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SRI International

IN VITRO MICROBIOLOGICAL MUTAGENICITY ASSAYS OF 3M COMPANY'S COMPOUND T-3727

Final Report

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SUMMARY

SRI International examined 3M Company's Compound T-3727 for mutagenic activity in the standard Ames Salmonella/microsome assay with strains TA1535, TA1537, TA1538, TA98, and TA100 of the bacterium Salmonella typhimurium. The assay was performed in the presence and absence of a rat-liver metabolic activation system. All tests were performed in compliance with the United States Food and Drug Administration Good Laboratory Practice Standards.

Compound T-3727 was reproducibly nonmutagenic when tested according to these procedures.

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QUALITY ASSURANCE UNIT

Final Report Statement

SRI International assures the quality and integrity of this study, In-Vitro Microbiological Mutagenicity Assays of Compound T-3727, for the 3M Company.

The study was inspected on March 12, 1985 during the colony counting phase. The findings of the Quality Assurance Unit inspection were reported at the time of the inspection to the Study Director. SRI management was informed of the inspection results on March 12, 1985. A data audit was performed on March 25, 1985. The Study Director and SRI management were informed of the audit results on March 25, 1985.

The final report was audited and reviewed on March 25, 1985. The results of the final report review were communicated to the Study Director and SRI management on March 25, 1985. The final report accurately describes the methods and standard operating procedures and reflects the raw data of the study. Any deviations from the approved protocol and standard operating procedures were made with proper authorization and documentation.

Sherry J. Heuer

Quality Assurance Director

March 25, 1985

Date

INTRODUCTION

SRI International examined 3M Company's Compound T-3727 for mutagenicity in the standard Ames Salmonella/microsome assay with strains TA1535, TA1537, TA1538, TA98, and TA100 of the bacterium Salmonella typhimurium. An Aroclor 1254-stimulated, rat-liver homogenate metabolic activation system was included in the assay procedure to provide metabolic steps that the microorganisms either are incapable of conducting or do not carry out under the assay conditions.

The assay procedure with S. typhimurium has proven to be 80 to 90% reliable in detecting carcinogens as mutagens, and it has about the same reliability in identifying chemicals that are not carcinogenic. However, because the assay systems do not always provide 100% correlation with carcinogenicity investigations in animals, neither a positive nor a negative response conclusively proves that a chemical is carcinogenic or noncarcinogenic to man.

Evaluation of experimental results from the Salmonella assay consists of comparing the number of histidine-independent colonies on the treated agar plates with the number observed on the control plates. Because all the plated Salmonella indicator organisms undergo a few cell divisions in the presence of the test chemical, the test is semiquantitative in nature. The plate test procedure does not permit quantitative determination of the number of cells surviving the chemical treatment. It is the demonstration of a mutagenic dose-response relationship that is important in establishing mutagenicity.

The test chemicals are assayed at several dose levels within a nontoxic dose range--with the exception of the highest dose level, which sometimes exhibits toxicity. Toxicity is evidenced by several phenomena: clearing of the background bacterial lawn growth, formation of pinpoint

colonies consisting of surviving cells, and a decrease in the number of revertant colonies below the spontaneous background.

A chemical is considered a mutagen in the Salmonella assay if it elicits a reproducible, dose-related increase in the number of histidine revertants per plate in one or more tester strains.

The assays with Compound T-3727 were begun on 20 February 1985 and testing was completed on 27 February 1985. Copies of the final report will be kept in our files (Building M, Room 213) and in SRI's Records Center. The raw data will be retained in Building 205, Room 13, for one year after the laboratory notebook has been filled and then will be stored in SRI's Records Center. All that remains of Compound T-3727 will be kept for six months in our chemical storage room (Building M, Room 217) and then returned to 3M Company.

MATERIALS

- Test Article
  - Name: T-3727
  - Date Received: 19 February 1985
  - Description: Light-amber waxy solid
  - Storage Conditions: Stored at room temperature in a secondary container
  - Special Testing Conditions: None
  - Stability: Assured by Sponsor

- Indicator Organisms
  - Species: *Salmonella typhimurium* LT2
  - Strains: TA1535, TA1537, TA1538, TA98, and TA100 for *S. typhimurium*
  - Source: Dr. Bruce Ames, University of California, Berkeley

- Metabolic Activation

  Aroclor 1254-induced, rat liver S-9; SRI Batch F-4;
  ~ 26.5 mg/ml protein

- Negative (Solvent) Control Material

  Acetone, CAS No. 67-64-1
  Date Opened: 14 December 1984
  Expiration Date: 14 December 1985
  Manufacturer: American Scientific Products, McGraw Park, IL
  Purity: 99.7%
  Lot No.: KTEA

- Positive Control Chemicals

  9-Aminoacridine, CAS No. 90-45-9
  Manufacturer: Pfaltz and Bauer, Stamford, CT

  2-Anthramine, CAS No. 613-13-8
  Manufacturer: Sigma Chemical Co., St. Louis, MO

  2-Nitrofluorene, CAS No. 607-57-8
  Manufacturer: Aldrich Chemical Co., Milwaukee, WI

  Sodium Azide, CAS No. 26628-22-8
  Manufacturer: Difco Laboratories, Detroit, MI

- Counters Used

  - New Brunswick Scientific BioTran II® Automated Colony Counter, Model C111, SRI No. 0030 6126 00

  - New Brunswick Scientific Bactronic® Colony Counter, Model C110, SRI No. 0012 3108 00

METHODS

Salmonella typhimurium Strains TA1535, TA1537, TA1538, TA98, and TA100

The *Salmonella typhimurium* strains used at SRI are all histidine auxotrophs by virtue of mutations in the histidine operon. When these histidine-dependent cells are grown on minimal medium agar plates containing a trace of histidine, only those cells that revert to histidine independence (*his*$^+$) are able to form colonies. The small amount of histidine allows all the plated bacteria to undergo a few divisions; in many cases, this growth is essential for mutagenesis to occur. The *his*$^+$ revertants are easily visible as colonies against the slight background growth. The spontaneous mutation frequency of each strain is relatively constant, but when a mutagen is added to the agar, the mutation frequency is increased, usually in a dose-related manner.

We obtained our *S. typhimurium* strains from Dr. Bruce Ames of the University of California at Berkeley. In addition to having mutations in the histidine operon, all the indicator strains have a mutation (*rfa*) that leads to a defective lipopolysaccharide coat; they also have a deletion that covers genes involved in the synthesis of the vitamin biotin (*bio*) and in the repair of ultraviolet (uv)-induced DNA damage (*uvrB*). The *rfa* mutation makes the strains more permeable to many large molecules, thereby increasing the mutagenic effect of these molecules. The *uvrB* mutation renders the bacteria unable to use the accurate excision repair mechanism to remove certain chemically or physically induced DNA lesions and thereby enhances the strains' sensitivity to some mutagenic agents. Strain TA1535 is reverted to *his*$^+$ by many mutagens that cause base-pair substitutions. Strain TA100 is derived from TA1535 by the introduction of the resistance transfer factor, plasmid pKM101. This plasmid is believed to cause an increase in error-prone DNA repair that leads to many more mutations for

a given dose of most mutagens. In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of the plasmid in the cell. The presence of this plasmid also makes strain TA100 sensitive to some frameshift mutagens [e.g., ICR-191, benzo(a)pyrene, aflatoxin B_1, and 7,12-dimethylbenz(a)anthracene]. Strains TA1537 and TA1538 are reverted by many frameshift mutagens. Strain TA98 is derived from TA1538 by the addition of the plasmid pKM101, which makes it more sensitive to some mutagenic agents.

All indicator strains are kept frozen in nutrient broth supplemented with 10% sterile glycerol at -80°C in 1-ml aliquots containing about 10^9 cells. New frozen stock cultures are made every three months from single colony isolates that have been checked for their genotypic characteristics (his, rfa, uvrB, bio) and for the presence of the plasmid. For each experiment, the frozen 1-ml cell cultures are allowed to thaw at room temperature before inoculation in 50 ml of glucose minimal liquid medium supplemented with an excess of biotin and histidine. The cultures are grown at 37°C, unshaken for 4 hours, then gently shaken (100 rpm) for 11 to 14 hours. All strains are genetically analyzed whenever experiments are performed.

Aroclor 1254-Stimulated Metabolic Activation System

Some carcinogenic chemicals (e.g., of the aromatic amine type or the polycyclic hydrocarbon type) are inactive unless they are metabolized to active forms. In animals and man, an enzyme system in the liver or other organs (e.g., lung or kidney) is capable of metabolizing a large number of these chemicals to carcinogens. Some of these intermediate metabolites are very potent mutagens in the S. typhimurium test. Ames has described the liver metabolic activation system that we use. In brief, adult male Sprague-Dawley rats (200 to 250 g) are given a single 500-mg/kg intraperitoneal injection of Aroclor 1254 (a mixture of polychlorinated biphenyls). This treatment enhances the synthesis of enzymes involved in the metabolic conversion of chemicals. Four days after the injection, the animals' food

is removed but drinking water is provided ad libitum. On the fifth day, the rats are killed and the liver homogenate is prepared as follows.

The livers are removed aseptically and placed in a preweighed, sterile glass beaker. The organ weight is determined, and all subsequent operations are conducted in an ice bath. The livers are washed with an equal volume of cold, sterile 0.15 M KCl, minced with sterile surgical scissors in three volumes of 0.15 M KCl (3 ml/g of wet organ), and homogenized with a Potter-Elvehjem apparatus. The homogenate is centrifuged for 10 minutes at 9000 × g, and the supernatant, referred to as the S-9 fraction, is quickly frozen on dry ice and stored at -80°C.

The metabolic activation mixture for each experiment consists of, for 50 ml:

- 5.0 ml of S-9 fraction
- 1.0 ml of $MgCl_2$ (0.4 M) and KCl (1.65 M)
- 0.25 ml of glucose-6-phosphate (1 M)
- 2.0 ml of NADP (0.1 M)
- 25.0 ml of sodium phosphate buffer (0.2 M, pH 7.4)
- 16.75 ml of sterile H_2O.

The amount of S-9 fraction delivered to each plate is 50 µl.

Plate Incorporation Assay

Prior to testing, the test article is serially diluted from an initial stock. In some cases, a preliminary experiment is conducted to find a suitable dose range for testing. The article is usually tested over a minimum of six dose levels, the highest nontoxic dose level being 10 mg/plate unless solubility, mutagenicity, or toxicity dictates a lower upper limit. When extracts are made, various undiluted aliquots are tested, usually over a dose range of 5 to 100 or 200 µl/plate. When liquids are tested, occasionally the sample is not diluted and various aliquots are used. All assays are repeated at least once on a separate day.

The plate incorporation assay is performed in the following way. To a sterile 13 × 100-mm test tube placed in a 43°C heating block we add:

(1) 2.00 ml of 0.6% agar containing 0.6% NaCl, 0.05 mM biotin, and 0.05 mM histidine

(2) 0.05 ml of indicator organisms (about 10^8 bacteria)

(3) 0.05 ml of a solution of the test article

(4) 0.50 ml of metabolic activation mixture (if appropriate).

This mixture is stirred gently and then poured on plates containing about 25 ml of minimal glucose agar. After the top agar has set, the plates are incubated for 48 hours at 37°C. The number of his$^+$ revertant colonies is counted using a BioTran II automated colony counter when possible. When accurate counts cannot be obtained (e.g., because of precipitate), the plates are counted manually using an electric probe colony counter.

Concurrent sterility, negative (solvent), and positive controls are run with every experiment. Sterility controls include plating out separately steps (3) and (4). For negative controls, we use steps (1), (2), (4), and 0.05 ml of the solvent used for the test article, if appropriate. For positive controls, we test each bacterial culture using the steps (1), (2), (3), and (4) with the following mutagens:

- Sodium azide for the base-pair substitution mutants TA1535 and TA100
- 9-Aminoacridine for the frameshift mutant TA1537
- 2-Nitrofluorene for the frameshift mutants TA1538 and TA98
- 2-Anthramine for all tester strains, in the presence of metabolic activation.

Statistical Analysis

No statistical analysis is performed. Results are a tabulation of the number of colonies appearing on the plates.

Criteria for Interpretation

Positive. A test article is considered a mutagen when it produces a reproducible, dose-related increase in the number of revertants in one or more strains. This increase must occur for at least three dose levels.

Negative. A test article is considered a nonmutagen when no dose-related increase in the number of revertants is observed in at least two independent experiments. The maximum dose level tested for nontoxic compounds is 10 mg/plate (unless dictated otherwise by solubility problems). For toxic compounds, only the highest dose level tested should show evidence of toxicity.

Inconclusive. When a test article cannot be identified clearly as a mutagen or nonmutagen in the standard plate assay, the results are classified as inconclusive.

References

Ames, B. N., E. G. Gurney, J. A. Miller, and H. Bartsch. Carcinogens as frameshift mutagens: Metabolites and derivatives of 2-acetylaminofluorene and other aromatic amine carcinogens. Proc. Natl. Acad. Sci. USA 69, 3128-3132 (1972).

Ames, B. N., W. E. Durston, E. Yamasaki, and F. D. Lee. Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. Proc. Natl. Acad. Sci. USA 70, 2281-2285 (1973).

Ames, B. N., J. McCann, and E. Yamasaki. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat. Res. 31, 347-364 (1975).

McCann, J., E. Choi, E. Yamasaki, and B. N. Ames. Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72, 5135-5139 (1975).

McCann, J., and B. N. Ames. Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals: Discussion. Proc. Natl. Acad. Sci. USA 73, 950-954 (1976).

Mortelmans, K. E., and B.A.D. Stocker. Segregation of the mutator property of plasmid R46 from its ultraviolet-protecting property. Mol. Gen. Genet. 167, 317-327 (1979).

RESULTS AND DISCUSSION

3M Company's Compound T-3727 was screened for mutagenic activity in the Ames Salmonella/microsome in vitro mutagenicity assay using the five standard strains of Salmonella typhimurium: TA1535, TA1537, TA1538, TA98, and TA100. The assays were performed in duplicate, both in the presence and absence of a rat-liver metabolic activation system. Three plates per dose level were tested. Acetone was used as the solvent.

The microbial mutagenicity testing of this sample was performed on 20 and 27 February 1985. Dose levels ranging from 10 to 5000 μg/plate were used for both assays (Tables 1 and 2). No dose-related increases in the number of histidine-independent revertants were observed in either assay. A black precipitate was noted at 5000 μg/plate; therefore, these plates were hand-counted.

We conclude that Compound T-3727 was reproducibly nonmutagenic when tested according to these procedures.

Table 1

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM

COMPOUND T-3727

Experiment Date: 20 February 1985

Compound	Metabolic Activation	Compound Added per Plate	Histidine Revertants per Plate													
			TA1535			TA1537			TA1538			TA98			TA100		
Negative Control																
Acetone	-	50 µl	20	22	32	3	10	3	17	18	17	18	26	32	125	124	129
	+	50	17	8	8	11	7	6	28	25	21	41	38	33	130	129	149
Positive Controls																	
Sodium Azide	-	1 µg	690	661	752										589	574	567
9-Aminoacridine	-	50				520	662	787									
2-Nitrofluorene	-	5							1440	1244	1552	942	895	1144			
2-Anthramine	-	1							23	34	27	37	36	34	150	136	151
	+	1							182	177	164	187	160	240	437	463	428
	-	2.5	37	37	27	8	11	6									
	+	2.5	405	347	338	87	89	95									
Compound T-3727	-	10 µg	25	15	25	10	7	5	19	19	17	30	19	26	117	125	116
	-	50	30	21	26	9	6	5	14	13	17	29	23	38	111	145	115
	-	100	28	32	20	8	8	6	8	16	10	22	26	26	109	122	96
	-	500	23	21	24	8	7	8	14	10	12	23	13	23	137	122	108
	-	1000	28	16	29	5	7	7	13	16	17	29	17	19	123	127	111
	-	5000*	25	23	17	6	12	6	11	12	7	23	24	22	109	127	122
	+	10	13	15	18	15	9	9	21	25	22	53	28	31	106	153	116
	+	50	10	15	12	5	10	8	12	24	17	37	44	35	111	124	118
	+	100	16	16	12	6	13	13	25	27	23	25	29	23	120	130	115
	+	500	5	11	9	5	15	9	25	24	22	28	24	27	112	136	99
	+	1000	12	10	6	11	19	4	24	20	21	41	33	36	141	118	126
	+	5000*	12	17	8	5	9	11	21	17	19	31	20	43	146	120	125

*Precipitated at this dose level; hand-counted.

Table 2

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM

COMPOUND T-3727

Experiment Date: 27 February 1985

Compound	Metabolic Activation	Compound Added per Plate	Histidine Revertants per Plate														
			TA1535			TA1537			TA1538			TA98			TA100		
Negative Control																	
Acetone	–	50 µl	10	22	19	7	6	10	8	11	11	19	21	18	98	92	90
	+	50	8	15	7	16	6	8	8	18	19	18	26	26	103	91	89
Positive Controls																	
Sodium Azide	–	1 µg	464	492	477										500	549	544
9-Aminoacridine	–	50				111	293	346									
2-Nitrofluorene	–	5							1253	1240	1102	783	689	737			
2-Anthramine	–	1							8	15	22	40	21	22	128	125	129
	+	1							193	158	151	141	168	158	382	378	334
	–	2.5	24	21	20	8	10	6									
	+	2.5	215	212	220	68	72	68									
Compound T-3727	–	10 µg	21	26	16	3	3	7	16	16	20	23	19	20	122	97	96
	–	50	20	24	17	8	5	7	18	14	9	28	19	20	97	120	101
	–	100	23	14	22	6	3	8	11	9	12	21	19	15	115	84	88
	–	500	20	13	17	4	9	8	18	11	13	32	26	19	85	103	93
	–	1000	23	25	20	7	4	6	7	16	10	30	12	20	108	98	94
	–	5000*	19	13	22	7	4	9	10	8	4	30	23	21	82	92	109
	+	10	11	10	8	16	12	20	11	11	11	32	24	27	138	113	128
	+	50	10	8	8	11	8	6	15	15	9	26	34	19	130	79	132
	+	100	10	15	17	9	10	11	17	21	12	26	27	18	114	94	113
	+	500	12	11	14	6	12	9	16	9	15	24	25	21	115	103	108
	+	1000	6	8	7	7	12	13	16	20	12	18	16	26	124	114	114
	+	5000*	10	7	11	11	9	9	20	13	17	32	26	36	122	114	127

*Precipitated at this dose level; hand-counted.