cells[a] M_1+	% Cells[a] M_2	Monolayer Confluency (%)
Negative Control						
Culture Medium	-	-	1	2	97	90
	-	+	1	2	97	95
Solvent Control						
Dimethylsulfoxide	1%	-	7	6	87	85
	1%	+	1	1	98	90
Positive Control						
Mitomycin C	0.5 µg	-	100	0	0	70
Cyclophosphamide	20.0 µg	+	100	0	0	85
Test Material						
Pyridate	33.3 µg[b]	-	35	12	53	70
	100 µg[c]	+	70	8	22	10

[a]Percent cells in first (M_1), between first and second (M_1+), or second (M_2) divisions.

[b]Highest nonactivated dose scored; doses ≥100 µg/mL were completely cytotoxic. Results for lower doses (1.0, 3.3, and 10.0 µg/mL) were comparable to the negative and solvent control values.

[c]Highest S9-activated dose scored; doses ≥333.3 µg/mL were completely cytotoxic. Results for lower doses (3.3, 10.0, and 33.3 µg/mL) were comparable to the negative and solvent control values.

TABLE 2. Representative Results from Duplicate Cultures of the CHO Cell *in vitro* Cytogenetic Assay with Pyridate

Substance	Dose (µg/mL)	S9 Activation	No. of Cells Scored	No. of Aberrations per Cell	% Cells with Aberrations	% Cells with >1 Aberration
Pooled Negative Control						
Medium and Dimethylsulfoxide	-	-	200	0.005	0.5	0.0
		+	200	0.010	1.0	0.0
Positive Control						
Mitomycin C	0.5	-	100	0.510	33.0*	14.0
	1.0	-	100	>0.760	46.0*	16.0
Cyclophosphamide	25.0	+	100	>0.730	37.0*	18.0
	50.0	+	100	>1.000	53.0*	23.0
Test Material						
Pyridate	50.0[a]	-	200	0.005	0.5	0.0
	50.0[b]	+	200	0.005	0.5	0.0
	100.0[c]	+	200	0.005	0.5	0.0

[a]Highest dose scored; cytotoxicity evident at this level and 100% cytotoxicity at 100 µg/mL. Results for lower doses (5, 10, and 25 µg/mL) were comparable to the pooled negative control values. Cells were harvested ~12 hours post-treatment.

[b]Cell harvest at 10 hours; lower levels (10 and 25 µg/mL) were comparable to the pooled negative control.

[c]Delayed cell harvest at 17.5 hours; cytotoxicity apparent at this level and 100% cytotoxicity at 250 µg/mL.

*Significantly higher ($p < 0.05$) than the pooled negative control values as determined by chi-square analysis.

2. S9-Activated Test Material: At the highest concentration (250 µg/mL), the test material was 100% cytotoxic. Cytotoxicity, as indicated by a decrease in mitotic cells, was also observed at 100 µg/mL. Cells exposed to 10, 25, 50, and 100 µg/mL were analyzed. The results show that exposure to 10, 25, and 50 µg/mL followed by a normal cell harvest and, in a delayed harvest protocol, to 100 µg/mL did not increase the frequency of aberrations in CHO cells. Marked increases in the number of cells with aberrations, percent cells with aberrations, and percent cells with >1 aberration were, however, scored for both concentrations of the S9-activated positive control (25 and 50 µg/mL CP). Representative results are presented in Table 2.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The study authors concluded, "The test article, Pyridate technical, was considered negative for chromosomal aberration induction in Chinese hamster ovary cells both under the activation and nonactivation conditions of this assay, according to our evaluation criteria."

B. A quality assurance statement was signed and dated December 15, 1986.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

We assess that the study was properly conducted and that the authors interpreted the data correctly. Pyridate was assayed both in the presence and absence of S9 activation to a cytotoxic level with no evidence of a clastogenic effect. It was demonstrated that the test system could adequately detect clastogenicity either with or without metabolic activation using appropriate positive controls, cyclophosphamide or mitomycin C, respectively.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 2-6; Protocol deviations, CBI p. 19.