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REPORT

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In Vitro Microbiological Mutagenicity Tests to Assess the Potential Mutagenic Effect of Nano TiO2

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Abstract

IZ Nano TiO2 was examined for a 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 no evidence showing any mutagenic potential of the test substance was obtained in this bacterial test system at the dose levels used.

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 Guidelines for The Standards of Mutagenicity Tests Using Microorganisms (Notification No. 77 of Labor Standards Bureau, Ministry of Labor, September 1, 1988) 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 October 16 to November 21, 2003.

This is a translation of the original report, No. 203101041-002, written in Japanese.

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In Vitro Microbiological Mutagenicity Tests to Assess the Potential Mutagenic Effect of Nano TiO2

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 Guidelines for The Standards of Mutagenicity Tests Using Microorganisms (Notification No. 77 of Labor Standards Bureau, Ministry of Labor, September 1, 1988).

2. Test substance

Ionic Zone Nano TiO2 PCO Liquid

Character: Opalescent liquid with whitishsediment

3. Materials and methods

1) Preparation of the test solution

The test substance was added directly to the test system. Negative control was Water for injection [Otsuka Pharmaceutical Factories Co., Ltd](Lot No. 2H91N) alone.

2) Dose levels

Dose-range-finding test:

500, 400, 300, 200, 100 μL/plate

Mutation test:

500, 400, 300, 200, 100 μL/plate

3) Positive controls and solvents

a) Positive controls for each strain

w/o S9			with S9		
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
2-AA: 2-aminoanthracene

b) Positive control substances and solvents

Substance		Supplier	Lot No.	Purity (%)	Solvent
Positive control	AF-2	Wako	SEL1402	99.0	DMSO
	NaN_3	Wako	TCP3725	99.1	Water
	9-AA	ICN Biomedicals	2436F	98.8	DMSO
	2-AA	Wako	TCM6742	92.1	DMSO
Solvent	DMSO	Dojin	NB123	>99.0	–
	Water	Otsuka	2H91N	–	–

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

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 BIOASSAY RESEARCH CENTER	September 19, 2002	February 5, 2003
TA98	JAPAN BIOASSAY RESEARCH CENTER	September 19, 2002	February 5, 2003
TA1535	JAPAN BIOASSAY RESEARCH CENTER	September 19, 2002	February 5, 2003
TA1537	JAPAN BIOASSAY RESEARCH CENTER	September 19, 2002	February 5, 2003
WP2*uvrA*	JAPAN BIOASSAY RESEARCH CENTER	September 19, 2002	February 5, 2003

b) Storage conditions of test strains

Storage conditions	0.2 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 Med. Co., Ltd.]

5) Preparation of cell culture

Several microliters of a cell suspension having been frozen was put into 15 mL of Nutrient broth No. 2 [OXOID](Lot No. 276098) in an Erlenmeyer flask. It was cultured at 37 ℃ for 10 hours on a rotator. The grown cells were counted with a turibidimeter 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.]
Number of rotation	100 r/min^{-1}
Apparatus and Volume	Erlenmeyer flask with baffled (100 mL)

6) S9 and S9 Mix

a) Source of S9

Manufacturer	ORIENTAL YEAST CO., LTD.	Storage temperature	-80 ℃
Date of preparation	May 9, 2003	Name and model of storage apparatus	Deep freezer MDF-293AT [Sanyo Med. Co., Ltd.]
Date obtained	July 23, 2003		
Lot No.	03050902		

b) Preparation of S9

Animal used		Inducing substances	
Species	Rat	Name	Phenobalbital (PB) 5,6-Benzofravon (5,6-BF)
Strain	Sprague-Dawley		
Sex	Male	Administration route	Intraperitoneal injection
Age	7 weeks old		
Body weight	233.5 g±8.4 g	Administration schedule and dose (mg/kg)	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 S9 Mix

Constituents	Amount in 1 mL S9Mix	Constituents	Amount in 1 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	July 23, 2003	Product name	INA AGAR BA-30A
Date obtained	August 7, 2003	Manufacturer	INA FOOD INDASTRY Co., Ltd.
Lot No.	ANI560GS	Lot No.	30325
Each plate contained about 30 mL of the minimal glucose agar medium.			
The minimal glucose agar medium: constituent of per 1 L			
$MgSO_4 \cdot 7H_2O$	0.2 g	Citric acid $\cdot H_2O$	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

The top agar consists of, for 100 mL:

Bacto agar [DIFCO](Lot No. 2148552)	0.6	g
NaCl	0.5	g

The top agar was autoclaved and mixed with a 0.1 volume of sterile 0.5 mmol/L histidine HCl·H_2O-0.5 mmol/L biotin-0.5 mmol/L tryptophan solution.

9) Experimental procedures

The liquid pre-incubation method was adopted.

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

The following procedure was carried out on each test strain.

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 second experiments, while triplicate for negative and positive controls. After the top agar had solidified, the plates were incubated for 48 hours at 37 ℃.

b) With metabolic activation

The methodology was 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 or clearing or diminution of the background lawn with a stereo-microscope.

12) Sterility tests on the test substance and the S9mix

A 0.5-mL portion of the test solution and 0.1 mL of the 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 treatment groups is compared with those obtained for negative and positive control groups. The effect of metabolic activation is assessed by comparing the results obtained both in the presence and absence of the 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 solvent control 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 was observed as compared with the negative control in any experiment.

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 and 2-aminoanthracene markedly increased the revertant colonies.

5. Conclusion

We concluded that no evidence showing any mutagenic potential of the test substance was obtained in this bacterial test system.

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

Test substance: Ionic Zone Nano TiO2 PCO Liquid

With or without S9Mix	Conc. of test substance (L/plate)	The number of revertant colony (colonies/plate)									
		Base-pair substitution type						Frameshift type			
		TA100		TA1535		WP2*uvrA*		TA98		TA1537	
S9Mix (-)	Negative control	96		9		23		17		8	
		107		8		28		15		15	
		91	(98)	9	(9)	23	(25)	15	(16)	7	(10)
	100	94		7		19		21		6	
		76	(85)	8	(8)	26	(23)	19	(20)	9	(8)
	200	89		11		14		14		11	
		94	(92)	8	(10)	23	(19)	20	(17)	8	(10)
	300	93		9		26		17		15	
		77	(85)	12	(11)	29	(28)	8	(13)	12	(14)
	400	78		7		23		16		8	
		85	(82)	11	(9)	31	(27)	18	(17)	10	(9)
	500	84		14		24		17		14	
		88	(86)	11	(13)	17	(21)	22	(20)	5	(10)
S9Mix (+)	Negative control	95		3		26		28		24	
		96		10		33		20		23	
		105	(99)	8	(7)	26	(28)	29	(26)	15	(21)
	100	114		9		24		34		15	
		99	(107)	7	(8)	40	(32)	32	(33)	20	(18)
	200	110		6		25		34		17	
		115	(113)	15	(11)	20	(23)	27	(31)	23	(20)
	300	106		8		20		34		20	
		103	(105)	8	(8)	27	(24)	32	(33)	10	(15)
	400	111		6		24		22		11	
		104	(108)	13	(10)	27	(26)	26	(24)	7	(9)
	500	95		8		30		21		9	
		94	(95)	14	(11)	27	(29)	25	(23)	17	(13)
Positive control not requiring S9Mix	Chemicals Conc. (g/plate)	AF-2 0.01		NaN 0.5		AF-2 0.01		AF-2 0.1		9-AA 80	
	Colonies /plate	331		534		86		321		187	
		307		521		75		301		147	
		320	(319)	538	(531)	95	(85)	287	(303)	115	(150)
Positive control requiring S9Mix	Chemicals Conc. (g/plate)	2-AA 1		2-AA 2		2-AA 10		2-AA 0.5		2-AA 2	
	Colonies /plate	1194		294		187		467		467	
		1056		323		190		430		461	
		1131	(1127)	296	(304)	220	(199)	389	(429)	450	(459)

2-AA : 2-aminoanthracene
AF-2 : 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
NaN : Sodium Azide
9-AA : 9-aminoacridine

( ) Mean
Negative control : Water for injection

Table 2 Test results of the mutation test

Test substance: Ionic Zone Nano TiO2 PCO Liquid

With or without S9Mix	Conc. of test substance (L/plate)	The number of revertant colony (colonies/plate)									
		Base-pair substitution type						Frameshift type			
		TA100		TA1535		WP2*uvrA*		TA98		TA1537	
S9Mix (-)	Negative control	85		13		25		11		6	
		100		9		22		20		5	
		90	(92)	15	(12)	25	(24)	19	(17)	8	(6)
	100	91		8		18		11		7	
		96	(94)	10	(9)	18	(18)	15	(13)	4	(6)
	200	98		12		29		14		7	
		100	(99)	12	(12)	22	(26)	24	(19)	5	(6)
	300	89		9		19		16		4	
		107	(98)	3	(6)	19	(19)	14	(15)	4	(4)
	400	94		8		18		19		6	
		90	(92)	9	(9)	22	(20)	15	(17)	6	(6)
	500	85		7		25		16		3	
		92	(89)	7	(7)	16	(21)	17	(17)	10	(7)
S9Mix (+)	Negative control	115		9		26		28		16	
		111		6		29		15		11	
		80	(102)	8	(8)	34	(30)	23	(22)	15	(14)
	100	102		6		34		24		11	
		104	(103)	7	(7)	21	(28)	19	(22)	17	(14)
	200	122		16		37		25		14	
		106	(114)	10	(13)	21	(29)	24	(25)	23	(19)
	300	94		11		16		19		21	
		83	(89)	9	(10)	25	(21)	14	(17)	17	(19)
	400	92		11		29		15		22	
		90	(91)	10	(11)	39	(34)	14	(15)	14	(18)
	500	84		11		30		19		18	
		85	(85)	12	(12)	28	(29)	27	(23)	9	(14)
Positive control not requiring S9Mix	Chemicals Conc. (g/plate)	AF-2 0.01		NaN 0.5		AF-2 0.01		AF-2 0.1		9-AA 80	
	Colonies /plate	298		580		105		288		98	
		350		547		92		292		125	
		329	(326)	537	(555)	107	(101)	271	(284)	90	(104)
Positive control requiring S9Mix	Chemicals Conc. (g/plate)	2-AA 1		2-AA 2		2-AA 10		2-AA 0.5		2-AA 2	
	Colonies /plate	1281		302		199		516		415	
		957		323		181		453		412	
		1248	(1162)	317	(314)	242	(207)	452	(474)	427	(418)

2-AA : 2-aminoanthracene
AF-2 : 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
NaN : Sodium Azide
9-AA : 9-aminoacridine

( ) Mean
Negative control : Water for injection

6. References

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