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

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Apr. 30 1990

OFFICE OF
PEST CIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT: Cyanazine - Mutagenicity Data Submitted under MRID
Nos. 001650-51, 403045-02, -03, -04, and -05
ID No. 066

Chemical (Caswell) No.: 188C
ED Record No.: 256,507
EED Project No.: 0-0464A

FROM: Irving Mauer, Ph.D., Geneticist
Toxicology Branch I - Insecticide, Rodenticide Support
Health Effects Division (H7509C) *Signature 4/2/90*

TO: Stephen Dapson, Ph.D.
Toxicology Branch II - Herbicide, Fungicide, and
Antimicrobial Support
Health Effects Division (H7509C)

THRU: Karl P. Baetcke, Ph.D., Chief
Toxicology Branch I - Insecticide, Rodenticide Support
Health Effects Division (H7509C) *Signature 4/2/90*

Registrant: Shell, Houston, TX and E.I. du Pont de Nemours,
Wilmington, DE

Request

In response to your request, please find attached primary
reviews of the following mutagenicity studies:

1. Genetic Toxicity Assay of BLADEX® Herbicide: Gene
Mutation Assay in Mammalian Cells in Culture, L5178Y,
Mouse Lymphoma Cells, [L5178Y/TK], performed at
Shell's Westhollow Research Center, Houston, TX,
Study No. 61282, Final Report issued August 12, 1986
(EPA MRID No. 00165051).

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2. Assessment of Cyanazine in the In Vitro Unscheduled DNA Synthesis Assay in Rat Primary Hepatocytes [HPC/UDS], performed at duPont's Haskell Laboratory, Newark, DE, Study No. HLR 347-87, Final Report issued July 14, 1987 (EPA MRID No. 40304702).
3. Mutagenicity Testing of Cyanazine (INR-1957) in the Salmonella typhimurium Plate Incorporation Assay [Ames Assay], performed at duPont's Haskell Laboratory, Newark, DE, Study No. HLR 258-87, Final Report issued May 7, 1987 (EPA MRID No. 40304703).
4. Mutagenicity Evaluation of Cyanazine in the CHO/HPRT Assay [CHO/HPRT], performed at duPont's Haskell Laboratory, Newark, DE, Study No. HLR 747-86, Final Report issued January 7, 1987 (EPA MRID No. 40304704).
5. In Vitro Evaluation of Cyanazine (INR-1957) for Chromosome Aberrations in Human Lymphocytes, [HLC/CA], performed at duPont's Haskell Laboratory, Newark, DE, Study No. 328-87, Final Report issued June 18, 1987 (EPA MRID No. 40304705).

EB Conclusions

[Detailed reviews are appended to this memorandum.]

Study	Reported Results	EB Evaluation
1) Gene Mutation in LS178Y/TK Cells	Positive for induced forward mutation in a dose-responsive fashion in repeat assays without activation (viable dose range = 0.5 to 500 ug/mL).	ACCEPTABLE
2) DNA Damage/Repair (UDS) in HPC	Positive for unscheduled DNA synthesis in repeat assays in rat hepatocytes treated <u>in vitro</u> (viable dose range = 1 to 100 ug/mL).	ACCEPTABLE
3) Ames Assay	Although reported as negative for inducing reversions in Salmonella TA strains exposed up to 5000 ug/plate (causing 50% toxicity), many procedural and reporting deficiencies exist.	UNACCEPTABLE

2

007893

-3-

Study	Reported Results	TS Evaluation
4 Gene Mutation in CHO/HPRT Cells	Negative for inducing mutation in repeat assays in nonactivated and activated Chinese hamster ovary cells treated up to cytotoxic limits of solubility (1.4 mM).	ACCEPTABLE
5 Chromosomal Aberrations in Human Lymphocyte Cultures	Negative in repeat assays with human lymphocytes exposed in the presence/absence of activation to cytotoxic dose levels (250 to 350 μ g/ml).	ACCEPTABLE

Attachments (DERs)

3

007893

Reviewed By: Irving Mauer, Ph.D., Geneticist
Toxicology Branch I - IRS (H7509C)
Secondary Reviewer: Karl P. Baetcke, Ph.D., Chief
Toxicology Branch I - IRS (H7509C)

4-18-90

4/31/90

DATA EVALUATION RECORD

I. SUMMARY

MRID (ACC) No.: 00165051
ID No.: 066
RD Record No.: 256,507
Caswell No.: 138C
Project No.: C-0464A

Study Type: Mutagenicity - Forward gene mutation in
mammalian cells (LS178Y/TK)

Chemical: Cyanazine

Synonyms: Bladex®

Sponsor: Shell, Houston, TX

Testing Facility: Westhollow Research Center (Shell),
Houston, TX

Title of Report: Genetic Toxicity Assay of Bladex®
Herbicide: Gene Mutation Assay in
Mammalian Cells in Culture, LS178Y,
Mouse Lymphoma Cells.

Authors: M.G. Jannasch, V.L. Sawin

Study Number: 61282

Date of Issue: August 12, 1986

TB Conclusions:

Positive for induced forward mutation when tested up
to the limit of solubility (1000, 1600 µg/mL), with dose-
related responses in repeat assays, both in the presence
and absence of activation.

Classification (Core-Grade): ACCEPTABLE

4

007893

II. DETAILED REVIEW

- A. Test Material - Bladex technical (Shell Biological, Molokai, CA)

Description: (Not stated)
Batch (Lot): 16-16-0-2
Purity (%): (Not stated)
Solvent Carrier/Diluent: Dimethylsulfoxide (DMSO)

- B. Test Organism - Established mammalian cell line

Species: Mouse (lymphoma)
Strain: L5178Y/TX⁺/-

Source: Dr. Donald Clive, Burroughs-Wellcome Co., RTP (NC)

- C. Study Design (Protocol) - This study was designed to assess the mutagenic potential of cyanazine when administered in vitro to mouse lymphoma (L5178Y) cells. A standardized published protocol* was employed to conduct this assay (referenced in the Final Report). A statement affirming compliance with Agency GLPs was provided. A Statement of Quality Assurance measures (inspections audits) was also provided.

- D. Procedures Methods of Analysis - Duplicate cell cultures (cleansed of spontaneous mutants (TX⁻/+)), were exposed for 4 hours to test substance in repeat assays at concentrations ranging from 0.05 to 1600 µg/mL, both in the absence and presence of a mammalian metabolic activation system** purchased from Cytos Bionetics.

Following treatment, cells were maintained in fresh medium for 3 days, then cloned for 11 days in triplicate to determine mutagenicity and viability, the former in the presence of trifluorothymidine (TFT) which selects only for TX⁺ mutants. Cells were harvested after this selection period, and the number of mutant colonies determined electronically (IBM XT-Biotran II System) as mutant frequencies (MF), calculated as Mean No. of Colonies/Cloning Efficiency.

*D. Clive (1979). Validation and characterization of the L5178Y TX Mouse Lymphoma Mutagen Assay System. MUTATION RES. 59:61-108.

**Post-mitochondrial fraction (S9, 25%) prepared from rats induced with Aroclor 1254 (36.6 mg/mL protein), to which was added the appropriate NADP generating cofactor (S9 Mix).

In addition to a concurrent solvent control (DMSO), cells were also exposed to the mutagens ethylmethane-sulfonate (EMS) and 20-methylcholanthrene (MCA), as positive controls for, respectively, the unactivated and S9-supplemented series.

Standardized criteria for both assay acceptance as well as interpretation of results were provided. For the former, only control cultures showing a respectable viability (greater than 3×10^5 cells/mL) were accepted for evaluation, and the background mutant frequency must be equal or less than 150 mutants/ 10^6 surviving cells. A response to a test compound was considered positive if a dose-related increase in MF was registered at three or more dose levels, with at least one dose exhibiting a "significant" value (defined as a MF greater or equal to twice that of the solvent control). In the absence of both these criteria, the response was considered negative. Where one or the other criterion was met, a response would be considered equivocal, and further evaluations might be necessary. No other statistical methods beyond calculation of means and SDs were "considered necessary."

5. Results - [Values for calculating mutagenicity (MF) and viability (cell growth relative to solvent) were provided for individual plates (REPORT APPENDIX II) and summarized in the Final Report (Tables I and II, as well as presented graphically.)]

In the first assay reported (Assay I had to be aborted for technical reasons), cell cultures were exposed to a series of half-log dilutions of the test substance (0.05, 0.5, 5, 50, 160, 500, and 1600 $\mu\text{g/mL}$) up to the limit of solubility (the HDT, 1600 $\mu\text{g/mL}$), and demonstrated a dose-related increase in toxicity coincident with significant increases in MF (as shown in the extracted summary of results on page 5 that follows).

The repeat assay employed six one-third log dilutions, from 22 $\mu\text{g/mL}$ up to 1000 $\mu\text{g/mL}$, the highest dose also exhibiting precipitation (and hence not evaluated). As in the initial assay, increased values for induced mutation were recorded, especially in the presence of activation, but without any increased toxicity (same tabulation). Positive controls responded appropriately in both assays.

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Table: The Effect of Cyanazine in Mouse Lymphoma
(LS178Y) Cells in vitro^{1/}

Initial Assay					Repeat Assay				
Cyanazine Dose (ug/mL)	-S9		+S9		Dose (ug/mL)	-S9		+S9	
	MRG (%)	MF (x10 ⁻⁶)	MRG (%)	MF (x10 ⁻⁶)		MRG (%)	MF (x10 ⁻⁶)	MRG (%)	MF (x10 ⁻⁶)
Control (DMSO)	100	28	100	4	Control (DMSO)	100	43	100	46
0.05	91	38	110	60	22	79	65	471	59
0.50	80	56*	84	86*	47	70	70	52	124*
5.0	81	60*	83	83*	100	59	73	37	61*
50	77	67*	75	106*	220	79	66	123	55*
160	66	71*	69	113*	470	26	167*	--	--
500	50	87*	53	124*	1000 ^{2/}	--	--	--	--
1600	--	--	--	--	--	--	--	--	--
EMS 160	8	273*	--	--	--	9	1259*	--	--
MCA:1	--	--	76	151*	--	--	--	86	174*
3	--	--	45	311*	--	--	--	116	219*
5	--	--	19	525*	--	--	--	57	515*

^{1/} Extracted from "Mean Report Tables" and I of the Final Report.

^{2/} Precipitation observed. Cultures neither plated nor evaluated.

*Significant, i.e., $\neq$ 0% solvent control.

MRG, mean relative growth.

MF, mutation frequency.

7

007893

The authors concluded that technical grade Bladex was mutagenic in mouse lymphoma cells both with and without metabolic activation.

F. TB Evaluation - ACCEPTABLE

Attachment (Data Tables)

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ATTACHMENT I

Data Tables

9
2

Cyanazine

RIN 3332-96

Page _____ is not included in this copy.

Pages 16 through 11 are not included.

The material not included contains the following type of information:

- ☐ Identity of product inert ingredients.
- ☐ Identity of product 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.
- ☒ FIFRA registration data.
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Toxicology Branch I - IRS (H7509C)

Jefferson
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DATA EVALUATION RECORD

I. SUMMARY

MRID (Acc) No.: 40304702
ID No.: 366
RD Record No.: 256,507
Caswell No.: 188C
Project No.: 0-0464A

Study Type: Mutagenicity - DNA damage repair in vitro
(rat HPC/UDS)

Chemical: Cyanazine

Synonyms: Bladex[®]

Sponsor: E.I. du Pont de Nemours & Co., Wilmington, DE

Testing Facility: Haskell Lab. (DuPont), Newark, DE

Title of Report: Assessment of Cyanazine in the in vitro
Unscheduled DNA Synthesis Assay in Rat
Primary Hepatocytes.

Author: Daniel P. Vincent

Study Number: HLP 347-87

Date of Issue: July 14, 1987

TS Conclusions:

Positive for unscheduled DNA synthesis (as measured
by increased silver grain counts) in repeat assays with
rat hepatocyte cells cultured in vitro.

Classification (Core Grade): ACCEPTABLE

II. DETAILED REVIEW

A. Test Material - Bladex technical

Description: White solid
Batch (Lot): 16-16-0-0
Purity (%): 96
Solvent/Carrier/Diluent: Dimethylsulfoxide (DMSO)

B. Test Organism - Rodent

Species: Rat (hepatocytes)
Strain: CRL:CD (Sprague-Dawley)
Age: (Not stated)
Weights - Males (only): (Weights not stated)
Source: Charles River, Kingston, NY

C. Study Design (Protocol) - This study was designed to assess the genotoxic potential of cyanazine when administered in vitro to rat primary hepatocytes (HPC), and DNA damage/repair determined by measuring unscheduled DNA synthesis (UDS) as reflected in the incorporation of radioactive thymidine into DNA (grain counts) under incubation conditions (37 °C). A statement affirming compliance with Agency GLPs was provided. A Statement of Quality Assurance measures (inspections/audits) was also provided.D. Procedures/Methods of Analysis - Hepatocytes perfused from the liver of a male rat were allowed to attach for up to 2 hours to the wells of individually marked chamber slides (4 cultures per treatment level). Cultures were then exposed to a graded series of eight test article concentrations together with a constant concentration of tritiated thymidine (84.1 Ci/mmol, ^3H -TdR; sp. act. = 5 $\mu\text{Ci/mL}$). In addition to concurrent solvent controls (1% DMSO), other cultures were exposed to the mutagen, 2-acetylaminofluorene (AAF, 0.1 and 1.0 μM) as a positive control agent known to induce UDS in rat hepatocytes.

After 18 hours incubation, the slide preparations were rinsed free of treatment medium, dipped in 1 percent citrate (to swell the cells), fixed in ethanol:acetic acid, rinsed with water and dried. Under darkroom conditions, the slides were then dipped into a photographic emulsion (NTB-2) dried thoroughly, then stored in light-tight microscope slide boxes at -70 °C for 4 to 7 days. After removal, the slide preparations were developed with standard photographic chemicals, and finally stained with methyl-green Pyronin Y.

007893

Silver grains were counted under oil immersion in 15 morphologically normal cells per culture (100 per treatment) using a colony counter interfaced with a remote TV camera attached to a universal microscope. Net nuclear grain counts per nucleus (NNG) were determined by subtracting the highest cytoplasmic (background) count from nuclear counts. The mean NNG derived from all slides of the same treatment concentration were calculated (by a computer program), in order to determine UDS response for that dose level.

The entire assay was repeated.

Calculations of both within-trial and between-trial means (plus their SDs) were the only statistical analyses performed.

According to the criteria of response employed by this laboratory, a test sample would be considered positive if:

1. The average UDS response for any concentration of the test compound from both trials is 5 NNG or more above the control response, and this increase is at least 3 standard deviations above the control response; AND
2. There is a positive correlation between increasing concentrations of test compound and the average UDS response in the absence of a negative correlation between test concentration and average cytoplasmic grains.

On the other hand, a compound is considered NEGATIVE if:

1. The average UDSA response for any concentration of the test compound from both trials is not 5 NNG above the control response, or the increase is not at least 3 standard deviations above the control response; AND
2. There is no evidence for a concentration-related effect of the test compound.

If the test samples do not satisfy the above criteria they are classified on a case-by-case basis. In these cases, the investigators consider factors such as 1) increases in the percent of cells in repair, 2) the presence of a concentration-related response, and 3) the reproducibility of the data.

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The following criteria must be met for an assay to be acceptable by this laboratory for evaluation:

1. Hepatocyte viability must be 75 percent or greater.
1. A culture is rejected if fewer than 25 scorable cells is observed.
1. A negative control (solvent) culture is rejected if the mean NNG is -5 or greater.
1. A positive control culture is rejected if the mean NNG is less than +3.

A concentration is rejected if:

1. More than one-half of the cultures prepared for each test concentration have fewer than 25 scorable cells; and/or
1. The variability of responses in the scored cultures is excessive when there is clear evidence of cytotoxicity.

A trial is rejected if:

1. The UDS response for the negative control cultures averages -5 NNG or more; and/or
1. The UDS response for the positive control cultures averages -5 NNG or less.

The rejection of slides, concentrations, or trials implies the data will not be used as either biological or statistical significance is questionable.

1. Results - Cyanazine was assayed in repeat experiments at concentrations ranging from 1 to 1450 μ M. Cytotoxicity was assayed indirectly by elevation of lactic dehydrogenase (LDH) activity in the incubation medium which was observed in a dose-related fashion in both trials at concentrations between 1 and 5 μ M (Report Table I, attached to this DER). The HDT (1450 μ M) was so toxic that cells could not be evaluated for UDS in Trial 1; hence this dose level was not used in Trial 1.

Values of NNG cell 5 above control (which defines a positive for this test) were recorded in both trials, clearly increasing in a dose-related fashion in Trial 1, but flatter in dose-responsiveness in Trial 2 (Report Tables II and III, attached here). Declines in UDS

15

occurred at the highest doses (1000 μ M in Trial 1; 500 μ M and above in Trial 2), considered by the investigators as "probably due to cytotoxicity."

- F. TB Evaluation - ACCEPTABLE as positive for UDS in repeat experiments with rat hepatocytes exposed up to levels of cytotoxicity.

Attachments (Data Tables)

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ATTACHMENT I

Data Tables

17.

Cyanazine

RIN 3332-96

Page _____ is not included in this copy.

Pages 18 through 22 are not included.

The material not included contains the following type of information:

- ☐ Identity of product inert ingredients.
- ☐ Identity of product impurities.
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Irving Mauer
4-18-90
Karl P. Baetcke
2-31-90

DATA EVALUATION RECORD

I. SUMMARY

MRID (Acc) No.: 40304703
ID No.: 066
RD Record No.: 256,507
Caswell No.: 138C
Project No.: C-0464A

Study Type: Mutagenicity - Reverse gene mutation in
bacteria (Ames Assay)

Chemical: Cyanazine

Synonyms: INR-1957; Bladex®

Sponsor: E.I. du Pont de Nemours & Co., Wilmington, DE

Testing Facility: Haskell Lab (duPont), Newark, DE

Title of Report: Mutagenicity Testing of Cyanazine (INR-
1957) in the Salmonella typhimurium Plate
Incorporation Assay.

Author: Carl T. Arce

Study Number: 158-37

Date of Issue: May 7, 1987

TB Conclusions:

Reported as negative for increasing reversions in
four TA strains of Salmonella exposed up to 5000 µg/plate,
a moderately toxic level (50%). However, procedural and
reporting deficiencies exist in the Final Report (as
detailed in the TB Evaluation).

Classification (Core Grade): UNACCEPTABLE

23

II. DETAILED REVIEW

A. Test Material - INR-1957 (cyanazine technical)

Description: White solid
Batch (Lot): 4581-460 (16-16-0-0)
Purity (%): 96
Solvent/Carrier/Diluent: Dimethylsulfoxide (DMSO)

B. Test Organism - Bacterial cultures

Species: Salmonella typhimurium LT2
Strains: TA1535, TA97a, TA98, and TA100 (all his⁻)
Source: Dr. Bruce Ames (UCal, Berkeley)

- C. Study Design (Protocol) - This study was designed to assess the mutagenic potential of cyanazine when administered in vitro to Salmonella TA strains stated to be treated according to procedures published by Ames,* and adopted as SOP by Haskell Labs. A statement affirming compliance with Agency GLPs was provided. A Statement of Quality Assurance measures (inspections, audits) was also provided.

- D. Procedures/Methods of Analysis - Selection of doses was made by exposing TA98 (only) in the presence and absence of a mammalian metabolic activation system** to test substance up to the limit dose, 5000 ug/plate.

In the (single) main assay, duplicate plates of the four TA strains were exposed to test compound, concurrently with positive*** and negative (DMSO) controls and with/without the S9 mix, and revertant colonies counted after 48 hours treatment at 37 °C.

Statistical analysis was stated to have been performed on individual sets of data from "two acceptable trials performed in one strain either with or without activation."

*B.N. Ames et al. MUTATION RES. 113:173-215, 1983.

**Pat S9 from livers of rats pretreated with Aroclor 1254, and purchased from SITEK Res., Rockville, MD, plus NADP generating cofactors.

***Appropriate to the individual strains, and including: 2-aminoanthracene; 9-aminoacridine; N-methyl-N'-nitro-N-nitrosoguanidine; and 2-nitrofluorene.

[NB: However, as noted above, only one complete assay was reported, and apparently run.]

The following criteria were used to classify responses of testing a substance in this assay (page 9 of the Final Report):

"A test sample is classified as a POSITIVE when:

- "A. The number of induced revertants at one or more of the test sample concentrations studied are at least two times greater than the number of revertants in the solvent control. These dose levels must have a probability of less than 0.01 that the number of induced revertants are the same as the spontaneous revertant number.

AND

- "B. The probability is less than 0.01 that there is not a positive correlation between the number of revertants and increasing concentrations of the test sample.

"A test sample is classified as a NEGATIVE when:

- "A. The probability is greater than 0.05 that the numbers of revertants at each of the test sample concentrations studied are not greater than the number of revertants in the solvent control.

OR

- "B. The probability is greater than 0.05 that there is not a positive correlation between the numbers of revertants and increasing concentrations of the test sample.

"A test sample is classified as EQUIVOCAL when:

- "A. Neither the criteria for a positive or negative are satisfied."

- B. Results - The test article was slightly toxic to strain TA98 beginning at 500 μ g/plate, (relative cell viability = 79%), but toxicity reached only about 50 percent at the HDT (Report Table 1, attached to this DER).

In the main mutagenicity assay, no statistically significant increases in revertants were recorded in

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any of the four strains exposed to test article up to 5000 ug/plate (Report Tables II through IX, also attached here). By contrast, the positive controls responded appropriately with statistically significant ($p < 0.0001$) increases, ranging from 8X to 100X solvent control values. The author concluded that under the conditions of this assay, [this sample of] cyanazine was negative for mutagenicity in Ames testing.

F. TS Evaluation - Although apparently conducted according to standardized procedures for Ames Tests, reporting of this study is judged UNACCEPTABLE because of:

1. The lack of reporting essential procedural details (or including the protocol mentioned in text);
2. Not providing the genetic nature and description of the "novel" strain, TA97a, together with, or justification for, its substitution in place of the original (Ames) frame-shift strains TA1537 and TA1538; and
3. The assay was not repeated (and data presented) to confirm the initial (negative) result.

Attachments: Data Tables)

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ATTACHMENT I

Data Tables

27

Cyanazine

RIN 3332-96

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Toxicology Branch I - IRS (H7509C)

Irving Mauer
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DATA EVALUATION RECORD

I. SUMMARY

MRID (Acc) No.: 40304704
ID No.: 066
RD Record No.: 256,507
Caswell No.: 188C
Project No.: 0-0464A

Study Type: Mutagenicity - Forward mutation in mammalian cells in vitro (CHO/HPRT)

Chemical: Cyanazine

Synonyms: INR-1957, Bladex®

Sponsor: E.I. du Pont de Nemours & Co., Wilmington, DE

Testing Facility: Haskell Labs (duPont), Newark, DE

Title of Report: Mutagenicity Evaluation of Cyanazine in the CHO/HPRT Assay.

Author: Lauren B. Rickard

Study Number: 747-86

Date of Issue: January 7, 1987

TB Conclusions:

Negative for inducing HPRT mutants in repeat assays with both nonactivated and activated cultures of Chinese hamster ovary cells treated up to the cytotoxic limit of solubility (1.4 mM).

Classification (Core Grade): ACCEPTABLE

38

II. DETAILED REVIEW

A. Test Material - INR-1957 (cyanazine, technical grade)

Description: White solid
Batch (Lot): 15-16-0-0
Purity (%): 96
Solvent/Carrier Diluent: Dimethylsulfoxide (DMSO)

B. Test Organism - Established mammalian cell line

Species: Chinese hamster (ovary)
Strain: BH4
Source: Dr. A.W. Hsie, Oak Ridge National Lab (TN)

C. Study Design (Protocol) - This study was designed to assess the mutagenic potential of cyanazine when administered *in vitro* to Chinese hamster ovary cells (CHO) by standardized (published) procedures (Hsie et al., 1975, *et seq.*). A statement affirming compliance with Agency GLPs was provided. A Statement of Quality Assurance measures (inspections/audits) was also provided.D. Procedures/Methods of Analysis - Primary cytotoxicity studies were performed up to the level of solubility in culture medium (1.4 mM). From this test, doses were selected for the mutagenicity assay, in which cell cultures were exposed to up to 5 concentrations of test substance, for 18 to 19 hours in the absence of activation, but only for 5 hours in the presence of S9 plus up to 21 hours in fresh medium. Following treatment, cells were allowed to recover for 7 days in the presence of 6-thioguanine (6-TG, which permits only mutant cells to survive), then plated to assess cell survival, and reincubated for an additional 6 to 8 days.

At harvest the surviving colonies were stained and counted, and mutation frequency (MF) expressed as the number of mutant colonies per 10^6 surviving cells at the time of mutant selection (i.e., immediately after test treatment).

In addition to solvent controls (DMSO) run concurrently, cell cultures were also exposed to the mutagens, ethylmethanesulfonate (EMS) and 7,10-dimethyl-1,2-benz-anthracene (DMBA), as positive controls for unactivated and S9-supplemented series, respectively.

Because the data from such assays are highly variable (with a tendency to large experimental errors), MF data

59

were first transformed by the Snee and Irr (1981) formula: $Y = (MF+1)^{0.15}$ (to allow appropriate parametric analysis), then analyzed by ANOVA and the t-test for significance of individual doses on frequency. A second analysis was performed to evaluate (any) dose-response relationship (ANOVA, followed by the F-test).

Criteria for assay acceptability were strictly adhered to, based upon:

1. Cloning efficiency (CE): control CE should be > 50%.
2. Average spontaneous (background) mutant frequency (MF) should be between 3 and 20/10⁶.

Standard criteria for response were also stressed, such as (pages 8 and 9 of the Final Report):

"A test sample is classified as POSITIVE when:

- "A. The mutant frequency of one or more of the sample concentrations tested is significantly greater than that of the solvent control, where significance is judged at the 0.05 level; and
- "B. The correlation between the mutant frequency and the concentration of the test sample is significantly greater than 0, where significance is judged at the 0.01 level.

"A test sample is classified as NEGATIVE when:

- "A. The mutant frequency of none of the sample concentrations tested is significantly greater than the mutant frequency of the solvent control, where significance is judged at the 0.10 level; and
- "B. The correlation between the mutant frequency and the concentration of the test sample is not significantly greater than 0, where significance is judged at the 0.05 level.

"Test samples that cannot be classified by the above criteria are dealt with on a case-by-case basis."

3. Results - Precipitation occurred at concentrations above 1.4 mM, at which dose reductions in relative survival of 35.7 percent (-S9) and 13.5 percent (-S9) were recorded. Hence, in the mutagenicity

007393

assay, the following dose schedule of cyanazine was used: 0.1, 0.7, 1.0, 1.2, and 1.4 mM.

Statistical analysis of data generated with/without activation revealed no significant increases in MF (i.e., no induced EPRT mutants) at any concentration of cyanazine, and no apparent dose-response (Report Tables I through IV, attached to this DER). In contrast, the positive controls responded appropriately, with values ranging from 8 to 100 times solvent controls.

5. IB Evaluation - ACCEPTABLE. These repeat assays were conducted with appropriate controls and under conditions capable of demonstrating a genetic response in the absence of high levels of cytotoxicity and/or the limit of solubility, and thus the data generated may be considered valid for the negative results obtained.

Attachments Data Tables

007893

ATTACHMENT I

Data Tables

42

Cyanazine

RIN 3332-96

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Pages 43 through 49 are not included.

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Reviewed By: Irving Mauer, Ph.D., Geneticist
Toxicology Branch I - IRS (H7509C)
Secondary Reviewer: Karl P. Baetcke, Ph.D., Chief
Toxicology Branch I - IRS (H7509C)

007893

4-18-90

4/31/90

DATA EVALUATION RECORD

I. SUMMARY

MRID (Acc) No.: 40304705
ID No.: 066
RD Record No.: 254,507
Caswell No.: 188C
Project No.: 0-0464A

Study Type: Mutagenicity - Chromosome damage in vitro
(human lymphocyte cultures)

Chemical: Cyanazine

Synonyms: INR-1957, Bladex®

Sponsor: E.I. du Pont de Nemours & Co., Wilmington, DE

Testing Facility: Haskell Labs (duPont), Newark, DE

Title of Report: In vitro Evaluation of Cyanazine
(INR-1957) for Chromosome Aberrations in
Human Lymphocytes.

Author: Ralph G. Stahl, Jr.

Study Number: 328-87

Date of Issue: June 18, 1987

TB Conclusions:

Negative in repeat in vitro assays with human lympho-
cytes exposed up to cytotoxic levels (250, 350 ug/mL),
with/without activation.

Classification (Core Grade): ACCEPTABLE

50

II. DETAILED REVIEW

A. Test Material - INR-1257 (cyanazine, technical)

Description: White solid
 Batch (Lot): 16-16-0-0
 Purity (%): 96
 Solvent/Carrier/Diluent: Dimethylsulfoxide (DMSO)

B. Test Organism - Primary peripheral lymphocyte cultures

Species: Man
 Age: Adult
 Weights - Males: (Not stated)
 Females: (Not stated)
 Source: (Blood donors)

C. Study Design (Protocol) - This study was designed to assess the clastogenic (chromosome-breaking) potential of cyanazine when administered in vitro to cultures of human lymphocytes. The procedure employed was derived from published articles, and stated to be in compliance with both EPA Guidelines as well as those of the OECD. A statement affirming compliance with Agency GLPs was provided. A Statement of Quality Assurance measures (inspection/audits) was also provided.D. Procedures/Methods of Analysis - In a preliminary cytotoxicity test, venous blood cells from two female donors were established in suspension culture in 15 ml centrifuge tubes with 2% phytohemagglutinin (a plant-derived mitogen, or mitosis-stimulating agent). Forty-four hours later duplicate cultures were exposed for 3 hours to test article at five concentrations: 0.5, 15, 125, 100, and 350 $\mu\text{g/mL}$, both in the absence and in the presence of a mammalian activation system.* Following treatment, all cultures were reincubated in fresh medium containing $1 \times 10^{-5} \text{M}$ (3 $\mu\text{g/mL}$) 5-bromodeoxyuridine (BUdR - to differentiate sister-chromatids) for 24 to 26 hours, the last 2 hours of which in the presence of the metaphase-arresting agent, Colcemid (0.1 $\mu\text{g/mL}$). Cultures were harvested by gentle centrifugation (200X g), and the cell pellet briefly exposed to 0.075 M KCl (to expand metaphases), then processed by conventional cytological

*Rat liver S9-Mix, consisting of the hepatic post-mitochondrial (supernatant) fraction (S9) from male Sprague-Dawley male rats treated with the PCB, Aroclor 1254 (purchased from SITEK Research, Rockville, MD), plus NADP-generating cofactors).

007893

techniques onto glass slides for microscopy. Slides were processed for sister-chromatid differentiation [for the determination of cell-cycle delay] by the standardized (published) FPG (fluorescence plus-Giemsa) method (referenced in this Final Report), dried, then mounted under coverslips with Permount.

Slides from the highest concentration were compared with solvent controls for evidence of cell-cycle delay, by examining 50 to 100 metaphases per treatment for their status with respect to how many cell divisions had transpired (one = M1; one to two = M1+; two = M2, etc.). Cell-cycle delay was considered to have occurred where "marked" increases over control in M1 cells were recorded, and thus the average proliferation time (APT) had risen. For each culture, cell kinetic data were expressed as the mean number of cell cycles (MCC) and the APT, calculated as follows:

$$MCC = \frac{1(M1) + 1.5(M1+) + 2(M2) + 2.5(M2+) + 3(M3)}{\text{Total Number of Metaphases}}$$

$$APT = \frac{\text{Time in BUdR (hr)}}{MCC}$$

From this cytotoxicity test, four concentrations were chosen for the main (aberration) assays (35, 100, 250, and 350 $\mu\text{g/mL}$). Also, harvest times were selected (13 to 20 hours) to allow only 1 to 1.5 cell cycles to elapse after treatment. For the main assay, the same procedures were employed as used for the cytotoxicity test for culturing, treatment, and harvest, except that BUdR was omitted, and positive controls* were included.

For each trial, 50 normal ($2n = 46$) cells per replicate (100 metaphases per treatment) were scored microscopically under oil immersion (1000X) for the conventional array of structural chromosome aberrations (gaps, breaks, fragments, tri-/quadri-radials, interchanges, double minutes, rings, translocations, dicentrics, other complex rearrangements, pulverization, and multiple aberrations, i.e., 10 or more per cell - both chromatid and isochromatid).

*The clastogens, mitomycin-C (MMC, $7.5 \times 10^{-7}\text{M}$) and cyclophosphamide (CPA, $3.6 \times 10^{-5}\text{M}$) for, respectively, the nonactivated and S9-supplemented series.

52

007893

A test article is considered positive (clastogenic) by this laboratory if a statistically significant increased incidence of aberrations (acentric gaps) over solvent control is recorded at at least one test dose level (by Fisher's Exact Test), and/or a significant dose response is found (by the Cochran-Armitage Trend Test), both judged at the 95% confidence level, and using the following parameters:

- 1) Proportion of abnormal cells (cells containing at least one aberration).
- 2) Proportion of cells with more than one aberration.

Two-way ANOVA was used to evaluate dose x trial interaction. Conventional criteria for assay acceptability were applied to both trials.

3. Results - The highest dose of cyanazine employed in the cytotoxicity test was 350 $\mu\text{g/mL}$, stated to be derived from "previous cytotoxicity/solubility studies conducted with Chinese hamster ovary cells." Four lower dose levels were selected to complete a thirtyfold concentration range (Report Table 1, attached to this DER). At the two highest doses (250 and 350 $\mu\text{g/mL}$), the test substance was declared "slightly toxic" (a 12 to 15% shift in cell cycle kinetics (APT)) without activation (-S9), but was considered "not cytotoxic" (the temporal shift in proliferation was less than 3% at the HDT with activation (-S9)).

The array of aberration types observed in both trials have been accumulated in Report Appendices A-1 through A-4, and the frequencies of aberrations summarized in Report Tables 2 and 3 (also attached here). No statistically significant increases in test metaphase preparations were found in either trial, even at doses that showed some indirect evidence of cytotoxicity (reduced mitotic indices). By contrast, both positive indicators produced a significant ($p < 0.101$ to 0.0001) array of simple and multiple chromosome damage, approximately 10 to 30 times background (solvent controls).

The author concluded that cyanazine was not a clastogen under the conditions of this study.

007893

- F. TB Evaluation - ACCEPTABLE. Since this study applied all the appropriate procedures and controls to identify and sample any potential chromosome damage that could be induced, the negative results generated are valid.

Attachments (Data Tables)

54

007893

ATTACHMENT I

Data Tables

55

Cyanazine

RIN 3332-96

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