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

DEC 3 1987

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OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: EPA ID No. 067002 - Pine Oil - Review of Gene Mutation Study in the CHO/HGPRT Assay, Chromosomal Alteration Study in Mouse Micronucleus Cytogenetic Assay and Unscheduled DNA Synthesis in Rat Primary Hepatocytes

TOX Chem No.: 665
TOX Project No.: 7-1033
Record No.: 204086

FROM: John D. Doherty 11/30/87
Toxicology Branch
Hazard Evaluation Division (TS-769C)

TO: Geraldine W. Werdig
Chief, Data Call-In
Registration Division (TS-767C)

THRU: Edwin R. Budd
Section Head, Toxicology Branch
Hazard Evaluation Division (TS-769C)

12/1/87 12/3/87

The Chemical Specialties Manufacturers Association, on behalf of the Pine Oil Joint Venture, has submitted three mutagenicity studies with pine oil blend in response to a Data Call-In Notice for pine oil products. A summary of the mutagenic potential of pine oil prepared by Dr. Richard A. Parent was also submitted.

The studies were reviewed and are listed on the following page.

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Toxicology Branch (TB) notes, however, that the lot number, the percent purity and the manufacturer(s) of the pine oil sample were not indicated for any of the three mutagenicity studies presented. Thus for each of the three studies the following information must be provided:

i. lot number

ii. percent purity

iii. manufacturer*

*If the material was a blend from several manufacturers, information on the identification of the contributing manufacturers must be presented.

Studies Reviewed

Study	Results	Classification
(1) Gene Mutation Test:		
CHO/HGPRT Forward Mutation Assay In Vitro. Microbiological Assoc. Study No. T5366.332001 September 1, 1987	No evidence of mutagenic effects up to and including 250 nL/mL in the absence of metabolic activation and up to and including 400 nL/mL in the presence of metabolic activation (S-9).	UNACCEPTABLE* [Cellular toxicity not high enough to be acceptable.]
(2) Structural Chromosome Alteration Test:		
Micronucleus Assay in Mouse Microbiological Assoc. Study No. T5366.122 September 1, 1987	No evidence of micronucleus induction. Doses tested: 0, 116, 578, and 1155 mg/kg.	ACCEPTABLE
(3) Test for other Genotoxic Effect(s):		
Unscheduled DNA Synthesis (UDS)in Rat Hepatocytes. Microbiological Assoc. Study No. T5366.380016 September 3, 1987	No evidence of UDS up to and including 0.03 ul/mL. Higher levels cytotoxic.	ACCEPTABLE

*A second study will have to be presented.

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Reviewed By: J.D. Doherty 11/30/87
Section II, Toxicology Branch (TS-769C)
Secondary Reviewer: K. Dearfield 11/30/87
Scientific Mission Support Staff, Toxicology Branch (TS-769C)

DATA EVALUATION REPORT

Study Type: 84-2 - Gene Mutation in the Mammalian CHO/HGPRT Assay

TOX Chem No.: 665
MRID No.:

Accession No.: 403414-02

Test Material: Pine Oil Blend, CSMA 1687 (Lot Number not provided)

Synonyms:

Study No.: T5366.332001

Sponsor: Chemical Specialties Manufacturers Association (CSMA)

Testing Facility: Microbiological Associates, Inc.
Bethesda, MD

Title of Report: CHO/HGPRT Mutation Assay

Author: Li L. Yang, Ph.D.

Report Issued: September 1, 1987

Conclusions:

No evidence of mutagenic effect at dose levels up to and including 250 nL/mL in the nonactivation assay and up to and including 400 nL/mL in the S-9 activation assay. The test dose levels of pine oil blend used were not appropriately high enough to be acceptable. Refer to study conclusion in DER for discussion.

Classification: UNACCEPTABLE

Special Review Criteria (40 CFR 154.7):

Quality Assurance Statement:

A statement was provided (signed by Janine (?) K. Burke on September 1, 1987) stating that inspections were made on four occasions (protocol, scoring the plates, draft report, and draft to final report).

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REVIEW

The purpose of this study was to assess the mutagenic potential of pine oil blend (CSMA 1687) based on induction of forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus of Chinese hamster ovary (CHO) cells in vitro.

Procedure:

For this study CHO cells were obtained from Dr. Abraham Hsie of the Oak Ridge National Laboratories. They were of the strain CHO-K_1-BH_4. These experiments were run in both the presence and absence of metabolic activation (S-9 preparation derived from male Fischer rats). The dose levels to be used for the main study were selected following a preliminary toxicity test based on colony-forming efficiency. In the preliminary study, CHO cells were exposed (for 5 hours) to solvent (DMSO) and to nine concentrations (in duplicate) of pine oil blend (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 uL/mL) in both the presence and absence of metabolic activation. The day following initial exposure of the pine oil blend to the cells, the treated cells were trypsinized and reseeded (in triplicate in media without hypoxanthine) at a density of 100 cells/60 mm dish and the cloning efficiency determined 7 to 10 days later at which time they were fixed in methanol, stained with 10% aqueous Giesma, and counted.

The preliminary toxicity test indicated that the pine oil blend was more toxic in the absence of the S-9 mix than in its presence. The dose levels selected for the main study were 250, 200, 170, 130, and 100 nL/mL in the absence of the S-9 mix and 600, 500, 400, 300, and 200 nL/mL in the presence of the S-9 mix. Eventually, however, the 600 and 500 nL/mL dose levels proved to be too toxic and the dose levels selected were 400, 300, 200, 100, and 50 nL/mL.

The mutation assessment and assay were performed according to published methodologies (Machanoff et al., Chem. Biol. Interactions 34:1-10, 1981 and O'Neill et al., Mutation Research 45:91-101, 1977). Basically, this assay consisted of plating exponentially-growing CHO-K_1-BH_4 cells in Ham's F-12 medium at a density of 5×10^5 cells/25 cm^2 flask and incubating at 37 $\pm$ 1 °C in a humidified atmosphere of 5% CO_2 in air for 18 to 24 hours. At initiation of the chemical treatment, the cells were exposed (in duplicate, for 5 hours) to the concentrations of pine oil blend as above with or without the S-9 activation mixture. After the chemical treatment period, the media was aspirated, the cells washed, and then cultured in assay medium for an additional 18 to 24 hours at 37 $\pm$ 1 °C. At this time, the cells were subcultured to assess cytotoxicity and to initiate the phenotypic expression period.

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To assess for expression of the mutant phenotype, replicates from each assay condition were pooled and subcultured (in duplicate) at a density of approximately 10^6 cells/100 mm dish. Subcultures from these preparations were again pooled and replated, in quintuplicate, at a density of 2 x 10^5 cells/100 mm dish on hypoxanthine-free buffer also containing 10 uM 6-thioguanine. These assays were also assessed for cloning efficiency determinations by plating, at the time of selection, 100 cells/60 mm dish (in triplicate) and examining after 7 to 10 days of incubation.

In addition to the five concentrations of pine oil blend, positive controls were also run. Ethylmethanesulfonate (0.2 uL/mL) was used for the positive control in the activation study and benz(a)pyrene (4 ug/mL) was used as the positive control in the S-9 activation study.

The mutant frequency (MF) for each treatment condition was calculated by dividing the total number of mutant colonies by the number of cells selected, corrected for the cloning efficiency of cells prior to mutant selection. The MF is expressed as TG-resistant mutants per clonable cells.

The study report stated that the criteria for determination of a valid test were as follows:

"The cloning efficiency of the solvent and untreated controls must be no less than 50%. The spontaneous mutant frequency in the solvent and untreated controls must fall within the range of 0-20 mutants per 10^6 clonable cells.

"The positive control must induce a mutant frequency at least three times that of the solvent control."

Photocopies of the summary tables (from the study report) for the main assays in both the absence and presence of the S-9 metabolic activation mix are attached. These tables show that pine oil blend did not cause mutation frequency (mutants/ 10^6 clonable cells) of greater than twice the untreated control or DMSO control (although it was almost twice the DMSO control level). The positive controls for both the presence and absence of the S-9 mix responded with mutation frequencies of 39 and 20 times the DMSO control groups.

The confirmatory assays produced similar results, which also did not indicate that pine oil blend induced mutations in this assay.

Conclusion:

This study is UNACCEPTABLE. The test data presented demonstrate that pine oil blend was not shown to be mutagenic in the CHO/HGPRT mutation assay at dose levels up to and including 250 nL/mL in the absence of metabolic activation and up to and including 400 nL/mL in the presence of metabolic activation (S-9 preparation from rat liver).

According to Dr. Kerry Dearfield, TB specialist in genetic toxicology, the concurrent toxicity data for these assays do not have the appropriately high toxicity. For example, they do not even meet the testing laboratory's own recommended range of 10-30% relative survival [refer to the toxicity tables attached]. TB has determined that the toxicity curve will have to be reassessed to obtain the appropriate range for retesting in a definitive study. The low survival range needs to be assayed to actually determine the possible effects of pine oil blend, especially in light of so many elevated mutation frequencies at different concentrations noted in the data presented. TB notes that the results presented may not have reached statistical significance due to the fairly high frequencies of the untreated controls albeit at the high end of the acceptable range. It is advised that when the study is repeated that cultures with lower spontaneous backgrounds be initiated.

Attachments

Pine oil toxicology review

Page _____ is not included in this copy.

Pages 7 through 10 are not included in this copy.

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___ Identity of product inert ingredients

___ Identity of product impurities

___ Description of the product manufacturing process

___ Description of product 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

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006484

Reviewed By: J. Doherty 11/30/87
Section II, Toxicology Branch (TS-769C)
Secondary Reviewer: K. Dearfield 11/30/87
Scientific Mission Support Staff, Toxicology Branch (TS-769C)

DATA EVALUATION REPORT

Study Type: 84-2 - Mouse Micronucleus Assay (In Vivo)

TOX Chem No.: 665
MRID No.:

Accession No.: 403414-03

Test Material: Pine Oil Blend (CSMA 1687, Lot Number not provided)

Synonyms:

Study No.: T5366.122

Sponsor: Chemical Specialties Manufacturers Association (CSMA)

Testing Facility: Microbiological Associates, Inc.
Bethesda, MD

Title of Report: Micronucleus Cytogenetic Assay in Mice

Author: Donald L. Putman, Ph.D.

Report Issued: September 1, 1987

Conclusions:

No evidence that the pine oil blend induced chromosome aberrations in mouse bone marrow was presented. Levels tested: 0, 116, 578, and 1155 mg/kg intraperitoneally.

Classification: ACCEPTABLE

Special Review Criteria (40 CFR 154.7): N/A

Quality Assurance:

Four inspections (protocol review, dosing, draft report, and final report) were reported as being made by J.K. Burke.

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REVIEW

The purpose of this study was to assess the potential of pine oil blend (CSMA 1687) to increase the incidence of micronucleated polychromatic erythrocytes (MPCEs) in bone marrow of male and female mice.

Preliminary Toxicity Study:

In this study CD-1 mice (males and females, young adults 6-8 weeks old) obtained from the Charles River Breeding Laboratory were grouped into eight groups of five males and five females per group and dosed intraperitoneally with either 0 (corn oil control), 1000. 1300, 1600, 2000, 3000, 4000, or 5000 mg/kg of the test material in corn oil and observed for reactions and mortality.

Most mice dosed at 1600 mg/kg and above died (only a single female dosed with 2000 mg/kg survived). The LD_{50} was calculated by probit analysis to be 1444 mg/kg (approximately). All mice dosed with 1000 mg/kg survived and a single male and female mouse survived dosing at 1300 mg/kg.

The mortality was reported as deaths in 7 days but information on the time of death, onset, and duration of symptoms were not reported.

From these data it was determined that the dose levels of 1155, 578, and 116 mg/kg would be used for the definitive micronucleus assay. The high-dose level represents 80 percent of the LD_{50} for 7 days.

Micronucleus Assay:

For this aspect of the study, 13 experimental groups of 5 males and 5 females (except that there were 8 mice of each sex for the high-dose group) were dosed intraperitoneally (at the rate of 10 mL/kg body weight) with the test substances (in corn oil), the vehicle or the positive control (0.25 mg/kg of triethylenemelamine) in corn oil. Sacrifices were made at 24, 48, and 72 hours postdosing by CO_2 asphyxiation and the femurs tapped for their bone marrow. The bone marrow was drawn into a syringe containing fetal bovine serum and transferred to a centrifuge tube and pelleted at 1000 rpm. A sample of the bone marrow was spread onto a cover slide, fixed in methanol and stained with May-Gruenwald-Giemsa stain and mounted. The slides were read by a person not involved in the preparation or labeling process who scored 1000 cells for the presence of polychromatic erythrocytes (PCEs). The proportion of PCEs to total erythrocytes and the "number of micronucleated normocytes in the field of 1000 polychromatic erythrocytes were enumerated."

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The criteria used by the testing laboratory to determine if a test material is positive in this study were that "the mean incidence of micronucleated polychromatic erythrocytes must not exceed 5/1000 polychromatic erythrocytes (0.5%) in the negative (vehicle) control. The incidence of micronucleated polychromatic erythrocytes in the positive control group must be significantly increased relative to the negative control ($p \leq 0.05$, Kastenbaum-Bowman Tables)."

In the high-dose treatment group, two of the males and four of the females died with the clinical signs of toxicity starting at 4 hours postdosing. The symptoms included prostration, depressed activity, and tremors. No test material effects were reported in the mid- or low-dose pine oil treated mice.

Analysis of the PCEs indicated that in the control group there were from 3 to 13 MPCEs per 5000 PCEs scored. There were 129 to 195 MPCEs per 5000 PCEs scored for the positive control group. The test report asserted that "the negative and positive controls fulfilled the requirements for determination of a valid test." The MPCEs ranged from 3 to 19 per 5000 PCEs for the mice dosed with pine oil blend. The incidences for the pine oil blend treated groups were not statistically significant.

Conclusion:

This study is ACCEPTABLE. No evidence that the pine oil blend was mutagenic in this study was presented.

It should be noted, however, that studies of this type are limited in their usefulness if there is no evidence that the test material actually was present in the bone marrow to affect the cells in this structure. In the case of pine oil, TB has no specific reason to believe that the pine oil did not reach the bone marrow. The study establishes that under the conditions of this assay in mice and in the timeframe allowed, pine oil blend did not show evidence of causing chromosome damage.

Reviewed By: J. Doherty 11/30/87
Section II, Toxicology Branch (TS-769C)
Secondary Reviewer: K. Dearfield 11/30/87
Scientific Mission Support Staff, Toxicology Branch (TS-769C)

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DATA EVALUATION REPORT

Study Type: 84-2 - Mutagenicity-Other (Unscheduled DNA Synthesis)

TOX Chem No.: 665
MRID No.:

Accession No.: 403414-04

Test Material: Pine Oil Blend (CSMA 1687)

Synonyms:

Study No.: T5366.380016

Sponsor: Chemical Specialties Manufacturers Association (CSMA)

Testing Facility: Microbiological Associates
Bethesda, MD

Title of Report: Unscheduled DNA Synthesis in Rat Primary Hepatocytes with a Confirmatory Assay

Author: Rodger D. Curren, Ph.D.

Conclusions:

No evidence of mutagenic effects at dose levels up to and including 0.03 uL/mL. Higher dose levels are cytotoxic.

Classification: ACCEPTABLE

Special Review Criteria (40 CFR 154.7): N/A.

Quality Assurance Statement:

A statement was provided (signed by Bradley E. Casti (sp.?)) on September 4, 1984 stating that inspections were made on four occasions (protocol, counting of silver grains, draft report, and draft to final report).

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REVIEW

The purpose of this study was to evaluate the test article, Pine Oil Blend, CSMA 1687, for its ability to induce unscheduled DNA synthesis in rat primary hepatocytes as measured by autoradiographic methods in vitro.

Preliminary Cytotoxicity Test:

Rat primary hepatocyte cultures were prepared by the method of Williams (In Vitro 13:809-817, 1977) and replicate cultures were treated with 10 doses of pine oil blend ranging from 0.003 to 10 uL/mL. Eighteen to 20 hours after treatment, the cultures were centrifuged and the level of lactic dehydrogenase (LDH) activity in the culture fluid determined. Two replicate plates were used for LDH measurement at each dose level. The relative toxicities were obtained by comparing the LDH activity in the treated cultures to the LDH activities in the untreated control cultures.

The results of this study showed that pine oil blend was immiscible when added to culture medium at 3.0 or 10.0 uL/mL and was moderately to highly toxic at 0.1 uL/mL and above. It was slightly toxic (11%) at 0.03 uL/mL and essentially nontoxic at lower concentrations.

Unscheduled DNA Synthesis Test:

Three replicate plates seeded with 5 x 10^5 rat hepatocytes/plate were treated with pine oil blend concentrations ranging from 0.0003 to 10 uL/mL. The cells were incubated with ^{3}H-thymidine at a final concentration of 10 uCi/mL. The cells were treated for 18 to 20 hours after this time. They were washed with buffer, swelled in 10% sodium citrate and fixed in ethanol:acetic acid fixative. Samples of the fixative were placed on coverslips and allowed to dry before being coated with Kodak NTB emulsion and stored for 10 days at 4 °C in light-tight boxes with desiccant. The slides were then developed in Kodak D-19 developer, fixed in Kodak fixer and stained in hematoxylin-sodium-acetate-eosin stain.

The assay also included the positive control 7,12-dimethylbenzanthracene (DMBA) tested at both 3 and 10 ug/mL. Pine oil blend was diluted in DMSO where appropriate and DMSO was used as the control.

A confirmatory assay testing pine oil blend at concentrations ranging from 0.0001 to 1.0 uL/mL was also conducted. Both the initial and confirmatory assay included parallel cytotoxicity studies which utilized the LDH technique to assess for cytotoxicity.

The following criteria were used in scoring and for evaluation of the test results. [Note: Quoted from the study report.]

"Scoring:

"The slides were read "blind" on an Artek Colony Counter. Nuclear grains were counted in 50 cells in random areas on each of three coverslips per treatment where possible. If some slides were unscorable, more than 50 cells may have been counted on one or more of the remaining slides from that dose level. The net nuclear counts were determined by counting three nucleus-sized areas adjacent to each nucleus and subtracting the average cytoplasmic count from the nuclear count. Replicative synthesis was identified by nuclei completely blackened with grains and such cells were not counted. Nuclei exhibiting toxic effects of treatment, such as dark or uneven staining, disrupted membranes, or irregular shape, were not counted.

"Criteria for Evaluation of Test Results:

"The results of the individual assays were evaluated according to the criteria described below.

"A test article was judged positive if it induced a dose-related response and at least one dose produced a significant increase in the average net nuclear grains when compared to that of the control. In the absence of the dose response, a test article which showed a significant increase in the mean net nuclear grain count in at least two successive doses was considered positive. If a test article showed a significant increase in the net nuclear grain count at one dose level without any dose response, the test article was considered to have a marginal positive activity The test article was considered negative if no significant increase in the net nuclear grain counts at any dose level was observed.

"A statistical analysis (Casciano, D.A. and Gaylor, D.W. 1983. Mutat. Res. 122:81-86) of historical solvent and medium controls indicated that an increase of at least 5.4 net nuclear grain counts over background is necessary to conclude that the results of a particular dose level are statistically significant.

"Criteria for Evaluation of the Two Independent Assays:

"In the case where both the original and confirmatory assay are clearly positive or negative, the entire test will be judged similarly. If there are discrepancies between the results of the two studies, the Study Director will make a judgement based on the specifics of the two assays (e.g., a second assay may have (been) designed with more appropriate dose levels based on the results

of the first assay) whether to call the assays equivocal or positive. In the case where reproducible significant increases occur but no clear dose response can be demonstrated, the test article will be considered to have positive activity." [End of quotation.]

The parallel cytotoxicity studies for both the initial and confirmatory assays confirmed the initial cytotoxicity study. For example, dose levels above 0.03 uL/mL were cytotoxic and below this level could be evaluated for unscheduled DNA synthesis.

Copies of the results summary table for both the initial and confirmatory unscheduled DNA synthesis studies are attached. The results show that the positive control (DMBA) produced the expected positive results. Pine oil blend did not show evidence of producing a positive response in either assay.

Conclusion:

This study is ACCEPTABLE. Pine oil blend did not indicate evidence of a positive response at dose levels up to and including 0.03 uL/mL. Dose levels above this level are cytotoxic.

Attachments

Pine oil toxicology review

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