10-9-92

DOC 930147
FINAL

DATA EVALUATION REPORT

D-NC 302 (ASSURE)

Study Type: Mutagenicity: Gene Mutation in Cultured Mammalian Cells (Mouse Lymphoma Cells)

Prepared for:

Health Effects Division
Office of Pesticide Programs
Environmental Protection Agency
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Work Assignment Number: 1-118
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GUIDELINE SERIES 84: MUTAGENICITY
MAMMALIAN CELLS IN CULTURE GENE MUTATIONS

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Toxicology Branch II/HED (H-7509C)
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EPA Review Section (II), Date: 10-14-92
Toxicology Branch II/HED (H-7509C)

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: Gene mutation in cultured mammalian cells (mouse lymphoma cells)

EPA IDENTIFICATION Numbers:

Tox Chem. Number:

MRID Number: 419366-01

TEST MATERIAL: D-NC 320 technical

SYNONYM: Assure

SPONSOR: Nissan Chemical Industries, Ltd., Tokyo, Japan

STUDY NUMBER: 18

TESTING FACILITY: IIT Research Institute, Chicago, IL

TITLE OF REPORT: Mouse Lymphoma Assay for Forward Mutation of D-NC 302

AUTHOR: Ketels, K.V.

REPORT ISSUED: Amended final report: June 1991

CONCLUSIONS-EXECUTIVE SUMMARY: D-NC 320 in either the presence or absence of S9 activation did not induce a mutagenic response in mouse lymphoma cells over a concentration range of 5 to 1000 µg/mL -S9 or 20 to 500 µg/mL +S9; severe cytotoxicity (i.e., <10% survival) was achieved at 1000 µg/mL -S9 and 500 µg/mL +S9. However, the high background mutation frequencies (MF) for the nonactivated solvent control (204×10^{-6}) and S9-activated solvent control (137×10^{-6}) casts doubts on the ability of the test system to detect a potential weak mutagenic response induced by an unknown test material (see Section D, Reviewers' Discussion and Interpretation of Results). We conclude, therefore, that the study is unacceptable.

STUDY CLASSIFICATION: Unacceptable. The study does not satisfy Guideline requirements (§84-2a) for genetic effects, Category I, Gene Mutations.

A. MATERIALS:

1. Test Material: D-NC 302 technical

Description: Fine, light-brown powder
Identification Number: Lot number 302 DT 8501
Purity: 98.1%
Receipt date: May 23, 1986
Stability: Not reported
Contaminants: None listed
Solvent used: Dimethyl sulfoxide (DMSO)
Other provided information: The test material was stored in the dark at 4°C. Prior to use, test material solutions were prepared with the aid of a vortex mixer.

2. Control Materials:

Negative: Untreated cells grown in Fischer's medium containing 10% horse serum (F_{10P}).

Note: The negative control was included in the preliminary cytotoxicity test and the cytotoxicity phase of the mutation assay only.

Solvent/final concentration: Dimethyl sulfoxide (DMSO) at final concentrations of 10% (preliminary cytotoxicity test) and 1% (preliminary cytotoxicity and mutation assays).

Positive: Nonactivation (concentrations, solvent): Hycanthone was prepared in saline to yield a final concentration of 10 µg/mL.

Activation (concentrations, solvent): 2-Acetylaminofluorene (2-AAF) was prepared in DMSO to yield a final concentration of 100 µg/mL.

3. Activation: S9 derived from

x Aroclor 1254	x induced	x rat[a]	x liver
_____ phenobarbital	_____ noninduced	_____ mouse	_____ lung
_____ none		_____ hamster	_____ other
_____ other		_____ other	

[a]Sex and strain not specified

The S9 liver homogenate was obtained from MA Bioproducts, Walkerville, MD; no further information was provided.

S9 mix composition:

Component	Concentration
NADP	8000 µg/mL
Isocitric acid	15,000 µg/mL
S9 homogenate	33%

4. Test Cells: Mammalian cells in culture

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

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

5. Locus Examined:

__x__ thymidine kinase (TK)
Selection agent: _____ bromodeoxyuridine (BrdU)
(give concentration) _____ fluorodeoxyuridine (FdU)
1 µg/mL trifluorothymidine (TFT)

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

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

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

6. Test Compound Concentrations Used:

(a) Cytotoxicity assay: Twelve doses (2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000, and 10,000 µg/mL) were evaluated in the presence and absence of S9 activation.

(b) Mutation assay:

(1) Nonactivated conditions: Thirteen doses (1, 2, 5, 10, 20, 50, 100, 200, 400, 600, 800, 1000, and 1500 µg/mL) were assayed; cells treated with 5, 100, 400, 800, and 1000 µg/mL were cloned.

(2) S9-activated conditions: Thirteen doses (1, 2, 5, 10, 20, 50, 100, 200, 300, 350, 400, 450, and 500 µg/mL) were assayed; cells treated with 20, 200, 350, 400, and 500 µg/mL were cloned.

B. TEST PERFORMANCE:

1. Cell Treatments:

(a) Cells were exposed to test compound or negative, solvent, or positive control
__4__ hours (nonactivated) __4__ hours (activated)

(b) After washing, cells were cultured for 2 days (expression period) before cell selection

(c) After expression, $3x10^5$ cells/mL were cultured for 11 days in selection medium to determine numbers of mutants and 6 cells/mL were cultured for 11 days without selection medium to determine cloning efficiency.

2. Statistical Methods: The data were not evaluated for statistical significance.

3. Evaluation Criteria:

(a) Assay validity: For the assay to be considered valid, the following criteria must be satisfied: (1) the daily suspension growth of untreated cells must increase by ≥4-fold; (2) the relative suspension growth (RSG) and the cloning efficiency (CE) of the solvent control must exceed 70% of the untreated cells; (3) the mutation frequency (MF) for untreated and solvent-treated cells should fall within the range of $15\text{-}150x10^{-6}$; and (4) the MF of the positive controls must be ≥3-fold higher than the corresponding solvent control value.

b. Positive response: The test material was considered positive if it induced an increase in the MF that exceeded 2 times the MF of the solvent control at one or more doses with ≥10% total survival.

4. Protocol: See Appendix A.

C. REPORTED RESULTS:

1. Cytotoxicity Assays: C-NC 302 was soluble in DMSO at 10,000 µg/mL; however, all but the "very low doses" precipitated upon addition to culture medium. Results from the cytotoxicity test indicated that <10% of the cells survived exposure to the three highest nonactivated concentrations (2000, 5000, and 10,000 µg/mL). At the remaining levels, RSG was generally dose related and ranged from 11.0% at 1000 µg/mL to ≥66.5% at ≤500 µg/mL. Accordingly, 1500 µg/mL was selected as the high dose for the nonactivated phase of the mutation assay. In the presence of S9 activation, D-NC 302 was more cytotoxic, as indicated by the ≤2.4% RSP at test material levels ≥500 µg/mL. Below 500 µg/mL, RSG was ≥84.7%. Based on these findings, 500 µg/mL was chosen as the starting concentration for the S9-activated phase of testing.

2. Mutation Assays: Summarized results from the nonactivated and S9-activated mutation assay (Table 1) show that the MF for the nonactivated solvent control ($204x10^{-6}$) was outside of the acceptable range ($15\text{-}150x10^{-6}$) of both the reporting laboratory and the quality

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TABLE 1. Representative Results of the Mouse Lymphoma Forward Mutation Assay with D-NC 302

Substance	Dose/mL	S9 Activation	Percent Relative Suspension Growth	Mutant Colonies[a] ±S.D.	Viable Colonies[a,b] ±S.D. x10^4	Percent Relative Cloning Efficiency[a,b]	Mutation Frequency x10^{-6}[b,c]
Negative Control							
Untreated cells	--	-	89.6	ND	ND	ND	--
	--	+	81.7	ND	ND	ND	--
Solvent Control							
Dimethyl sulfoxide	1%	-	100.0	146.3±11.3	143.3±13.7	100 (72)	204
	1%	+	100.0	103.7±14.0	151.3±5.6	100 (76)	137
Positive Control							
Hycanthone	10 µg/mL	-	23.6	574.3±32.6	92.0±12.7	64	1249
2-Acetylaminofluorene	100 µg/mL	+	5.3	653.3±24.9	157.0±6.5	104	832
Test Material							
D-NC 302	800 µg/mL[d]	-	12.9	221.7±11.6	198.0±11.4	138	224
	1000 µg/mL[e]	-	7.1	213.7±16.3	144.7±10.8	101	295
	400 µg/mL[d]	+	16.9	155.3±11.9	192.3±12.4	127	162
	500 µg/mL	+	9.0	186.3±9.1	172.7±13.0	114	216

[a]Means and standard deviations from triplicate platings of single cultures; numbers in () are absolute cloning efficiences.
[b]Data extracted from CBI p. 44.

[c]Mutation Frequency (MF) = $\frac{\text{Mutant Colonies}}{\text{Viable Colonies}} \times 2.$

[d]Results for lower doses (5, 100, or 400 µg/mL -S9 or 20, 200, or 350 µg/mL +S9) did not suggest a mutagenic effect.

[e]Highest nonactivated dose (1500 µg/mL) was severely cytotoxic (<0.1% cell survival).

control guidelines adopted by Caspary et al. (1988)[1] for evaluation of the mouse lymphoma assay. Similarly, the S9-activated background control MF ($137x10^{-6}$) was only borderline acceptable. As further shown, the two highest nonactivated (1000 and 1500 µg/mL) and the highest S9-activated (500 µg/mL) doses were cytotoxic. There was, however, no indication of a mutagenic response at any dose either in the presence or absence of S9 activation.

Based on the overall results, the study author concluded that D-NC 302 was not mutagenic in the mouse lymphoma assay.

D. REVIEWERS' DISCUSSION AND INTERPRETATION OF RESULTS: We assess that the study provided no evidence that D-NC 302 was mutagenic, however, the high background MF for the nonactivated solvent control ($204x10^{-6}$), which according to the performing laboratory's criterion should have been rejected, compromised the ability of the test system to detect a potential weak mutagenic response induced by the test material. A similar argument can be made for the MF of the S9-activated solvent control ($137x10^{-6}$), which was within the acceptable range ($15-150x10^{-6}$) but was very high. Our claim that background frequencies have a profound influence on assay sensitivity is illustrated by the findings of Turner et al. (1984).[2] For example, Turner et al. presented data showing that 2-AAF at 50 µg/mL induced an ~11-fold increase in mutation (MF solvents = $61x10^{-6}$; MF 2-AAf = $683x10^{-6}$), while in the currently reviewed study, 2-AAF at approximately double this concentration (100 µg/mL) caused only an ~6-fold increase in the MF. Turner et al. (1984) also showed that 10 µg/mL hycanthone -S9 caused mutation at the TK locus that was ~24-fold higher than background (MF solvent = $52x10^{-6}$; MF hycanthone = $1266x10^{-6}$). In the currently reviewed study, 10 µg/mL hycanthone elicited an ~6-fold increase in mutation (MF solvent = $204x10^{-6}$; MF hycanthone = $1249x10^{-6}$). It was noteworthy that while the MFs for the nonactivated positive control were comparable in both studies, the degree of response was clearly a function of the spontaneous background mutation rate.

Based on these considerations, we conclude that the sensitivity of the test system to detect the potential mutagenic activity of an unknown substance was compromised. The study is, therefore, unacceptable.

E. QUALITY ASSURANCE MEASURES: Was test performed under GLPs? Yes. (A statement of GLP compliance and a statement listing changes in the final report were signed and dated June 14, 1991.)

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[1]Caspary, W.J., Lee, Y.J., Poulton, S., Myhr, B.C., Mitchell, A.D., Rudd, C.J. (1988). Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: Quality-control guidelines and response categories. Environ. Mol. Mutagen 12:19-36.

[2]Turner, N.T., Batson, A.G., Clive, D. (1984). Procedures for the L5187Y/TK+/- → TK-/- mouse lymphoma cell mutagenicity assay. In: Handbook of Mutagenicity Test Procedures. B.J. Kilbey, M. Legator, W. Nichols, C. Ramel, (eds). Elsevier 239-268.

F. CBI APPENDICES: Appendix A, Protocol, CBI pp. 23-30; Appendix B, Materials and Methods, CBI pp. 10-13.

CORE CLASSIFICATION: Unacceptable. The study does not satisfy Guideline requirements (§84-2a) for genetic effects, Category I (Gene Mutations).

APPENDIX A

PROTOCOL
CBI pp. 23-30

ASSURE

Page_____ is not included in this copy.

Pages 10 through 22 are not included.

The material not included contains the following type of information:

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