ToxEHS

Mutagenicities of 2-Propylaniline and 4-Propylaniline Determined by a Bacterial Reverse Mutation (*Ames*) Assay

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Abstract

Objective: Although the organic compounds, 2-propylaniline and 4-propylaniline are frequently used in many industrial sectors, and have little information about the potential genetic toxicity, and it is covered by the Occupational Safety & Health Act (OSHAct) in Korea.
Methods: The mutation test of 2-propylaniline and 4-propylaniline was evaluated in five different doses for each chemical through a well-known *Ames* bacterial mutation test. This test was performed regardless of metabolic activation.
Results: In this assay, we obtained positive results under all tested conditions, indicating that these two chemicals have mutagenic and potentially carcinogenic properties.
Conclusion: Both 2-propylaniline and 4-propylaniline were mutagenic under the conditions of these tests. This result means that all of these chemicals exhibit mutations and potential carcinogenicity.

Keywords: 2-Propylaniline, 4-Propylaniline, Mutagenicity, Reverse mutation

Introduction

Workers' concerns about chemical-induced genotoxicity and carcinogenicity have made genotoxic testing as an essential regulatory requirement for all new and existing chemicals[1]. There are regulations to inspect medicines, medical devices, pesticides, industrial chemicals/intermediates, household chemicals, food additives, etc.[2], and it is essential that genetic toxicity tests are carried out for all new chemical substances which are expected to be substantially exposed to humans.

Mutational studies of chemicals have increased interest in the relationship between mutagenicity and carcinogenicity, and thus the mutagenicity of *Salmonella* has helped predict carcinogenicity in mammals. The *Ames* test was the first to be proposed for all types of *in vitro* and *in vivo* genotoxicity tests[3,4], and international validation studies were started to verify or justify the various tests[5]. In 1976, the United Toxic Substances Control Act (TSCA) was passed, which indicated the need for genotoxicity testing of newly developed industrial chemicals and already commercialized chemicals[6]. In the tests required here, positive reactions were considered to represent putative carcinogens and were not approved for use without evidence of carcinogenicity. According to this rule, a test for chromosome-impairing chemicals was carried out because a test was needed to identify the chemical causing the mutation.

Initial test requirements are those in which a chemical is first tested *in vitro* and then "validated" *in vivo* if positive results are obtained, and the cancer or bacterial cell mutation to provide test data related to the positive result. Although these tests are currently used extensively in industry and regulatory decisions, there is relatively little data on their effectiveness. In addition, the evaluation and regulation of chemicals is important for maintaining workers' health and safe working environments[7], so 2-propylaniline and 4-propylaniline, which are increasingly used chemicals, are required to ensure that employers maintain a safe and healthy workplace. The two chemicals are regulated by Occupational Safety & Health Act (OSHAct; 1981)[8]. Because there is no such data in KOSHA's MSDS service[9], a lack of genotoxicity or the possible mutagenic potentials of these two chemicals, we conducted the widely used *Ames* test to assess the carcinogenic potentials of 2-propylaniline and 4-propylaniline. Our work also has additional importance, because we wanted to supplement the lack of information available about these chemicals, in accordance with the need to improve human health and wellbeing, as outlined in the OECD guideline[10].

Table 1. Physicochemical properties of 2-propylaniline and 4-propylaniline.

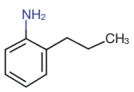
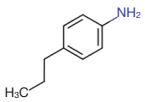
Chemical Name (CAS No.)	2-Propylaniline; $C_9H_{13}N$ (Molecular Weight 135.21) (1821-39-2) 			4-Propylaniline; $C_9H_{13}N$ (Molecular weight 135.21) (2696-84-6) 		
Purity	97.1%	Lot No.	65396BPV	98%	Lot No.	BCBS7363V
Melting point	No data	Flash point	98°C	No data	Flash point	104°C
Boiling point	222-224°C	Partition coefficient	No data	224-226°C	Partition coefficient	No data
Forms	Colorless to amber liquid	Water solubility	No data	Yellow to brown liquid	Water solubility	No data
Toxicity and GHS classification	Skin corrosion/irritation Category 2 (Skin irritation) Serious eye damage/eye irritation Category 2 (Eye irritation) Specific target organ toxicity (single exposure) category 3 (Respiratory system)			Skin corrosion/Skin irritation Category 2 (Skin irritation) Serious eye damage/eye irritation Category 2 (Eye irritation) Specific target organ toxicity (single exposure) category 3 (Respiratory system)		

Table 1 shows the physicochemical and toxicological information for these two chemicals.

Results and Discussion

Bacterial genotoxicity testing can be divided into three main categories; return mutation, mutation, and DNA repair defect detection[11]. These tests, which detect reverse mutations, are the only widely used tests and can generally be used at regulatory agency submissions. The bacterial reverse mutation assay plays an important role in detecting point mutations that cause many human genetic disorders and in confirming tumor initiation and development. Because the strain has various mutations that inactivate genes involved in the synthesis of essential amino acids of histidine (*Salmonella*) or tryptophan (*E. coli*), it can only grow in a culture medium supplemented with these amino acids.

The assay is conducted by mixing a bacterial suspension with molten top agar. Bacteria exposed to a mutagen may mutate back to the wild type and, hence, grow in the absence of the amino acid. When the bacteria are exposed to mutagenic chemicals, depletion of the limited amino acids of the agar will result in mutations that can restore (or reverse) the ability of the bacteria to synthesize amino acids and continue to grow. A related mutation involves the substitution of an individual base pairs or frame-shift mutations caused by the addition or deletion of some DNAs. According to the OECD guidelines for the toxicological testing of chemicals (TG 471), the *Ames* assay will test the chemicals with batteries of different conditions[10].

Tables 2 and 3 show the results of the bacterial reverse mutation (*Ames*) assay, using *Salmonella typhimurium* and *E. coli* treated with these two chemicals, respectively. It can be seen that the number of colonies/plates in each group is 2.0 times higher than the other groups in each table. Both treatment of 2-propylaniline and 4-propylaniline induced mutagenicity in *S. typhimurium* TA100, TA1535 and TA 1537 or *E. coli* WP2uvrA, regardless of metabolic activation. These results indicate that the two chemicals are mutagens under these individual experimental conditions. Mutations were induced in a reproducible and dose-dependent manner independent of the absence and presence of the S9 mixture in *Salmonella* and *E. coli* test strains. All positive control chemicals used in this study induced a significant increase in the frequency of revertant colonies and confirmed the activity of the S9-mix and the susceptibility of bacterial strains.

According to the classification provided by companies to European Chemicals Agency (ECHA) in CLP (Classification, Labelling and Packaging of substances and mixtures) notifications, this substance causes serious eye irritation, skin irritation and may cause respiratory irritation[12-15]. According to governmental notification No 2016-41 (Exposure Standards of Chemicals and Physicals Agents), it has 2 ppm or 10 mg/m^2 as TLVs (Threshold Limit Value; as aniline and its homologs)[16]. Also, these are target substances for special health examination of workers in the OSHAct in Korea[8]. Nevertheless, it did not offer any information

Table 2. Results of the main test using *Salmonella* and *E. coli* treated with 2-propylaniline. The lower panel is the positive control (without and with metabolic activation).

With/Without S9-mix	Concentration of test chemical (μg/plate)	Number of reverse mutations (colony number/plate)															
		Base-pair substitution type									Frameshift type						
		TA100			TA1535			WP2uvrA			TA98			TA1537			
S9 Mix (−)	0	119	105	108	8	9	7	35	49	37	34	20	22	4	5	3	
		(111±7)			(8±1)			(40±8)			(25±8)			(4±1)			
	312.5	89	82	98	6	5	8	43	53	38	32	26	21	3	2	3	
		(90±8)			(6±2)			(45±8)			(26±6)			(3±1)			
	625	58	67	53	2	7	6	27	22	25	13	10	8	2	1	1	
		(59±7)			(5±3)			(25±3)			(10±3)			(1±1)			
	1,000	0	0	0	1	0	0	19	24	13	9	5	15	68	171	0	
		(0±0)			(0±1)			(19±6)			(10±5)			(80±86)			
	1,250	0	0	0	0	0	0	0	22	75	2	1	2	0	50	0	
		(0±0)			(0±0)			(32±39)			(2±1)			(17±29)			
	2,500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	
		(0±0)			(0±0)			(0±0)			(0±0)			(1±1)			
	5,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	
		(0±0)			(0±0)			(0±0)			(0±0)			(1±1)			
S9 Mix (+)	0	91	106	86	3	8	8	63	55	77	36	35	26	13	15	22	
		(94±10)			(6±3)			(65±11)			(32±6)			(17±5)			
	312.5	102	100	119	9	6	10	57	72	71	33	37	31	15	17	11	
		(107±10)			(8±2)			(67±8)			(34±3)			(14±3)			
	625	70	74	85	9	7	1	52	52	64	19	11	15	10	8	9	
		(76±8)			(6±4)			(56±7)			(15±4)			(9±1)			
	1,000	0	0	0	0	21	393	25	20	24	19	3	2	1	8	9	
		(0±0)			(138±221)			(23±3)			(8±10)			(6±4)			
	1,250	0	1	0	64	0	0	23	17	19	1	0	0	13	0	22	
		(0±1)			(21±37)			(20±3)			(0±1)			(12±11)			
	2.500	0	1	0	0	0	0	0	2	5	0	0	0	0	0	0	
		(0±1)			(0±0)			(2±3)			(0±0)			(0±0)			
	5,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		(0±0)			(0±0)			(0±0)			(0±0)			(0±0)			
Positive control	Strain	AF-2			NaN_3			AF-2			AF-2			9-AA			
Without S9 Mix	Conc. (μg/plate)	0.01			0.5			0.01			0.1			80			
	Colony number/plate	477	508	475	362	370	419	406	418	427	356	382	376	1477	1412	1247	
		(487±19)			(384±31)			(417±11)			(371±14)			(1379±119)			
With S9 Mix	Strain	2-AA			2-AA			2-AA			2-AA			2-AA			
	Conc. (μg/plate)	1.0			2.0			10.0			0.5			2.0			
	Colony number/plate	1284	1306	1201	259	229	200	474	487	564	355	363	383	157	153	151	
		(1264±55)			(229±30)			(508±49)			(367±14)			(154±3)			

NaN_3, sodium azide; 9-AA, 9-aminoacridine; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; 2-AA, 2-aminoanthracene.

Table 3. Results of the main test using *Salmonella* and *E. coli* treated with 4-propylaniline. The lower panel is the positive control (without and with metabolic activation).

With/Without S9-mix		Concentration of test chemical (μg/plate)	Number of reverse mutations (colony number/plate)					
			Base-pair substitution type			Frameshift type		
			TA100	TA1535	WP2uvrA	TA98	TA1537	
S9 Mix (−)		0	79 112 96 (96±17)	10 4 5 (6±3)	32 40 38 (37±4)	16 15 15 (15±1)	4 3 4 (4±1)	
		62.5	83 104 119 (102±18)	7 4 7 (6±2)	34 31 37 (34±3)	17 11 15 (14±3)	6 5 6 (6±1)	
		125	99 115 106 (107±8)	5 7 6 (6±1)	31 33 38 (34±4)	20 18 21 (20±2)	1 7 6 (5±3)	
		250	73 74 65 (71±5)	5 3 6 (5±2)	40 42 35 (39±4)	15 8 8 (10±4)	3 4 2 (3±1)	
		500	3 234 815 (351±418)	0 820 9 (276±471)	14 16 26 (19±6)	6 7 598 (204±342)	0 11 550 (187±314)	
		1,000	0 0 0 (0±0)	0 0 0 (0±0)	0 0 0 (0±0)	0 2 0 (1±1)	0 0 0 (0±0)	
S9 Mix (+)		0	102 111 118 (110±8)	5 5 9 (6±2)	41 35 53 (43±9)	27 22 37 (29±8)	7 8 9 (8±1)	
		62.5	129 152 137 (139±12)	6 5 9 (7±2)	63 44 45 (51±11)	30 38 35 (34±4)	11 13 8 (11±3)	
		125	147 146 165 (153±11)	12 15 10 (12±3)	52 46 35 (44±9)	29 30 30 (30±1)	6 14 11 (10±4)	
		250	136 143 123 (134±10)	9 18 5 (11±7)	61 44 42 (49±10)	30 27 30 (29±2)	6 9 10 (8±2)	
		500	62 703 77 (281±366)	8 6 8 (7±1)	29 28 19 (25±6)	14 10 16 (13±3)	202 500 28 (243±239)	
		1,000	0 0 0 (0±0)	0 0 0 (0±0)	0 2 64 (22±36)	3 0 0 (1±2)	0 0 0 (0±0)	
Positive control	Without S9 Mix	Strain	AF-2	NaN_3	AF-2	AF-2	9-AA	
		Conc. (μg/plate)	0.01	0.5	0.01	0.1	80	
		Colony number/plate	468 439 458 (455±15)	310 269 256 (278±28)	190 172 159 (174±16)	401 367 436 (401±35)	1933 1176 1176 (1428±437)	
	With S9 Mix	Strain	2-AA	2-AA	2-AA	2-AA	2-AA	
		Conc. (μg/plate)	1.0	2.0	10.0	0.5	2.0	
		Colony number/plate	1319 1360 1219 (1299±73)	228 202 213 (214±13)	688 655 661 (668±18)	335 333 304 (324±17)	284 303 234 (274±36)	

NaN_3, sodium azide; 9-AA, 9-aminoacridine; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; 2-AA, 2-aminoanthracene.

on the KOSHA MSDS[8]. Our study represents the first trial for genetic toxicology of these chemicals. We suggest that future studies using DNA damage detection tools such as the 3-battery test that produce results at the molecular and cellular levels will further inform our initial positive outcome of these chemicals in the

Ames assay[17-19]. Some acknowledged the importance of germ cell mutagenicity, but it was clear that decisions based on carcinogenicity were made[20].

Since the late 1980s, genetic toxicity testing requirements from domestic (Korean) and international regulatory agency have been harmonized with formal OECD testing guidelines for chemicals and pesticides as International Conference on Harmonization (ICH) guidelines for pharmaceuticals[21]. These tests are developing by recognizing biological endpoints that have not been resolved by conventional scientific testing. *In vitro* and *in vivo* genotoxicity tests are the most commonly required tests at the regulatory agency for new substances, and other tests used as additional tests may potentially be useful in the future. They are routinely described by scientists conducting and evaluating these tests. They should reflect the latest official international testing guidelines and should be encouraged for all scientists considering these tests. It is also recommended for researchers who want to keep their previous and new tests.

Conclusion

In this assay, we obtained positive results under all tested conditions, indicating that these two chemicals have mutagenic and potentially carcinogenic properties. In conclusion, both chemicals are managed according to the OSHAct in Korea, which encompasses the appropriate handling of chemicals in the workplace and guidance regarding special health examinations.

Materials and Methods

Chemicals and Bacterial Strains

2-Propylaniline (Sigma-Aldrich, 97.1%, St. Louis, MO, USA, Lot No 65396BPV) and 4-propylaniline (Sigma-Aldrich, 98%, St. Louis, MO, USA, Lot No BCBS7363V) were dissolved in dimethyl sulfoxide (Sigma-Aldrich, 99.5%, St. Louis, MO, USA, Lot No. SHBH6859) and added to the test systems and/or diluted prior to treatment. Sodium azide (WAKO, 98%, Osaka, Japan, Lot No. JWF7959), 9-aminoacridine (9AA) (Aldrich, 98%, Lot No. 07620TDV), 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) (WAKO, 98%, Osaka, Japan, Lot No. STQ 3987) and 2-aminoanthracene (2AA) (WAKO, 95%, Osaka, Japan, Lot No. DCK 3519) were used as positive controls.

The five strains used in this study included four strains of *S. typhimurium* (TA100 Lot No. 4475D, TA1535 Lot No. 4487D, TA98 Lot No. 4486D and TA1537 Lot No. 4455D, Moltox, NC, USA) and one strain of *E. coli* (WP2uvrA, Moltox, NC, USA, Lot No. 4473D) that have been shown to be reliable and to yield reproducible responses between laboratories. An appropriate minimal agar plate (e.g., containing Vogel-Bonner glucose (VBG) minimal medium and glucose; Junsei, Tokyo, Japan, Lot No. 2014B1627) and an agar overlay, containing histidine and biotin or tryptophan (Bacto-agar, BD, NJ, USA, Lot No. 1059387) were used to allow for a few cell divisions (No. 2 Nutrient Broth, Oxoid, Cambridge, UK, Lot No. 1239615; Shaker bath, Precision, VA, USA, Model 50, 180 rpm).

Mutagenicity Assay

The most commonly used metabolic activation system is a cofactor-supplemented post-mitochondrial fraction (S9, Moltox, NC, USA, Lot No. 3763) prepared from the livers of rats treated with enzyme-inducing agents such as Aroclor 1254 or a combination of phenobarbitone and β-naphthoflavone. The test substance/test solution was preincubated with the test strain (containing approximately 10^8 viable cells) and a sterile buffer or the metabolic activation system (0.5 mL) for >20 min at 30-37°C prior to mixing with the overlay agar and pouring the mixture onto the surface of a minimal agar plate. According to GLP (Good Laboratory Practice) guideline[6], six doses (5000, 2500, 1250, 1000, 625 and 312.5 μg/plate with 2-propylaniline) and five doses (1000, 500, 250, 125, 62.5 μg/plate with 4-propylaniline) of each one of the test chemicals were used for the main assay. To test reliability and reproducibility, the agar-plating method was assessed by using triplicates of each dose. All plates representing different testing conditions and were incubated at 37°C for 48 hours. After the incubation period, the number of bacterial revertant colonies per plate was counted.

Data Analysis

Bacterial revertant colonies per plate were enumerated using an auto-colony counter (SCAN4000, Interscience International, Bois Arpents, France). The mutant frequency was expressed as the number of reverting colonies divided by several colonies in the negative control. A test chemical was assumed to have mutagenic potential if the mutant frequency was more than twice that of the negative control. A chemical was assumed to have possible mutagenic potential if the quotient was within a range from 1.7 to 1.9, in combination with a dose-effect relationship. No mutagenic potential was assumed if all quotients ranged between 1.0 (and lower) and 1.6.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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