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REPORT

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In Vitro Microbiological Mutagenicity Tests to Assess the Potential Mutagenic Effect of SAGAN COAT Photocatalyst Coating Solution TPX

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Abstract

SAGAN COAT Photocatalyst Coating Solution TPX was examined for its mutagenic activity in the pre-incubation Ames *Salmonella* microsome assay, using four strains of *Salmonella typhimurium* TA100, TA98, TA1535, TA1537 and *Escherichia coli* WP2*uvrA*. The assays were performed in both with and without rat-liver metabolic activation. No significant increases in the number of revertant colonies were observed in the tester strains, either with or without metabolic activation.

We concluded that the test substance showed no evidence of mutagenic potential at the dose levels used in this bacterial test system.

Statement of Study Director

I, the undersigned, hereby declare that the work described in this report was performed under my supervision, as Study Director, in compliance with Methods of Testing New Chemical Substances (Yakushokuhatsu No. 1121002 of the Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, Heisei 15・11・13 seikyoku No. 2 of the Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry and Kampokihatsu No. 031121002 of the Environmental Policy Bureau, Ministry of the Environment, November 21, 2003) with the exception of possible minor items, none of which is considered to have an impact on the validity of the data or the interpretation of the results in the report.

The experiments described in this report were carried out from July 6 to August 13, 2007.

This is a translation of the original report, No. 207062138-003, written in Japanese.

Study Director

Takanori Maeda August 27, 2007

Takanori Maeda Date
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In Vitro Microbiological Mutagenicity Tests to Assess the Potential Mutagenic Effect of SAGAN COAT Photocatalyst Coating Solution TPX

1. Purpose

The purpose of this study is to test the test substance for its mutagenic activity in the reverse mutation assay with four strains of *Salmonella typhimurium* TA100, TA98, TA1535, TA1537 and *Escherichia coli* WP2*uvrA*, as indicated by induction of mutant colonies in systems with and without rat-liver metabolic activation, in compliance with Methods of Testing New Chemical Substances (Yakushokuhatsu No. 1121002 of the Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, Heisei 15·11·13 seikyoku No. 2 of the Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry and Kampokihatsu No. 031121002 of the Environmental Policy Bureau, Ministry of the Environment, November 21, 2003)

2. Test substance

SAGAN COAT Photocatalyst Coating Solution TPX
Character: Yellowish suspended solution

3. Materials and methods

1) Preparation of the test solution

The test substance was dissolved in Dimethylsulfoxide (DMSO)[Dojindo Laboratories Co., Ltd](Lot No. TA026) to make a 50 mg/mL test solution. This was diluted with DMSO to prepare a series of the test solutions.

Negative control was DMSO alone.

2) Dose levels

Dose-range-finding test:
5000, 1250, 313, 78.1, 19.5 and 4.88 μg/plate
Mutation test:
5000, 2500, 1250, 625 and 313 μg/plate

3) Positive controls and solvents

a) Positive controls for each strain

S9Mix(-) S9Mix(+)

Strain	Chemical	Concentration (μg/plate)	Strain	Chemical	Concentration (μg/plate)
TA100	AF-2	0.01	TA100	2-AA	1
TA98	AF-2	0.1	TA98	2-AA	0.5
TA1535	NaN_3	0.5	TA1535	2-AA	2
TA1537	9-AA	80	TA1537	2-AA	2
WP2*uvrA*	AF-2	0.01	WP2*uvrA*	2-AA	10

AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide
NaN_3: Sodium Azide
9-AA: 9-Aminoacridine Hydrochloride
2-AA: 2-Aminoanthracene

b) Positive control substances and solvents

Substance		Supplier	Lot No.	Purity (%)	Solvent
Positive control	AF-2	Wako	SDJ4376	98.3	DMSO
	NaN_3	Wako	LTJ4146	99.4	Water
	9-AA	MP Biomedicals, LLC	2436F	98.8	DMSO
	2-AA	Wako	DPN4440	93.2	DMSO
Solvent	DMSO	Dojindo	TA026	>99.0	−
	Water	Otsuka	6K82N	−	−

Positive control solutions were stored at -80 ℃.
Wako: Wako Pure Chemical Industries, Ltd.
Dojindo: Dojindo Laboratories Co., Ltd.
Otsuka: Otsuka Pharmaceutical Factories Co., Ltd.
DMSO: Dimethylsulfoxide
Water: Water for injection (JP)

4) Test strains

Five strains, *Salmonella typhimurium* TA100, TA98, TA1535, TA1537 and *Escherichia coli* WP2*uvrA*, were used. All test strains in Nutrient broth No. 2 [OXOID] supplemented with 8 % sterile dimethylsulfoxide were kept frozen at -80 ℃. The strains were tested routinely for their biological as well as genetic characteristics (e.g. amino-acid requirements, presence of R-factor plasmid, etc.).

a) Test strains

Strain	Obtained from	Date obtained	Date of characteristic test
TA100	JAPAN INDUSTRIAL SAFETY & HEALTH ASSOCIATION JAPAN BIOASSAY RESEARCH CENTER	September 19, 2002	November 29, 2006
TA98			November 29 and December 5, 2006
TA1535			November 29, 2006
TA1537			November 29, 2006
WP2*uvrA*			November 29, 2006

b) Storage conditions of test strains

Storage conditions	0.1 mL each in plastic tubes
Volume of storage mixture	0.8 mL of cell suspension mixed with 0.07 mL of DMSO
Storage temperature	-80 ℃
Name and model of storage apparatus	Deep freezer MDF-293AT [SANYO Electric Biomedical Co., Ltd.]

5) Preparation of cell culture

Several microliters of a cell suspension having been frozen were put into 15 mL of Nutrient broth No. 2 [OXOID](Lot No. 392492) in an Erlenmeyer flask. It was cultured at 37 ℃ for 10 hours on a rotator. The grown cells were counted with a turbidimeter and the cell concentration was confirmed to be more than as 10^9/mL.

Name and model of incubator	BIO-SHAKER BR-40LF [TAITEC CO., LTD.]
Number of rotation	100 r/min^{-1}
Apparatus and volume	Erlenmeyer flask with baffled (100 mL)

6) S9 and S9Mix

a) Source of S9

Manufacturer	ORIENTAL YEAST CO., LTD.	Storage temperature	-80 ℃
Date of preparation	May 18, 2007	Name and model of storage apparatus	Deep freezer MDF-293AT [SANYO Electric Biomedical Co., Ltd.]
Date obtained	June 13, 2007		
Lot No.	07051802		

b) Preparation of S9

Animal used		Inducing substances	
Species	Rat	Name	Phenobalbital (PB) 5,6-Benzofravone (5,6-BF)
Strain	Sprague-Dawley		
Sex	Male	Administration route	Intraperitoneal injection
Age	7 weeks old		
Body weight	202.4 g±9.8 g	Administration schedule and dose	Day 1: PB30 mg/kg Day 2: PB60 mg/kg Day 3: PB60 mg/kg+5,6-BF80 mg/kg Day 4: PB60 mg/kg

c) Composition of S9Mix

Constituents	Amount in 1.0 mL S9Mix	Constituents	Amount in 1.0 mL S9Mix
S9	0.1 mL	NADH	4 μmol
$MgCl_2$	8 μmol	NADPH	4 μmol
KCl	33 μmol	Na-phosphate buffer (pH 7.4)	100 μmol
G-6-P	5 μmol		

7) Minimal glucose agar plate

Product name	TESMEDIA AN		
Manufacturer	ORIENTAL YEAST Co., Ltd.	Agar	
Date prepared	May 17, 2007	Product name	INA AGAR BA-30A
Date obtained	June 7, 2007	Manufacturer	INA FOOD INDUSTRY Co., Ltd.
Lot No.	ANI360EW	Lot No.	60322

Each plate contained about 30 mL of the minimal glucose agar medium.			
Minimal glucose agar medium: constituent per 1 L			
$MgSO_4 \cdot 7H_2O$	0.2 g	Citric acid·H_2O	2 g
K_2HPO_4	10 g	$NH_4H_2PO_4$	1.92 g
NaOH	0.66 g	Glucose	20 g
Agar	15 g		

8) Top agar

Top agar was prepared as follows:

Soft agar was autoclaved and mixed with sterile amino acid solution at a ratio of 10:1 (v:v).

Soft agar;

Bacto agar [DIFCO](Lot No. 6023052) 0.6 %

NaCl 0.5 %

Amino acid solution;

0.5 mmol/L L-histidine·0.5 mmol/L D-biotin·0.5 mmol/L L-tryptophan

9) Experimental procedures

The liquid pre-incubation method was adopted.

Two independent experiments were conducted; the first experiment was for dose-range-finding and the second experiment was for reproducibility.

The following procedure was carried out on each of the test strains.

a) Without metabolic activation

Each dose level of the test substance, 0.5 mL of sterile 0.1 mol/L sodium phosphate buffer (pH 7.4) and 0.1 mL of a bacterial suspension were added to each of one set of sterile 12 mm×75 mm disposable tubes. The tubes were kept standing with shaking for 20 minutes in a 37 ℃ water-bath. Next, 2 mL of top agar was added to each tube. The contents were poured onto the surface of minimal glucose agar plates.

Duplicate cultures were made per dose in both the first and the second experiments, while triplicate were made for the negative and the positive controls. After the top agar solidified completely, the plates were incubated for 48 hours at 37 ℃.

b) With metabolic activation

The method was same as described in a) except that 0.5 mL of liver homogenate S9Mix was added to each tube in place of sterile buffer.

10) Colony counting

Revertant colonies were counted with the naked eye.

11) Cytotoxic effects on bacteria

The cytotoxicity of the test substance was checked by reduction in number of revertants, clearing or diminution of the background lawn using a stereo-microscope.

12) Sterility tests on the test substance and the S9Mix

A 0.1 mL of the test solution and 0.5 mL of S9Mix were placed on the minimal agar plate, which were incubated for 48 hours at 37 ℃ to check any contamination with exogenous microorganism.

13) Statistical analysis

Statistical analysis was not performed.

14) Assessment of results

The mean number of revertant colonies for all of the treatment groups is compared with the mean number obtained from the negative control group. The effect of metabolic activation is assessed by comparing the results obtained both in the presence and the absence of S9Mix for each treatment group.

A compound is deemed to provide evidence of mutagenic potential if (1) a significant dose-related increase in the number of revertant colonies is obtained in two separate experiments, and (2) the increase in the number of revertant colonies is at least twice the concurrent negative control group value.

4. Results and discussion

The revertant colony counts are shown in Tables 1 and 2. No marked increase in the number of revertant colonies of the treatment groups was observed as compared with that of the negative control group in any experiments.

In the sterility tests, bacterial growth was not observed on the minimal agar plate with the test substance and S9Mix.

Positive control chemicals, such as 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide, Sodium Azide, 9-Aminoacridine Hydrochloride and 2-Aminoanthracene, markedly increased the revertant colonies.

5. Conclusion

We concluded that the test substance showed no evidence of mutagenic potential in this bacterial test system.

Table 1 Test results of the dose-range-finding test

Test substance: SAGAN COAT Photocatalyst Coating Solution TPX

With or without S9Mix	Conc. of test substance (μg/plate)	The number of revertant colony (colonies/plate)									
		Base-pair substitution type						Frame shift type			
		TA100		TA1535		WP2*uvrA*		TA98		TA1537	
S9Mix (-)	Negative control	80 84 76	(80)	10 5 8	(8)	31 13 20	(21)	15 14 13	(14)	11 4 9	(8)
	4.88	118 93	(106)	12 10	(11)	19 27	(23)	20 19	(20)	8 7	(8)
	19.5	95 86	(91)	7 11	(9)	23 19	(21)	14 19	(17)	9 5	(7)
	78.1	94 90	(92)	14 14	(14)	25 14	(20)	23 10	(17)	10 13	(12)
	313	92 98	(95)	9 8	(9)	20 17	(19)	22 22	(22)	3 3	(3)
	1250	80 81	(81)	10 9	(10)	23 19	(21)	12 17	(15)	5 3	(4)
	5000	93 75	(84)	8 12	(10)	19 29	(24)	8 14	(11)	8 7	(8)
S9Mix (+)	Negative control	69 96 127	(97)	10 8 7	(8)	22 23 23	(23)	19 26 21	(22)	14 15 15	(15)
	4.88	112 103	(108)	10 15	(13)	15 18	(17)	20 16	(18)	15 19	(17)
	19.5	93 92	(93)	10 14	(12)	21 24	(23)	29 25	(27)	18 18	(18)
	78.1	97 106	(102)	5 9	(7)	25 21	(23)	28 28	(28)	11 13	(12)
	313	105 90	(98)	12 8	(10)	18 23	(21)	23 24	(24)	18 20	(19)
	1250	83 104	(94)	9 15	(12)	23 12	(18)	34 30	(32)	12 14	(13)
	5000	98 95	(97)	17 7	(12)	25 19	(22)	25 26	(26)	20 18	(19)
Positive control not requiring S9Mix	Chemicals	AF-2		NaN_3		AF-2		AF-2		9-AA	
	Conc. (μg/plate)	0.01		0.5		0.01		0.1		80	
	Colonies /plate	208 220 234	(221)	473 432 468	(458)	50 60 71	(60)	153 151 160	(155)	113 117 96	(109)
Positive control requiring S9Mix	Chemicals	2-AA		2-AA		2-AA		2-AA		2-AA	
	Conc. (μg/plate)	1		2		10		0.5		2	
	Colonies /plate	1167 732 1242	(1047)	327 344 322	(331)	189 201 234	(208)	466 441 475	(461)	198 203 209	(203)

2-AA: 2-Aminoanthracene
AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide
NaN_3: Sodium Azide
9-AA: 9-Aminoacridine Hydrochloride

( ): Mean
Negative Control: DMSO

Table 2 Test results of the mutation test

Test substance: SAGAN COAT Photocatalyst Coating Solution TPX

With or without S9Mix	Conc. of test substance (μg/plate)	The number of revertant colony (colonies/plate)									
		Base-pair substitution type						Frame shift type			
		TA100		TA1535		WP2*uvrA*		TA98		TA1537	
S9Mix (-)	Negative control	106		6		28		19		8	
		94		12		24		21		9	
		89	(96)	7	(8)	16	(23)	17	(19)	5	(7)
	313	90		11		23		12		7	
		111	(101)	11	(11)	15	(19)	23	(18)	3	(5)
	625	97		15		24		27		6	
		95	(96)	7	(11)	24	(24)	22	(25)	9	(8)
	1250	108		12		22		19		3	
		85	(97)	10	(11)	24	(23)	17	(18)	11	(7)
	2500	94		9		23		16		3	
		105	(100)	13	(11)	27	(25)	19	(18)	11	(7)
	5000	87		14		17		32		4	
		105	(96)	10	(12)	17	(17)	21	(27)	13	(9)
S9Mix (+)	Negative control	116		10		30		21		18	
		131		10		21		38		14	
		139	(129)	14	(11)	32	(28)	36	(32)	11	(14)
	313	121		8		21		33		13	
		116	(119)	6	(7)	32	(27)	29	(31)	16	(15)
	625	122		7		31		26		19	
		141	(132)	14	(11)	31	(31)	29	(28)	10	(15)
	1250	111		6		32		23		14	
		112	(112)	9	(8)	24	(28)	27	(25)	11	(13)
	2500	145		13		17		30		14	
		147	(146)	6	(10)	37	(27)	19	(25)	11	(13)
	5000	142		14		19		35		15	
		107	(125)	14	(14)	24	(22)	25	(30)	11	(13)
Positive control not requiring S9Mix	Chemicals	AF-2		NaN_3		AF-2		AF-2		9-AA	
	Conc. (μg/plate)	0.01		0.5		0.01		0.1		80	
	Colonies /plate	277		475		81		191		141	
		290		532		76		201		154	
		286	(284)	553	(520)	71	(76)	174	(189)	145	(147)
Positive control requiring S9Mix	Chemicals	2-AA		2-AA		2-AA		2-AA		2-AA	
	Conc. (μg/plate)	1		2		10		0.5		2	
	Colonies /plate	942		292		219		435		190	
		1110		317		207		417		211	
		1065	(1039)	301	(303)	243	(223)	412	(421)	198	(200)

2-AA: 2-Aminoanthracene
AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide
NaN_3: Sodium Azide
9-AA: 9-Aminoacridine Hydrochloride

( ): Mean
Negative Control: DMSO

6. References

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