



DANAK

Reg. No. 145

scantox

TEST REPORT

Lab No 28709
Issued: 19 June 1998
Page 1 of 15

SPONSOR

AstaCarotene AB
Idrottsvägen 4
S-134 40 Gustavsberg
Sweden

HAEMATOCOCCUS PLUVIALIS

***IN VITRO* MAMMALIAN CELL GENE
MUTATION TEST PERFORMED WITH
MOUSE LYMPHOMA CELLS (L5178Y)**

AUTHOR:

C. Nicholas Edwards, BSc, PhD

The report shall not be reproduced except in full without approval by Scantox
SCANTOX DK-4623 Lille Skensved Denmark Tel +45 56 86 15 00 Fax +45 56 82 12 02

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The investigation described in this report "Haematococcus pluvialis - *In Vitro* Mammalian Cell Gene Mutation Test Performed with Mouse Lymphoma Cells (L5178Y)" was carried out under my supervision and responsibility and in accordance with the principles of Good Laboratory Practice (GLP) according to OECD principles of GLP, May 1981, Doc C(81)30 (Final) Annex 2, which are essentially in conformity with:

EEC Principles of Good Laboratory Practice, Directive 87/18/EEC,
United States Food and Drug Administration, Title 21, CFR, Part 58,
Japanese Ministry of Health and Welfare, PAB Notification No. 313.

The report is a complete and accurate account of the methods employed and the data obtained.

SCANTOX
19 June 1998

CNEwards

C. Nicholas Edwards, BSc, PhD

QUALITY ASSURANCE STATEMENT

The Quality system at Scantox complies with the OECD principles of Good Laboratory Practice and the European Standards EN45001.

Short term routine studies of the type described in this report "Haematococcus pluvialis - *In Vitro* Mammalian Cell Gene Mutation Test Performed with Mouse Lymphoma Cells (L5178Y)" are inspected by the Quality Assurance Unit in compliance with the principles of Good Laboratory Practice. Process-based inspections are carried out regularly. Documented inspection reports are communicated to the study director and to the management.

Date of most recent inspection: 20 April 1998

Date of report to study director and management 20 April 1998

This report has been audited by the Quality Assurance Unit and was found to be an accurate description of the methods and procedures used during the conduct of the study and an accurate reflection of the raw data.

Date of final audit: 19 June 1998

19 June 1998



Susanne Benn Nissen, MSc
QA Auditor

PERSONNEL RESPONSIBLE FOR THE STUDY

Study Director:



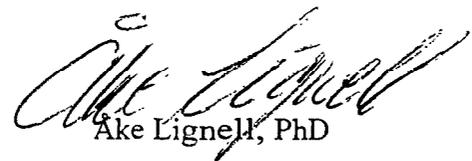
C. Nicholas Edwards, BSc, PhD

Quality Assurance



Susanne B. Nissen, MSc

Sponsor Monitor:



Åke Lignell, PhD

TABLE OF CONTENTS

SUMMARY	6
INTRODUCTION.....	7
Objective	7
General description of the test system.....	7
MATERIALS AND METHODS	8
Test article	8
Vehicle	8
Cells and culture methods	8
Frozen stock cultures.....	8
Rat liver postmitochondrial fraction	9
S-9 mix	9
TEST PROCEDURES	9
Treatment of cultures	9
Evaluation of results.....	11
Archives	11
RESULTS	12
CONCLUSION	12

TABLES

1 Test 1	13
2 Test 1 (repeat) without S-9 mix.....	14
3 Test 2.....	15

SUMMARY

Haematococcus pluvialis was tested in an *in vitro* mammalian cell gene mutation test using mouse lymphoma (L5178Y) cells. The test was performed in accordance with the OECD guideline "*In Vitro* Mammalian Cell Gene Mutation Test" No 476 (1997) and the corresponding EU guideline (1988).

The test material was tested at a range of concentrations up to 5000 µg/ml in two independent tests. The highest concentration (5000 µg/ml) is the maximum required by the OECD and EU guidelines for materials of low toxicity. In the absence of S-9 mix, the cells were treated for 4 hours in the first test and 24 hours in the second test. In the presence of S-9 mix (a metabolic activation system prepared from the livers of rats pre-treated with Aroclor[®] 1254), the cells were treated for 3 hours in both tests.

No biologically or statistically increase in mutation frequency was observed in cultures treated with the test material in either test, compared to the negative control cultures.

The sensitivity of the test was demonstrated by large increases in mutation frequency observed in cultures treated with the positive control agents.

It is concluded that Haematococcus pluvialis was not mutagenic in this *in vitro* mammalian cell gene mutation test performed with mouse lymphoma cells in the presence and absence of S-9 mix.

INTRODUCTION

Objective

The *in vitro* mammalian cell gene mutation test is a short term test for the evaluation of possible mutagenic effects of chemicals. This test was conducted in accordance with the OECD guideline "*In Vitro* Mammalian Cell Gene Mutation Test" No. 476 (1997) and the corresponding EU guideline (1988).

The experimental work was performed between 23 March 1998 and 13 May 1998.

General description of the test system

The test is performed using mouse lymphoma (L5178Y) cells which are heterozygous for the thymidine kinase gene (TK^{+/−}). This cell line has been grown for many years in suspension culture. Shortly before the mutagenicity tests, any pre-existing TK^{−/−} cells in the cultures are removed: the cultures are grown for 1-2 days in medium containing thymidine (9 µg/ml), hypoxanthine (15 µg/ml), methotrexate (0.3 µg/ml) and glycine (22.5 µg/ml) (THMG-medium). After a recovery period of 1 - 3 days in medium containing the above mentioned chemicals except methotrexate (THG-medium), all the cells have the genotype TK^{+/−}.

Some chemicals do not exert a mutagenic effect in this system unless they have been changed to active metabolites by mammalian enzymes. The activation is accomplished by the addition of S-9 mix to the treatments. S-9 mix consists of salts, co-factors and an enzyme-rich postmitochondrial fraction prepared from the livers of rats pre-treated with Aroclor® 1254.

Cultures of mouse lymphoma (L5178Y) cells are treated with the test article at a range of concentrations for an exposure period of 4 or 24 hours without metabolic activation and 3 hours with metabolic activation. They are then sub-cultured for 3 days to allow phenotypic expression of mutations which may have been induced in the TK-gene. The cell cultures are seeded (cloned, 2 cells/well) in culture medium in 96-well microtiter plates at the end of the treatment period to measure cytotoxic effects of the test material and at the end of the expression period to determine the number of colony forming units present. They are also cloned in medium containing trifluorothymidine (TFT) (2000 cells/well) at the end of the expression period. Heterozygous TK^{+/−} cells are poisoned by TFT and do not grow, but any cells that have mutated to TK^{−/−} can grow and form colonies in medium containing TFT. The mutation frequency can be determined from the number of cell clones in each type of medium after 10 days incubation.

MATERIALS AND METHODS

Test article

Haematococcus pluvialis
Batch No: 971215:6 (Heat treated)
Stability: Six months

The test article, a red-brown powder, was received from the Sponsor on 5 March 1998.

The Sponsor was responsible for all test article preparation and characterisation. On arrival at Scantox the test article was labelled with the Lab No of this study and stored at approximately -20°C in the dark. The test results relate to the above mentioned test article supplied by the Sponsor.

Vehicle

Formulations of the test article were prepared in cell culture medium freshly before use.

Cells and culture methods

The assay was performed with the mouse lymphoma cell line L5178Y (Ba 21) received from Dr B.-M. Jörgensen-Burmann, Astra, S-15185 Södertälje, Sweden.

The cells were grown as suspension cultures with gentle mixing in RPMI 1640 medium supplemented with 10% horse serum, 200 µg/ml sodium pyruvate and 50 µg/ml gentamycin. The medium for cloning and the THG-medium were supplemented with 50% conditioned medium.

The doubling time during optimal growth was approximately 12 hours. Sterile NUNC plastic flasks and microtiter plates were used and incubation was performed in a CO₂-incubator (5% CO₂) at 37°C. During normal maintenance and during the phenotypic expression period, the cells were diluted daily and kept at a density of about 2×10^5 to 1.5×10^6 cells/ml.

Frozen stock cultures

Frozen stock cultures were kept in a liquid nitrogen tank at -196°C. New stock cultures were made periodically by adding DMSO (1 ml) to cell suspensions (10 ml, at approx. 10^6 cells/ml). The cell suspensions were cooled slowly to -20°C and then the vials were transferred to the liquid nitrogen tank (-196°C). Thawing was performed in a 37°C water bath.

Before new stock cultures were used in assays, the cells were checked for general morphology and growth characteristics.

Rat liver postmitochondrial fraction

SPF Wistar rats of the stock Mol:WIST were obtained from Møllegaard Breeding and Research Centre A/S, Ejby, DK-4623 Lille Skensved. Rats weighing approximately 200 g were used for induction of liver enzymes. A single intraperitoneal injection of Aroclor® 1254 at a dose of 500 mg/kg body weight was given to each rat. The animals were killed with carbon dioxide 5 days after being injected and following a 16-hour period of fasting.

All steps in preparation of the liver homogenate were performed on ice using aseptic techniques and cold sterile solutions. The livers were removed, rinsed briefly in 0.15 M KCl, and minced in 0.15 M KCl solution (3 ml per gram wet liver). After homogenization, the homogenate was centrifuged at 9000 g for 15 minutes. The supernatant (postmitochondrial fraction) was decanted, frozen and stored at -196°C until use.

S-9 mix

Postmitochondrial fraction	1.8 ml
Hepes buffer (1 M, pH 7.2)	0.3 ml
MgCl ₂ (50 mM)	1.5 ml
KCl (330 mM)	1.5 ml
Glucose-6-phosphate (0.1 M)	0.75 ml
NADP (0.1 M)	0.6 ml
0.9% NaCl	2.4 ml
Distilled H ₂ O	6.0 ml
Phenol red, sodium salt (0.5 mg/ml)	0.15 ml

The postmitochondrial fraction was added after adjustment of pH to 7.2 with NaOH or HCl. The S-9 mix was prepared shortly before use, kept on ice, and warmed to 37°C immediately before addition. S-9 mix was added at 0.5 ml per 2.5 ml incubation mixture, corresponding to a final concentration of 2% postmitochondrial fraction.

TEST PROCEDURE

Two independent tests were performed. The part of the first test without S-9 mix was repeated because of toxicity of the test material.

Treatment of cultures

In the absence of S-9 mix, the cultures were treated by adding aliquots of the test material formulations (1 ml) to cell suspensions in normal medium to give a final total volume of 15 ml in each culture (3×10^5 cells/ml). In the presence of S-9 mix, the same number of cells were mixed with aliquots of the test material formulation (0.5 ml) and S-9 mix (0.5 ml) in a final total volume of 3 ml (1.5×10^6 cells/ml).

The final concentrations of the test material in each test were as follows:

First test, without S-9 mix:	313, 625, 1250, 2500, 3500 and 5000 µg/ml
with S-9 mix:	625, 1250, 2500 and 5000 µg/ml
Repeat first test, without S-9 mix:	625, 1250, 2500, 3500, 4000, 4500 and 5000 µg/ml
Second test, without S-9 mix:	625, 1250, 2500, 3500, 4000, 4500 and 5000 µg/ml
with S-9 mix:	625, 1250, 2500 and 5000 µg/ml.

The highest test concentration selected (5000 µg/ml) is the highest required by the OECD and EU test guidelines for materials of low toxicity. Several closely-spaced concentrations were used in the range 2500 to 5000 µg/ml in the absence of S-9 mix because a steep dose-toxicity relationship was observed in the first test.

Duplicate cultures were used at each test concentration. Untreated and positive controls were included in each test.

The positive control used without S-9 mix was ethylnitrosourea (ENU, 50 and 150 µg/ml) and the positive control used with S-9 mix was dimethylbenzanthracene (DMBA, 3.3 and 10 µg/ml). Single cultures were used at each dose level.

The treated cultures were incubated at 37°C with gentle shaking for 4 hours (first test) or 24 hours (second test) in the absence of S-9 mix, or for 3 hours (both tests) in the presence of S-9 mix.

At the end of the appropriate incubation period, the cultures were centrifuged (10 min at 764 g), the supernatants were discarded, and the cells were resuspended in fresh medium with 10% conditioned medium (15 ml) to give a cell density of approximately 3×10^5 cells/ml.

The cultures were then incubated at 37 °C in an atmosphere with 5% CO₂ for 3 days. During this phenotypic expression period, the cultures were counted daily and diluted to 3×10^5 cells/ml.

At the end of the exposure period, a small sample of cells was taken from each culture, diluted and seeded into a microtiter plate for determination of the cloning efficiency (2 cells/well). At the end of the expression period the cultures were diluted and a sample of cells was seeded in two microtiter plates (2000 cells/well) in medium containing 10 µg/ml trifluorothymidine (TFT) to count mutant clones, while another sample was seeded in one microtiter plate in normal medium (2 cells/well) for determination of the cloning efficiency.

After 10 days incubation (37°C, 5% CO₂) the number of cell clones in each plate was counted. The mutant clones counted on plates containing TFT were scored as 'large' or 'small, dense' clones. There is evidence to suggest that most large clones result from gene mutations, while most small clones result from large deletions (chromosome aberrations). The mutation frequencies were determined for each culture. The relative total growth was also calculated for each culture as the relative suspension growth (the increase in cell numbers during the treatment and expression periods) multiplied by Day 3 cloning efficiency.

Evaluation of results

The cloning efficiency (CE) and the mutation frequency (MF) were calculated for each culture using the formulae:

$$\begin{aligned} \text{CE} &= \{-\ln(X/N)\}/2 \\ \text{MF} &= \{-\ln(X/N)\}/(2000 \times \text{CE}) \end{aligned}$$

where X = number of empty wells and N = number of wells seeded).

The calculated mutation frequencies of the groups treated with the test material were compared to the negative control groups using the Analysis of Variance test (general linear model, least square mean). The statistical analyses were made with SAS[®] Procedures (Version 6.12) described in "SAS/STAT[®] User's Guide, Version 6, Fourth Edition, Vol. 2", 1989, SAS Institute Inc., Cary, North Carolina 27513, USA.

Archives

For a period of 10 years the following material relating to the study will be retained in the archives of Scantox:

- Protocols, protocol amendments and correspondence
- Test material receipts
- All original data
- Final report

At the end of the storage period Scantox will contact the Sponsor for instructions whether the material should be transferred, retained or destroyed.

RESULTS

The results are presented in Tables 1-3.

Toxicity

In the absence of S-9 mix, cultures treated at 5000 µg/ml in the first test showed severe toxicity (the cloning efficiency at the end of the exposure period was 6% of the negative control value and cell numbers fell during the expression period) and they were not plated to determine Day 3 cloning efficiency or mutation frequency. At the lower concentrations (313-3500 µg/ml), little toxicity was observed (relative total growth was similar to the control values at each concentration). This part of the test was repeated with additional test concentrations in the range 3500-5000 µg/ml to investigate the steep concentration/toxicity relationship. In the repeat test, the test material was less toxic and the relative total growth at 5000 µg/ml was 35% of the negative control value. A similar reduction in relative total growth (to 39% of the control value) was observed at this test point in the second test.

In the presence of S-9 mix, moderate reductions in relative total growth were observed in cultures treated at 5000 µg/ml in both tests (to 77% of the negative control value in the first test and 48% in the second test).

The highest concentration tested (5000 µg/ml) is the highest required by the OECD and EU guidelines for materials of low toxicity. Some insoluble material present in the treatment formulations of the test material was carried through the centrifugation steps at the end of the exposure and remained in the cultures through the expression and cloning phases of the tests.

Mutation

The mutation frequencies of the negative and positive controls were within the expected ranges.

No biologically or statistically significant increases in mutation frequency were observed in cultures treated with the test material, compared to the negative control values, in the presence or absence of S-9 mix, in either test.

The sensitivity of the test and the efficacy of the S-9 mix were demonstrated by large increases in mutation frequency in the positive control cultures.

CONCLUSION

It is concluded that *Haematococcus pluvialis* was not mutagenic in this *in vitro* mammalian cell gene mutation test performed with mouse lymphoma cells in the presence and absence of S-9 mix.

Haematococcus pluvialis
In Vitro Mammalian Cell Gene Mutation Test
 Performed with Mouse Lymphoma Cells (L5178Y)

TEST 1, WITHOUT S-9 MIX:

DOSE ($\mu\text{g/ml}$)	CLONING EFFICIENCY		CLONES IN TFT (192 WELLS)		MUTATION FREQUENCY (per 10000 cells)		
	DAY 0 PLATING	DAY 3 PLATING	LARGE	SMALL	LARGE	SMALL	TOTAL
0	0.34	0.71	8	7	0.30	0.26	0.57
0	0.19	0.76	8	8	0.28	0.28	0.57
3500	0.18	0.78	8	8	0.27	0.27	0.55
3500	0.19	0.76	7	7	0.24	0.24	0.50
2500	0.17	0.74	12	8	0.44	0.29	0.75
2500	0.19	1.00	9	7	0.24	0.19	0.44
1250	0.19	0.71	7	8	0.26	0.30	0.57
1250	0.24	0.71	8	7	0.30	0.26	0.57
625	0.19	0.69	7	9	0.27	0.35	0.63
625	0.17	0.67	9	6	0.36	0.24	0.60
313	0.16	0.69	7	7	0.27	0.27	0.55
313	0.25	0.65	8	7	0.33	0.28	0.62
ENU (1)	0.12	0.53	61	31	3.58	1.65	6.11
ENU (2)	0.10	0.44	84	33	6.57	2.15	10.74

TEST 1, WITH S-9 MIX:

DOSE $\mu\text{g/ml}$	CLONING EFFICIENCY		CLONES IN TFT (192 WELLS)		MUTATION FREQUENCY (per 10000 cells)		
	DAY 0 PLATING	DAY 3 PLATING	LARGE	SMALL	LARGE	SMALL	TOTAL
0	0.32	0.76	8	5	0.28	0.17	0.46
0	0.24	0.71	10	6	0.37	0.22	0.61
5000	0.15	0.74	11	3	0.40	0.11	0.51
5000	0.14	0.87	12	7	0.37	0.21	0.60
2500	0.22	0.71	11	6	0.41	0.22	0.65
2500	0.17	0.65	11	8	0.45	0.33	0.80
1250	0.17	0.67	12	6	0.48	0.24	0.73
1250	0.16	0.62	5	4	0.21	0.17	0.39
625	0.24	0.76	11	7	0.39	0.24	0.65
625	0.22	0.67	9	7	0.36	0.28	0.65
DMBA (3)	0.12	0.46	41	21	2.59	1.25	4.21
DMBA (4)	0.04	0.25	50	27	5.99	3.01	10.17

(1) Positive control, 50 $\mu\text{g/ml}$ ENU(2) Positive control, 150 $\mu\text{g/ml}$ ENU(3) Positive control, 3.3 $\mu\text{g/ml}$ DMBA(4) Positive control, 10 $\mu\text{g/ml}$ DMBA $p > 0.05$, versus negative controls (positive controls were not included in the statistical analysis)

Haematococcus pluvialis
In Vitro Mammalian Cell Gene Mutation Test
 Performed with Mouse Lymphoma Cells (L5178Y)

TEST 1 (REPEAT). WITHOUT S-9 MIX:

DOSE µg/ml	CLONING EFFICIENCY		CLONES IN TFT (192 WELLS)		MUTATION REQUENCY (PER 10000 CELLS)		
	DAY 0 PLATING	DAY 3 PLATING	LARGE	SMALL	LARGE	SMALL	TOTAL
0	0.57	0.84	11	9	0.35	0.29	0.66
0	0.74	0.87	8	8	0.25	0.25	0.50
5000	0.40	0.87	10	9	0.31	0.28	0.60
5000	0.67	0.76	7	8	0.24	0.28	0.54
4500	0.50	0.71	11	6	0.41	0.22	0.65
4500	0.50	0.87	10	8	0.31	0.25	0.57
4000	0.43	0.81	10	9	0.33	0.30	0.64
4000	0.57	0.74	8	6	0.29	0.22	0.51
3500	0.44	0.81	9	9	0.30	0.30	0.61
3500	0.53	0.81	10	9	0.33	0.30	0.64
2500	0.60	0.84	10	9	0.32	0.29	0.62
2500	0.60	0.71	9	9	0.34	0.34	0.69
1250	0.69	0.78	8	7	0.27	0.24	0.52
1250	0.67	0.76	9	7	0.32	0.24	0.57
625	0.78	0.76	9	7	0.32	0.24	0.57
625	0.49	0.76	8	8	0.28	0.28	0.57
ENU (1)	0.60	0.34	59	40	5.46	3.47	10.78
ENU (2)	0.35	0.34	72	43	6.99	3.77	13.59

(1) Positive control, 50 µg/ml ENU

(2) Positive control, 150 µg/ml ENU

p>0.05, versus negative controls (positive controls were not included in the statistical analysis)

Haematococcus pluvialis
In Vitro Mammalian Cell Gene Mutation Test
 Performed with Mouse Lymphoma Cells (L5178Y)

TEST 2. WITHOUT S-9 MIX:

DOSE µg/ml	CLONING EFFICIENCY		CLONES IN TFT (192 WELLS)		MUTATION REQUENCY (PER 10000 CELLS)		
	DAY 0 PLATING	DAY 3 PLATING	LARGE	SMALL	LARGE	SMALL	TOTAL
0	0.46	0.78	8	7	0.27	0.24	0.52
0	0.58	0.74	8	6	0.29	0.22	0.51
5000	0.25	0.63	6	3	0.25	0.12	0.38
5000	0.26	0.65	7	6	0.28	0.24	0.54
4500	0.21	0.63	8	5	0.34	0.21	0.55
4500	0.24	0.63	7	5	0.29	0.21	0.51
4000	0.30	0.71	9	6	0.34	0.22	0.57
4000	0.45	0.74	9	5	0.33	0.18	0.51
3500	0.50	0.81	7	7	0.23	0.23	0.47
3500	0.58	0.81	8	6	0.26	0.20	0.47
2500	0.55	0.76	8	7	0.28	0.24	0.54
2500	0.38	0.76	9	6	0.32	0.21	0.54
1250	0.43	0.81	8	6	0.26	0.20	0.47
1250	0.40	0.78	9	9	0.31	0.31	0.63
625	0.46	0.67	7	7	0.28	0.28	0.56
625	0.38	0.67	9	6	0.36	0.24	0.60
ENU (1)	0.10	0.50	70	70	4.49	4.49	12.95
ENU (2)	0.19	0.17	43	43	7.68	7.68	17.99

TEST 2. WITH S-9 MIX:

DOSE µg/ml	CLONING EFFICIENCY		CLONES IN TFT (192 WELLS)		MUTATION REQUENCY (PER 10000 CELLS)		
	DAY 0 PLATING	DAY 3 PLATING	LARGE	SMALL	LARGE	SMALL	TOTAL
0	0.41	0.96	12	10	0.34	0.28	0.63
0	0.39	0.90	11	11	0.33	0.33	0.68
5000	0.19	0.81	12	8	0.40	0.26	0.68
5000	0.23	0.71	10	9	0.37	0.34	0.73
2500	0.31	0.90	11	10	0.33	0.30	0.65
2500	0.35	0.71	7	7	0.26	0.26	0.53
1250	0.49	0.93	11	10	0.32	0.29	0.62
1250	0.38	0.87	10	7	0.31	0.21	0.54
625	0.44	0.81	9	9	0.30	0.30	0.61
625	0.38	0.87	11	8	0.34	0.25	0.60
DMBA (3)	0.30	0.67	65	59	3.07	2.73	7.71
DMBA (4)	0.32	0.48	71	65	4.84	4.33	12.92

(1) Positive control, 50 µg/ml ENU

(2) Positive control, 150 µg/ml ENU

(3) Positive control, 3.3 µg/ml DMBA

(4) Positive control, 10 µg/ml DMBA

p>0.05, versus negative controls (positive controls were not included in the statistical analysis)