AR226-0684

IN VITRO MICROBIOLOGICAL MUTAGENICITY ASSAYS OF 3M COMPANY'S COMPOUND T-3651

Final Report

February 1985

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SUMMARY

SRI International examined 3M Company's Compound T-3651 for mutagenic activity in the standard Ames Salmonella/microsome assay with strains TA1535, TA1537, TA1538, TA98, and TA100 of the bacterium Salmonella typhimurium. Compound T-3651 was also screened for recombinogenic activity in the yeast Saccharomyces cerevisiae D3 assay. All assays were performed in the presence and absence of a rat-liver metabolic activation system.

Compound T-3651 was reproducibly nonmutagenic and nonrecombinogenic when tested according to these procedures.

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INTRODUCTION

SRI International examined 3M Company's Compound T-3651 for mutagenicity in the standard Ames Salmonella/microsome assay with strains TA1535, TA1537, TA1538, TA98, and TA100 of the bacterium Salmonella typhimurium. Compound T-3651 was also tested for recombinogenic activity in the yeast Saccharomyces cerevisiae D3 assay. An Aroclor 1254-stimulated, rat-liver homogenate metabolic activation system was included in the assay procedures 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. The assay procedure with S. cerevisiae is about 60% reliable in detecting carcinogens as agents that increase mitotic recombination. 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 non-toxic 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 yeast Saccharomyces cerevisiae D3 is a eukaryotic microorganism capable of detecting mitotic recombination, as expressed through a mutation leading to a defective enzyme in the adenine-metabolizing pathway, resulting in a red-pigmented colony. In this assay, the yeast cells are exposed to several concentrations of the test chemical, usually ranging from a concentration that results in no killing to one that causes 50% killing. Any concentration that induces 90% killing is considered toxic. When the number of genetically altered colonies per milliliter (yield) and the ratio of altered colonies to survivors (frequency) from the treated cells are unequivocally larger than those of the solvent-treated controls, we conclude that the exposure of the cells to the compound induces mitotic recombination. If this event is dose-related, the observation is termed a positive response.

MATERIALS

Test Articles

- Names: T-3651
- Date Received: 28 November 1984
- Description: Amber liquid (foams at high concentrations)
- Storage Conditions: Room temperature
- Special Testing Conditions: None
- Stability: Assured by Sponsor

Indicator Organisms

- Species: *Salmonella typhimurium* LT2; *Saccharomyces cerevisiae*
- Strains: TA1535, TA1537, TA1538, TA98, and TA100 for *S. typhimurium*; D3 for *S. cerevisiae*
- Source: Dr. Bruce Ames, University of California, Berkeley, for the *Salmonella*; Dr. F. K. Zimmermann, W. Germany, for the yeast

Metabolic Activation

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

Negative (Diluent) Control Material

Sterile water

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

1,2:3,4-Diepoxybutane, CAS No. 1464-53-5
Manufacturer: Pfaltz and Bauer, Stamford, CT

Sterigmatocystin, CAS No. 10048-13-2
Manufacturer: Aldrich Chemical Co., Milwaukee, WI

Counters Used

- New Brunswick Scientific BioTran II® Automated Colony Counter, Model C111, SRI Nos. 0030 6126 00 and 0012 3318 00

- New Brunswick Scientific Bactronic® Colony Counter, Model C110, SRI Nos. 0013 0788 00 and 0030 1471 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 resis-

tance 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/diluent), 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/diluent used for the test article, if appropriate. For positive controls, we test each bacterial culture with the following mutagens using steps (1), (2), (3), and (4):

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.

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.

Saccharomyces cerevisiae D3

The yeast S. cerevisiae D3 is a diploid microorganism heterozygous for a mutation leading to a defective enyzme in the adenine-metabolizing pathway. When grown on medium containing adenine, cells homozygous for this mutation produce a red pigment. These homozygous mutants can be generated from the heterozygotes by mitotic recombination. The frequency of this recombinational event may be increased by incubating the organisms with various carcinogenic or recombinogenic agents. The recombinogenic activity of a compound or its metabolite is determined from the number of red-pigmented colonies appearing on test plates.

A stock culture of S. cerevisiae is stored at 4°C. For each experiment, broth containing 0.05% $MgSO_4$, 0.15% KH_2PO_4, 0.45% $(NH_4)_2SO_4$, 0.35% peptone, 0.5% yeast extract, and 2% dextrose is inoculated with a loopful of the stock culture and incubated overnight at 30°C, with shaking.

The in vitro yeast mitotic recombination assay in suspension is conducted as follows. The overnight culture is centrifuged and the cells are resuspended at a concentration of 10^8 cells/ml in 67 mM phosphate buffer (pH 7.4). To a sterile test tube are added:

1.0 ml of the resuspended culture
0.5 ml of either the metabolic activation mixture or buffer
0.2 ml of the test chemical
0.3 ml of buffer.

Several doses of the test chemical are tested (up to 5% w/v or v/v) in each experiment, and appropriate solvent/diluent controls are included. 1,2:3,4-Diepoxybutane without metabolic activation and sterigmatocystin with activation are used as positive controls.

The suspension mixture is incubated at 30°C for 4 hours on a roller drum. The sample is then diluted serially in sterile physiologic saline, and 0.2 ml of the 10^{-5} and 10^{-3} dilutions is spread on plates containing

the same ingredients as the broth plus 2.0% agar; five plates are spread with the 10^{-3} dilution and three plates are spread with the 10^{-5} dilution. The plates are incubated for 3 days at 30°C, followed by at least 1 day at 4°C to enhance the development of the red pigment indicative of adenine-deficient homozygosity. Plates containing the 10^{-3} dilution are scanned with a dissecting microscope at 10× magnification, and the number of mitotic recombinants (red colonies or red sectors) is recorded. The surviving fraction of organisms is determined from the total number of colonies appearing on the plates of the 10^{-5} dilution.

Criteria for Interpretation

Positive. A positive response in this assay is indicated by a dose-related increase of more than threefold in the absolute number of mitotic recombinants per milliliter and in the relative number of mitotic recombinants per 10^5 survivors.

Negative. When no reproducible recombinogenic activity is obtained in any of the assays performed, the test results are considered to be negative.

Inconclusive. When a test article cannot be identified clearly as causing a positive or a negative response, the results are classified as inconclusive.

Statistical Analysis

No statistical analysis was performed for any of the assays. The results of the plate incorporation assay are a tabulation of the number of colonies appearing on the plates. The results of the *S. cerevisiae* D3 assay are tabulated after calculating the number of mitotic recombinants per 10^5 survivors. All calculations are expressed with two significant digits.

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RESULTS AND DISCUSSION

3M Company's Compound T-3651 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. Sterile water was used as the diluent.

The microbial mutagenicity testing of this sample was performed on 12 and 18 December 1984. Dose levels ranging from 10 to 5000 µg/plate were used for all Ames assays (Tables 1 and 2). Compound T-3651 foamed when it was diluted with water to form the stock solution of 100 mg/ml; further dilutions were performed once the foam settled. No dose-related increases in the number of histidine-independent revertants were observed with the compound in either assay.

Compound T-3651 was also screened for recombinogenic activity in the yeast Saccharomyces cerevisiae D3 assay for mitotic recombination. The assays were performed on 10 and 29 January 1985, both with and without metabolic activation (Tables 3 and 4). Sterile water was used as the diluent. At the high doses, T-3651 foamed and appeared to react with water. No dose-related increases in the number of mitotic recombinants per 10^5 survivors were observed with the test compound in either assay.

In conclusion, Compound T-3651 was reproducibly nonmutagenic and nonrecombinogenic when tested according to these procedures.

Table 1

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM

COMPOUND T-3651

Experiment Date: 12 December 1984

Compound	Metabolic Activation	Compound Added per Plate	Histidine Revertants per Plate									
			TA1535		TA1537		TA1538		TA98		TA100	
Negative Control												
Water	-	50 µl	19	24	10	10	14	11	25	39	159	146
	+	50	19	7	10	6	17	21	42	38	155	165
Positive Controls												
Sodium Azide	-	1 µg	460	376							615	621
9-Aminoacridine	-	50			223	670						
2-Nitrofluorene	-	5					1554	1441	1027	983		
2-Anthramine	-	1					28	19	47	31	180	149
	+	1					336	321	394	360	622	693
	-	2.5	17	16	10	17						
	+	2.5	249	226	128	92						
Compound T-3651	-	10 µg	23	24	9	9	21	15	21	20	143	139
	-	50	13	18	1	7	21	20	21	19	151	149
	-	100	13	11	4	4	18	12	15	17	139	115
	-	500	21	20	9	15	17	24	37	20	124	141
	-	1000	14	24	7	10	15	23	32	19	136	146
	-	5000	26	17	5	8	10	14	26	25	115	120
	+	10	14	12	6	8	20	11	32	26	178	182
	+	50	9	9	12	6	15	15	31	37	147	161
	+	100	15	14	13	8	17	9	28	32	137	152
	+	500	17	8	6	12	10	14	34	37	177	168
	+	1000	8	16	4	6	10	12	26	43	162	154
	+	5000	7	11	12	13	11	21	31	30	170	156

Table 2

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM

COMPOUND T-3651

Experiment Date: 18 December 1984

Compound	Metabolic Activation	Compound Added per Plate	Histidine Revertants per Plate									
			TA1535		TA1537		TA1538		TA98		TA100	
Negative Control												
Water	-	50 µl	21	26	7	8	10	10	24	25	128	150
	+	50	8	16	8	8	19	17	28	32	116	123
Positive Controls												
Sodium Azide	-	1 µg	557	641							652	636
9-Aminoacridine	-	50			331	305						
2-Nitrofluorene	-	5					1115	1105	708	653		
2-Anthramine	-	1					22	12	19	27	105	105
	+	1					137	122	171	151	319	343
	-	2.5	18	20	7	16						
	+	2.5	257	252	30	36						
Compound T-3651	-	10 µg	23	23	9	10	12	12	33	20	122	122
	-	50	29	24	5	7	19	12	21	15	108	101
	-	100	21	27	4	5	16	15	14	15	111	128
	-	500	25	17	7	10	20	8	20	29	103	94
	-	1000	18	16	7	8	17	7	13	18	93	123
	-	5000	23	24	9	8	18	11	17	20	106	115
	+	10	9	10	7	7	25	20	20	19	101	104
	+	50	6	10	4	8	15	17	36	28	111	108
	+	100	8	6	11	12	28	12	28	26	142	98
	+	500	15	7	12	5	28	26	32	36	130	106
	+	1000	9	10	7	6	23	18	28	25	102	99
	+	5000	14	10	6	12	19	13	23	30	103	94

Table 3

IN VITRO ASSAYS OF SACCHAROMYCES CEREVISIAE D3

COMPOUND T-3651

Experiment Date: 10 January 1985

Compound	Metabolic Activation	Percent Concentration (w/v)	Surviving Cells per ml ($\times 10^{-7}$)	Survivors (%)	Mitotic Recombinants per ml ($\times 10^{-3}$)	Mitotic Recombinants per 10^5 Survivors*
Negative Control						
Water	-		6.7	100	4	6.0
	+		5.8	100	2	3.4
Positive Controls						
1,2:3,4-Diepoxy-butane	-	0.025	6.2	93	971	1600
Sterigmatocystin	-	0.005	6.5	97	4	6.2
	+	0.005	7.1	100	210	300
Compound T-3651	-	0.05	7.1	100	10	14
	-	0.1	7.8	100	6	7.7
	-	0.5	6.9	100	12	17
	-	1.0	7.1	100	2	2.8
	-	5.0	6.7	100	3	4.5
	+	0.05	5.8	100	4	6.9
	+	0.1	6.9	100	8	12
	+	0.5	7.0	100	6	8.6
	+	1.0	7.5	100	5	6.7
	+	5.0	7.3	100	5	6.8

*All calculations are expressed to two significant figures.

Table 4

IN VITRO ASSAYS OF SACCHAROMYCES CEREVISIAE D3

COMPOUND T-3651

Experiment Date: 29 January 1985

Compound	Metabolic Activation	Percent Concentration (w/v)	Surviving Cells per ml ($\times 10^{-7}$)	Survivors (%)	Mitotic Recombinants per ml ($\times 10^{-3}$)	Mitotic Recombinants per 10^5 Survivors*
Negative Control						
Water	-		6.2	100	5	8.1
	+		6.4	100	8	13
Positive Controls						
1,2:3,4-Diepoxy-butane	-	0.025	6.3	100	933	1500
Sterigmatocystin	-	0.005	6.1	98	8	13
	+	0.005	6.3	98	235	370
Compound T-3651	-	0.05	6.5	100	10	15
	-	0.1	6.9	100	7	10
	-	0.5	6.4	100	8	13
	-	1.0	7.1	100	2	2.8
	-	5.0	6.5	100	13	20
	+	0.05	6.2	97	10	16
	+	0.1	6.4	100	4	6.3
	+	0.5	6.6	100	13	20
	+	1.0	6.8	100	12	18
	+	5.0	6.5	100	5	7.7

*All calculations are expressed to two significant figures.