SRI International

IN VITRO MICROBIOLOGICAL MUTAGENICITY ASSAYS OF 3M COMPANY COMPOUND T-2816CoC

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By: Kristien E. Mortelmans, Ph.D.
Director, Microbial Genetics Department
and
Anne Pomeroy, Microbiological Technician

Prepared for:

3M COMPANY
Medical Department
General Offices, 3M Center
St. Paul, Minnesota 55101

Attention: W. C. McCormick
Toxicology Services

SRI Project LSC-8958

Approved:

David C. L. Jones, Director
Toxicology Laboratory

W. A. Skinner, Executive Director
Life Sciences Division

SRI International
333 Ravenswood Ave. • Menlo Park, California 94025
(415) 326-6200 • Cable: SRI INTL MPK • TWX: 910-373-1246

SUMMARY

SRI International examined 3M Company's Compound T-2816CoC for mutagenic activity with strains TA1535, TA1537, TA1538, TA98, and TA100 of Salmonella typhimurium in the standard Ames Salmonella/microsome assay and with the yeast Saccharomyces cerevisiae D3. Each assay was performed in the presence and in the absence of a rat liver metabolic activation system. Compound T-2816CoC was not mutagenic or recombinogenic in any assay performed.

INTRODUCTION

SRI International examined 3M Company's Compound T-2816CoC for mutagenicity by *in vitro* microbiological assays with strains TA1535, TA1537, TA1538, TA98, and TA100 of the bacterium *Salmonella typhimurium* in the standard Ames *Salmonella*/microsome assay and with the yeast *Saccharomyces cerevisiae* D3. An Aroclor 1254-stimulated, rat liver homogenate metabolic activation system was included in the assay procedures to provide metabolic steps that the bacteria 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 hazardous or nonhazardous to man.

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 aromatic molecules, thereby increasing the mutagenic effect of these molecules. The uvrB mutation causes decreased repair of some types of chemically or physically damaged DNA and thereby enhances the strains' sensitivity to some mutagenic agents. Strain TA1535 is reverted to his$^+$ by many mutagens that cause base-pair substitutions. 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 at 4°C on minimal agar plates supplemented with an excess of biotin and histidine. The plates with the plasmid-carrying strains also contain ampicillin (25 μg/ml) to ensure stable maintenance of the plasmid pKM101. New stock culture plates are made every 4 to 6 weeks 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, an inoculum from the stock culture plates is grown overnight at 37°C in nutrient broth (Oxoid, CM67).

Aroclor 1254-Stimulated Metabolic Activation System

Some carcinogenic chemicals (e.g., of the aromatic amino 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 rats (250 to 300 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 (1 ml/g of wet organ), minced with sterile surgical scissors in three volumes of 0.15 M KCl, and homogenized with a Potter-Elvehjem apparatus. The homogenate is centrifuged for 10 minutes at 9000 x g, and the supernatant, referred to asthe S-9 fraction, is quickly frozen in dry ice and stored at -80°C.

The metabolic activation mixture for each experiments consists of, for 10 ml:

- 1.00 ml of S-9 fraction
- 0.20 ml of $MgCl_2$ (0.4 M) and KCl (1.65 M)
- 0.05 ml of glucose-6-phosphate (1 M)
- 0.40 ml of NADP (0.1 M)
- 5.00 ml of sodium phosphate buffer (0.2 M, pH 7.4)
- 3.35 ml of H_2O.

Assays in Agar

To a sterile 13 x 100 mm test tube placed in a 43°C heating block, we add in the following order:

(1) 2.00 ml of 0.6% agar*
(2) 0.05 ml of indicator organisms
(3) 0.50 ml of metabolic activation mixture (if appropriate)
(4) 0.05 ml of a solution of the test chemical.

This mixture is stirred gently and then poured onto minimal agar plates.† After the top agar has set, the plates are incubated at 37°C for 3 days. The number of *his*$^+$ revertant colonies is counted and recorded.

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*The 0.6% agar contains 0.05 mM histidine, 0.05 mM biotin, and 0.6% NaCl.

†Minimal agar plates consist of, per liter, 15 g of agar, 10 g of glucose, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2 g of citric acid monohydrate, 10 g of K_2PO_4, and 3.5 g of $NaHNH_4PO_4 \cdot 4H_2O$.

For negative controls, we use steps (1), (2), and (3) and 0.05 ml of the solvent used for the test chemical. For positive controls, we test each culture by specific mutagens known to revert each strain, using steps (1), (2), (3) and (4).

Saccharomyces cerevisiae D3

The yeast S. cerevisiae D3 is a diploid microorganism heterozygous for a mutation leading to a defective enzyme 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:

- 1.00 ml of the resuspended culture
- 0.50 ml of either the metabolic activation mixture or buffer
- 0.20 ml of the test chemical
- 0.30 ml of buffer.

Several doses of the test chemical are tested in each experiment, and appropriate controls are included.

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^{-3} and 10^{-5} 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 2 days at 30°C, followed by 2 days at 4°C to enhance the development of the red pigment indicative of adenine-deficient homozygosity. Plates containing the 10^{-5} dilution are scanned with a dissecting microscope at 10 X 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.

The number of mitotic recombinants is calculated per 10^5 survivors. A positive response in this assay is indicated by a dose-related increase of more than 3-fold in the absolute number of mitotic recombinants per milliliter as well as in the relative number of mitotic recombinants per 10^5 survivors.

RESULTS AND DISCUSSION

Compound T-2816CoC was tested for mutagenicity in the Ames Salmonella/microsome assay and with the yeast Saccharomyces cerevisiae D3 in the presence and in the absence of a metabolic activation system. The compound was tested at least twice on separate days in both assays. The results are presented in Tables 1 through 5.

In the Ames Salmonella/microsome assay, T-2816CoC was initially tested in a preliminary assay with strain TA100 over a wide range of concentrations, from 10 to 5,000 µg/plate. Toxicity was observed at a dose of 5,000 µg/plate (Table 1). Ethanol was used as the solvent in all assays.

The results of our tests of T-2816CoC with five strains of S. typhimurium in the Ames Salmonella/microsome assay are presented in Tables 2 and 3. No toxicity or dose-related increase in the number of revertants was observed in these assays.

The results of the microbiological assays with S. cerevisiae D3 on T-2816CoC are presented in Tables 4 and 5. The compound was tested at concentrations from 0.05 to 5.0%. No toxicity or significant dose-related increase in the number of mitotic recombinants above background was observed.

We therefore conclude that Compound T-2816CoC was not mutagenic with S. typhimurium or recombinogenic with S. cerevisiae D3.

Table 1

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM
COMPOUND T-2816Coc
Experiment Date: 13 February 1980

Compound	Metabolic Activation	Amount of Compound Added per Plate	Histidine Revertants per Plate TA100
Negative Control			
Ethanol	-	50 µl	114
	+	50	111
Positive Controls			
2-Anthramine	-	1.0 µg	102
	+	1.0	427
Sodium Azide	-	0.5 µg	367
Compound T-2816Coc	-	10.0 µg	127
	-	50.0	123
	-	100.0	93
	-	500.0	102
	-	1,000.0	97
	-	5,000.0	toxic
	+	10.0 µg	146
	+	50.0	116
	+	100.0	110
	+	500.0	108
	+	1,000.0	108
	+	5,000.0	toxic

Table 2

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM
COMPOUND T-2816CoC
Experiment Date: 20 February 1980

Compound	Metabolic Activation	Amount of Compound Added per Plate	Histidine Revertants per Plate									
			TA1535		TA1537		TA1538		TA98		TA100	
Negative Control												
Ethanol	-	50.0 µl	19	14	15	7	18	18	30	29	100	117
	-	50.0	26	21	5	14	9	8	31	27	120	91
	+	50.0	31	27	20	13	24	24	44	55	92	108
	+	50.0	25	32	21	20	55	26	67	56	140	114
Positive Controls												
2-Anthramine	-	1.0 µg					12	13	38	31	125	115
	+	1.0					320	330	299	319	542	550
	-	2.5	21	26	8	15						
	+	2.5	201	197	110	109						
Sodium Azide	-	0.5 µg									260	245
	-	1.0	293	370								
9-Aminoacridine	-	50.0 µg			180	193						
2-Nitrofluorene	-	5.0 µg					530	490	402	399		
Compound T-2816oC	-	5.0 µg	17	18	9	11	8	18	39	37	111	114
	-	10.0	32	20	7	9	8	15	38	28	93	89
	-	50.0	28	31	15	16	17	15	36	38	105	111
	-	100.0	28	18	16	9	14	15	33	34	110	99
	-	500.0	26	19	6	9	15	16	27	40	104	108
	-	1,000.0	29	28	6	8	18	12	37	32	97	103
	+	5.0	18	30	17	22	27	37	42	99	98	114
	+	10.0	26	24	12	13	36	27	61	55	122	120
	+	50.0	19	30	8	12	30	27	39	44	140	99
	+	100.0	26	38	16	12	19	28	49	53	126	101
	+	500.0	39	31	18	23	35	37	42	61	113	117
	+	1,000.0	36	30	6	9	19	42	51	63	113	115

Table 3

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM
COMPOUND T-2816CoC
Experiment Date: 25 February 1980

Compound	Metabolic Activation	Amount of Compound Added per Plate	Histidine Revertants per Plate									
			TA1535		TA1537*		TA1538		TA98		TA100*	
Negative Control												
Ethanol	-	50.0 µl	20	16	7	9	16	13	45	48	90	96
	+	50.0	36	31	19	3	24	19	77	69	137	139
Positive Control												
2-Anthramine	-	1.0 µg					13	19	43	40	126	96
	-	2.5	21	26	9	9						
	+	1.0					163	134	250	230	662	706
	+	2.5	219	176	137	138						
Sodium Azide	-	0.5 µg									403	369
	-	1.0	304	331								
9-Aminoacridine	-	50.0 µg			357	506						
2-Nitrofluorene	-	5.0 µg					934	813	517	461		
Compound T-2816Coc	-	5.0 µg	25	24	8	7	17	15	33	44	111	87
	-	10.0	19	26	7	8	9	7	35	54	137	114
	-	50.0	38	34	4	3	21	13	54	53	111	97
	-	100.0	32	35	6	9	10	11	56	41	122	87
	-	500.0	33	33	12	6	7	10	46	51	98	104
	-	1,000.0	22	26	6	7	14	18	41	48	97	123
	+	5.0 µg	38	37	17	9	36	33	70	62	126	116
	+	10.0	23	35	8	20	21	31	46	68	124	117
	+	50.0	30	39	9	15	20	21	55	53	140	124
	+	100.0	27	34	21	13	27	34	47	53	93	89
	+	500.0	34	28	16	14	28	23	50	57	110	97
	+	1,000.0	25	33	9	13	22	28	59	52	116	110

*Retested on 6 March; control values of 25 February were invalid.

Table 4

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE
COMPOUND T-2816Coc
Experiment Date: 13 February 1980

Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Survivors		Mitotic Recombinants	
			Cells per ml ($\times 10^{-7}$)	Percent	Per ml ($\times 10^{-5}$)	Per 10^5 Survivors
Negative Control						
Ethanol	-		6.3	100	6.0	9.0
	+		5.6	100	7.0	12
Positive Control						
1,2,3,4-Diepoxybutane	-	0.025	6.4	102	980	1,500
	+	0.025	7.3	116	1,000	1,400
Compound T-2816Coc	-	0.05	3.3	50	7.0	21
	-	0.1	6.6	105	5.0	7.6
	-	0.5	5.7	90	15	26
	-	1.0	5.0	79	7.0	14
	-	5.0	2.9	46	5.0	17
	+	0.05	3.4	61	3.0	8.8
	+	0.1	6.4	114	7.0	11
	+	0.5	5.3	95	11	21
	+	1.0	5.7	102	4.0	7.0
	+	5.0	5.2	93	11	21

Table 5

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE
COMPOUND T-2816Coc
Experiment Date: 21 February 1980

Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Survivors		Mitotic Recombinants	
			Cells per ml ($\times 10^{-7}$)	Percent	Per ml ($\times 10^{-3}$)	Per 10^5 Survivors
Negative Control						
Ethanol	-		5.4	100	4.5	8.5
	+		5.5	100	6.0	11
Positive Control						
1,2,3,4-Diepoxybutane	-	0.025	5.8	107	1,100	1,900
	+	0.025	5.7	104	1,100	1,900
Compound T-2816Coc	-	0.05	9.4	174	10	11
	-	0.1	9.4	174	16	17
	-	0.5	8.0	148	12	15
	-	1.0	8.1	150	9.0	11
	-	5.0	9.7	180	8.0	8.2
	+	0.05	8.0	160	9.0	11
	+	0.1	8.1	147	9.0	11
	+	0.5	8.6	156	5.0	5.8
	+	1.0	8.4	153	10	12
	+	5.0	8.4	153	4.0	4.8