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Original Article

Comparison of stabilities of nitrenium ions and *in vitro* and *in vivo* genotoxic potential, between four aniline derivatives

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ABSTRACT — Stabilities of nitrenium ions estimated by *in silico* analyses and *in vitro* and *in vivo* genotoxicity were compared for four aniline derivatives, 2-chloro-4-methylaniline (2C4MA), 4-chloro-2-methylaniline (4C2MA), 2-chloro-4,5-difluoroaniline (2C4,5DFA) and 4-trifluoromethylaniline (4TFMA). The AM1 values as an index of stability of the nitrenium ions of 2C4MA, 4C2MA, 2C4,5DFA and 4TFMA were -5.38, -4.67, 8.36 and 16.6 kcal/mol, respectively, indicating that the potential of mutagenicity is high for 2C4MA and 4C2MA and low for 2C4,5DFA and 4-TFMA. The specific mutagenicity determined in Ames tests with S9 mix for 2C4MA and 4C2MA was 4,067 and 12,500 revertants/mg/plate, respectively. The specific mutagenicity could not be determined for 2C4,5DFA because the results of the Ames tests were equivocal. Among the four aniline derivatives, only 4TFMA showed positive results with and without S9 mix in the Ames tests and the specific mutagenicity of 4TFMA were 1,590 and 1,910 revertants/mg/plate for TA100 with and without S9 mix, respectively. These results indicated that the mutagenic potential is high for 2C4MA and 4C2MA and is low for 2C4,5DFA and 4TFMA. *In vivo* genotoxicity is positive for 2C4MA, 4C2MA and 2C4,5DFA and is negative for 4TFMA. The results of *in silico* analyses and *in vitro* and *in vivo* genotoxicity tests were consistent for aniline derivatives with strong mutagenicity (2C4MA and 4C2MA) but were not for those with weak mutagenicity (2C4,5DFA and 4TFMA). Careful assessment for the risk of carcinogenicity is necessary for aniline derivatives with weak mutagenicity by combining *in silico* analyses and *in vitro* and *in vivo* genotoxicity tests.

Key words: AM1, Nitrenium ions, Ames test, Comet assay, Aniline derivatives

INTRODUCTION

Aniline and its derivatives are widely used not only as intermediates for dyestuffs and a variety of polyurethane products but also as materials for drug synthesis. Despite their usefulness, aniline derivatives have various toxic effects and some of them have been reported to be mutagenic and carcinogenic. Especially, the monosubstituted type of aniline derivative including *o*-toluidine, *p*-toluidine, *o*-anisidine and *p*-phenyl-aniline, have been reported to be carcinogenic in rodents. Because of the frequent use of aniline derivatives as building blocks in the synthesis of new drugs, it is important to estimate their mutagenic or carcinogenic potential before their usage and to select aniline derivatives with low mutagenic potential as materials for drug synthesis. For this purpose, *in vitro* and *in vivo* genotoxicity tests and *in silico* analyses have been used for the screening of aniline derivatives.

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Among the screening methods for estimation of mutagenic potential of chemicals, the usefulness of *in silico* analyses based on structure-activity relationships (SAR) has been increasing. An *in silico* method based on the so-called "nitrenium hypothesis" was developed by Ford (1992) for the estimation of the mutagenic potential of aromatic amines including aniline derivatives (Ford and Griffin, 1992; Ford and Herman, 1992). This hypothesis asserts that the mutagenic effect for this class of molecules is mediated through the transient formation of a nitrenium ion and that the stability of this cation is correlated with the mutagenic potential. Bentzien (2010) applied the original nitrenium hypothesis to a set of primary aromatic amines to understand how this hypothesis can best be used to select primary amine molecules that are less likely to be mutagenic (Bentzien *et al*., 2010).

In order to validate the usefulness of *in silico* analyses for estimation of mutagenic potential of chemicals, correlation analyses between *in silico* analyses and *in vitro* and/or *in vivo* mutagenicity tests are necessary using a series of chemicals with weak to strong mutagenicity.

In the present study, we conducted *in silico* analyses based on the nitrenium hypothesis and *in vitro* and *in vivo* genotoxicity tests for four aniline derivatives, 2-chloro-4-methylaniline (2C4MA), 4-chloro-2-methylaniline (4C2MA), 2-chloro-4,5-difluoroaniline (2C4,5DFA) and 4-trifluoromethylaniline (4TFMA) to investigate relationships between the stability of the nitrenium ions and the genotoxic potential. *In silico* analyses and bacterial reverse mutation tests (Ames test) were conducted for these four aniline derivatives in our study. *In vivo* genotoxicity tests (comet assay) were also conducted for 2C4MA and 2C4,5DFA. 4C2MA has been reported to have a mutagenic potential and carcinogenicity based on the results of an *in vivo* comet assay in rats and carcinogenicity studies in rodents (Sekihashi *et al*., 2002; Zimmer *et al*., 1980). 4TFMA has been reported not to induce mutagenicity, clastogenicity or carcinogenicity based on the results of *in vivo* genotoxicity tests including an unscheduled DNA synthesis (UDS) test in rats, a bone marrow micronucleus test in rats, a bone marrow chromosomal aberration test in hamsters and carcinogenicity studies in rodents (Pharmacology Review(s), Food and Drug Administration, Center for Drug Evaluation and Research, 2012, Application, Number: 202992Orig1s000).

MATERIALS AND METHODS

Chemicals

2-Chloro-4-methylaniline (2C4MA) was from Wako Pure Chemical Industries, Ltd. (Japan), 4-chloro-2-methylaniline (4C2MA) and 4-trifluoromethylaniline (4TFMA) were from Sigma-Aldrich (USA) and 2-chloro-4, 5-difluoroaniline (2C4,5DFA) was from Alfa Aecar (USA). The purity of all compounds was in all cases > 99%. Chemical structures of the tested aniline derivatives are shown in Fig. 1.

Calculation of AM1 values as an index of stabilities of nitrenium ions by *in silico* methods

The stabilities of the nitrenium ions were calculated according to the methods reported by Ford (1992) and Bentzien (2010) and are shown as AM1 values (stability energies for the nitrenium ions, kcal/mol) for each test compound (Bentzien *et al*., 2010; Ford and Griffin, 1992; Ford and Herman, 1992). The AM1 values were calculated using semi-empirical AM1 calculations with MOPAC after generating three dimensional structures using Ligprep and Confgen (Schrödinger Release, 2014, 2014-4, Ligprep, 3.2; Schrödinger Release, 2014, 2014-4, Confgen, 3.0; Stewart, 1990). When the AM1 value was negative, the nitrenium ion is more stable compared to that of aniline and the mutagenic potential of the compound was regarded as high (Ames positive). When the AM1 value was positive, the nitrenium ion is less stable compared to that of aniline and the mutagenic potential of the compound was regarded as low (Ames negative).

Bacterial reverse mutation tests (Ames test)

Based on the results of preliminary studies, two tester strains, *Salmonella typhimurium* TA98 and TA100, were more sensitive to the aniline derivatives used in the

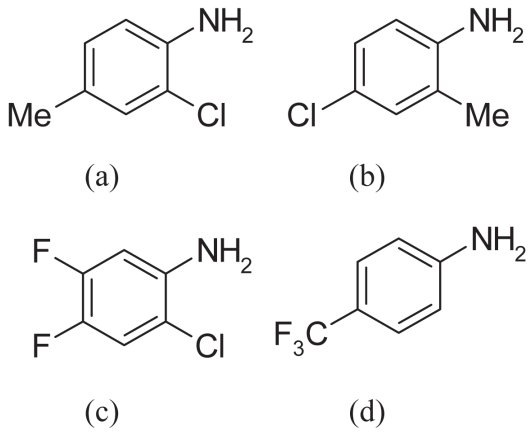

Fig. 1. Chemical structures of the tested aniline derivatives. a: 2-chloro-4-methylaniline, b: 4-chloro-2-methylaniline, c: 2-chloro-4,5-difluoroaniline, d: 4-trifluoromethylaniline.

present study than other tester strains and were used in this study. The tester strains used in the study were obtained from the Institute for Fermentation, Osaka (Japan). The S9 mix (S9 mix for Ames test, Kikkoman Corporation, Japan) contained 10% S9 prepared from the livers of rats treated with phenobarbital and 5,6-benzoflavone.

The solvent used for all the compounds was dimethyl sulfoxide (DMSO, 100 μL/plate). In accordance with the International Conference on Harmonization (ICH) S2(R1) Guidance, the test was performed by the pre-incubation method with or without S9 mix. The S9 mix, 0.5 mL, (when S9 mix was added) or 0.1 mol/L sodium phosphate buffer solution, 0.5 mL (pH 7.4; when S9 mix was not added) and the bacterial culture solution, 0.1 mL, were added to a test tube (internal diameter 13 mm × 100 mm) containing 0.1 mL of the negative control compound, the test compound or the positive control formulations. The mixtures were pre-incubated at 37°C for 20 min while shaking. After the pre-incubation period, 2 mL of top agar were added and the mixtures were vortex-mixed and seeded onto plates. Three plates per treatment were used. The plates were incubated at 37 ± 1°C for 48 hr or more and the revertant colonies were counted and examined. The number of revertant colonies was counted manually for the negative control and test compound plates or using a colony analyzer for the positive control compound plates. The mean number of revertant colonies for each treatment plate was then calculated. The presence or absence of inhibition of the background lawn due to any antibacterial effect and precipitation of the test compound was observed grossly or under a stereomicroscope.

The results were judged as positive if the mean number of revertant colonies showed a dose dependent increase which reached 2-fold over that of the negative control at one or more concentrations. Evaluation was based on mean values with no statistical comparisons being used. Specific mutagenicity (revertants/mg/plate) was also calculated for each test compound using the following formula;

Specific mutagenicity = (Number of revertant colonies for the test compound indicating positive result-number of revertant colonies for the negative control compound) / dose level (mg/plate) indicating positive result

In vivo comet assay

Seven week old male Crl:CD (SD) rats were purchased from Charles River Japan Inc. (Kanagawa, Japan). The animals were housed individually in stainless steel bracket cages kept in an air-conditioned room with a 12-hr light-dark cycle (lighting from 7:00 a.m. to 7:00 p.m.) at a temperature of 20 to 26°C, a relative humidity of 30 to 75% and a ventilation rate of 10 to 20 times per hour. The rats were quarantined for 1 week and were allowed free access to a commercial pelleted diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) during the quarantine period. UV-treated tap water was available for drinking ad libitum. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the Toxicology Research Laboratories, Central Pharmaceutical Research Institute, Japan Tobacco Inc. This study was conducted in accordance with the Japanese Law for the Humane Treatment and Management of Animals (Law No. 105, as revised in 2013, issued in October 1, 1973).

Comet assays were conducted for 2C4MA and 2C4,5DFA. The procedures for the comet assay in this study were based on the published method (Burlinson *et al*., 2007; Hartman *et al*., 2003; Tice *et al*., 2000).

The highest dose levels of 2C4MA and 2C4, 5DFA used in the comet assay were the maximum tolerated doses (MTDs) determined based on the results of preliminary studies. 2C4MA or 2C4,5DFA was administered orally once daily for 3 days to Sprague-Dawley rats (seven week old at the initiation time of first dosing, male, five rats per group) at the dose levels of 100, 300 and 500 mg/kg for 2C4MA and 500 and 1000 mg/kg for 2C4,5DFA. The animals in the negative control groups were given the vehicle for 2C4MA (20 wt% polyoxyethylene hydrogenated castor oil 60/5 wt% propylene glycol solution) or the vehicle for 2C4,5DFA (0.5 w/v% methylcellulose aqueous solution), in the same manner. The animals in the positive control group were given ethyl methanesulfonate (EMS) at a dose level of 200 mg/kg. Water for injection was used as the vehicle for EMS. The specimen preparations of the liver and kidney were performed 3 hr after the last dosing.

For the liver, portions of the tissue (about 5 mm × 5 mm × 5 mm cube from the left lobe) were collected and were washed with the cold mincing buffer (20 mM EDTA/10% DMSO/Hanks' balanced salt solution without Ca, Mg). The liver cubes were minced with scissors about 100 times to release the hepatocytes. Then, the hepatocytes were suspended with 3 mL of the cold mincing buffer by gently pipetting about 15 times. The suspension was strained through a cell strainer (Falcon Corporation, pore size: 40 μm). The suspension was diluted with the cold mincing buffer to the appropriate concentration (approximately 20×10^4 cells/mL).

For the kidney, portions of the tissue (about 5 mm × 5 mm × 5 mm cube composed mostly of the cortex) were collected and were washed with the cold mincing buff-

er (20 mM EDTA/10% DMSO/Hanks' balanced salt solution without Ca, Mg). The kidney cubes were minced with scissors about 100 times to release the renal cortex cells. Then, the renal cortex cells were suspended with 3 mL of the cold mincing buffer by gently pipetting about 15 times. The suspension was strained through a cell strainer (Falcon Corporation, pore size: 40 μm). The suspension was diluted with the cold mincing buffer to the appropriate concentration (approximately 20×10^4 cells/mL).

Each prepared sample of the liver or kidney (40 μL of the cell suspension) was mixed with Comet Assay® LMAgarose (0.5%) (360 μL) and the resultant mixture (40 μL) was placed onto each well of a slide (CometSlide HT, 20 Well Slide, Trevigen® Inc.). Three wells per animal were assigned to the slides. Seven slides per organ were prepared. Each slide was placed in the tray containing lysis solution and left for approximately 25.0 to 25.5 hr under the refrigerated and light-protected conditions.

The specimens were rinsed with cold purified water and placed onto the electrophoresis bath poured with the electrophoresis buffer (pH 13.2) and were left for 20 min. The conditions of the electrophoresis were set at 0.7 V/cm constant voltages and approximately 300 mA. The specimens were subjected to electrophoresis for 20 min while keeping the temperature of the electrophoresis buffer at 5.0 to 6.0°C for the electrophoresis for the liver and kidney. The specimens were immersed in a neutralization buffer (10 mM Tris HCl buffer (pH 7.6), LSI Medience Corporation) for 10 min and then rinsed in absolute ethanol for 10 min or longer and allowed to air dry. The dried specimens were stored at room temperature. The specimens were dehydrated with ethanol (99.5% or more) following neutralization.

Five microliters of SYBR® Gold nucleic acid gel stain (Invitrogen Corporation) diluted 10000-fold with TE (10 mM Tris HCl buffer (pH 7.6)/1 mM EDTA) buffer were dropped on each slide, which was then covered with a coverslip. The images of the DNA migration of the cells were examined using a fluorescence microscope (AHBT-RFL-1, ORYMPUS Corporation). The images were imported to a computer through a CCD camera attached to the microscope and analyzed using a Comet assay analyzer (Comet Assay IV system, Perceptive Instruments). One hundred cells per organ (50 cells per slide), i.e. 500 cells per group (five animals), were analyzed. The remarkable hedgehog images of the damage were not analyzed.

The percentage of DNA in the tail to total DNA (% tail DNA: Tail % intensity) was used as an indicator of DNA damage. The % tail DNA in the positive control group should increase with a statistically significant difference from the negative control group (0.5%MC aqueous solution-treated group).

Statistical analyses were performed using the MiTOX Computer System (Mitsui Zosen Systems Research Inc.). The levels of significance were set at 5 and 1% (two-tailed). DNA damage by the test compound was determined based on the presence or absence of significant differences in the % tail DNA between the matched negative control compound and each of the test compound-treated groups.

RESULTS

Stabilities of Nitrenium Ions (AM1 Values)

AM1 values as the index of the stability of the nitrenium ions of 2C4MA, 4C2MA, 2C4,5DFA and 4TFMA were calculated to be -5.38, -4.67, 8.36 and 16.6 kcal/mol, respectively, and the potential for mutagenicity of 2C4MA and 4C2MA were considered to be high (Positive results in the Ames tests) while that for 2C4,5DFA and 4TFMA were considered to be low (Negative results in the Ames tests) in terms of stability of the nitrenium ion (Table 1).

Bacterial reverse mutation test (Ames test)

2C4MA

Inhibition of bacterial growth was observed at 1000 μg/plate for both strains with or without S9 mix (Table 2).

Table 1. AM1 values as an index of the stabilities of nitrenium ions of four aniline derivatives.

Compound	Stabilities of nitrenium ions (AM1)	
	kcal/mol	Potential
2C4MA	-5.38	high
4C2MA	-4.67	high
2C4,5DFA	8.36	low
4TFMA	16.6	low

Table 2. Bacterial reverse mutation test of 2-chloro-4-methylaniline with or without S9 mix.

Compound	Dose (μg/plate)	S9 mix	Number of revertant colonies per plate										
			Frameshift type					Base-pair substitution type					
			TA98					TA100					
DMSO	(0.1 mL)	+	28					99					
			32		[	30	]	94		[	97	]	
2C4MA	3	+	33					113					
			29		[	31	]	119		[	116	]	
	10	+	30					138					
			29		[	30	]	135		[	137	]	
	30	+	46					215					
			47		[	47	]	223		[	219	]	#
	100	+	58					273					
			60		[	59	]	292		[	283	]	#
	300	+	67					382					
			67		[	67	] #	357		[	370	]	#
	1000	+	672	*				122	*				
			717	*	[	695	]	119	*	[	121	]	
2AA	0.5	+	325					--					
			328		[	327	]						
	1	+	--					463					
								401		[	432	]	
DMSO	(0.1 mL)	–	22					101					
			24		[	23	]	105		[	103	]	
2C4MA	3	–	24					97					
			21		[	23	]	111		[	104	]	
	10	–	20					98					
			23		[	22	]	103		[	101	]	
	30	–	23					100					
			19		[	21	]	97		[	99	]	
	100	–	22					96					
			21		[	22	]	101		[	99	]	
	300	–	25					93					
			20		[	23	]	114		[	104	]	
	1000	–	2	*				1	*				
			3	*	[	3	]	2	*	[	2	]	
AF-2	0.01	–	--					503					
								510		[	507	]	
	0.1	–	582					--					
			631		[	607	]						

[ ]: The figures in the parentheses indicate the mean number of revertant colonies., --: Not treated,
*: Growth inhibition (thin lawn), #: The results were judged as positive when the mean number of revertant colonies showed a dose-dependent increase and reached 2-fold over that of the negative control (DMSO).

The number of revertant colonies in the two strains (TA98 and TA100) with S9 mix increased dose-dependently from 10 μg/plate (TA100) or 30 μg/plate (TA98). 2C4MA increased the number of revertant colonies to approximately twice or more when compared with the negative control at 300 μg/plate for TA98 and 30 to 300 μg/plate for TA100 with S9 mix (Table 2). Without S9 mix, 2C4MA did not increase the number of revertant colonies in either strain (Table 2). Specific mutagenicity calculated based on the data from TA100 (with S9 mix) was 4,067 revertants/mg/plate (Table 7). The positive controls induced a large number of revertant colonies in both strains with or without S9 mix (Table 2).

4C2MA

Inhibition of bacterial growth was observed at

Table 3. Bacterial reverse mutation test of 4-chloro-2-methylaniline with or without S9 mix.

Compound	Dose (μg/plate)	S9 mix	Number of revertant colonies per plate									
			Frameshift type					Base-pair substitution type				
			TA98					TA100				
DMSO	(0.1 mL)	+	28					99				
			32		[	30	]	94		[	97	]
4C2MA	3	+	34					137				
			31		[	33	]	140		[	139	]
	10	+	31					220				
			32		[	32	]	224		[	222	] #
	30	+	53					251				
			48		[	51	]	268		[	260	] #
	100	+	57					278				
			59		[	58	]	279		[	279	] #
	300	+	89					318				
			98		[	94	] #	310		[	314	] #
	1000	+	76	*				288	*			
			72	*	[	74	]	298	*	[	293	]
2AA	0.5	+	325					--				
			328		[	327	]					
	1	+	--					463				
								401		[	432	]
DMSO	(0.1 mL)	–	22					101				
			24		[	23	]	105		[	103	]
4C2MA	3	–	24					100				
			20		[	22	]	106		[	103	]
	10	–	22					98				
			19		[	21	]	108		[	103	]
	30	–	23					108				
			21		[	22	]	95		[	102	]
	100	–	22					93				
			23		[	23	]	97		[	95	]
	300	–	24					111				
			18		[	21	]	93		[	102	]
	1000	–	1	*				1	*			
			0	*	[	1	]	1	*	[	1	]
AF-2	0.01	–	--					503				
								510		[	507	]
	0.1	–	582					--				
			631		[	607	]					

[ ]: The figures in the parentheses indicate the mean number of revertant colonies., --: Not treated,
*: Growth inhibition (thin lawn), #: The results were judged as positive when the mean number of revertant colonies showed a dose-dependent increase and reached 2-fold over that of the negative control (DMSO).

1000 μg/plate for both strains with or without S9 mix (Table 3).

The number of revertant colonies in both strains (TA98 and TA100) with S9 mix increased dose-dependently from 3 μg/plate (TA100) or 30 μg/plate (TA98). 4C2MA increased the number of revertant colonies to approximately twice or more when compared with the negative control at 300 μg/plate for TA98 and 10 to 300 μg/plate for TA100 with S9 mix (Table 3). Without S9 mix, 4C2MA did not increase the number of revertant colonies in either strain (Table 3). Specific mutagenicity calculated based on the data from TA100 (with S9 mix) was 12,500 revertants/mg/plate (Table 7). The positive controls induced a large number of revertant colonies in both strains with or without S9 mix (Table 3).

2C4,5DFA

Inhibition of bacterial growth was observed at 3000 µg/plate and above for both the strains with or without S9 mix (Table 4).

The mean number of revertant colonies in either strain with S9 mix did not exceed 2-fold when compared with that in the negative control with S9 mix (Table 4). However, the mean number in TA100 with S9 mix at 100, 300 and 1000 µg/plate was 1.6, 1.4 and 1.6-fold higher, respectively, when compared with that in the negative control with S9 mix (Table 4). Without S9 mix, 2C4,5DFA did not increase the number of revertant colonies in either strain (Table 4). Specific mutagenicity was not calculated for this compound because the results of Ames test were negative (Table 7). The positive controls induced a large number of revertant colonies in both strains with or without S9 mix (Table 4).

4TFMA

Inhibition of bacterial growth was observed at 1000 µg/plate and above for both strains with S9 mix (Table 5). Without S9 mix, inhibition of bacterial growth was observed at 300 µg/plate and above for TA100 and 1000 µg/plate and above for TA98 (Table 5).

The number of revertant colonies in TA100 with S9 mix increased dose-dependently from 30 µg/plate and the number of revertant colonies was approximately twice or more when compared with the negative control at 100 and 300 µg/plate (Table 5). Without S9 mix, the number of revertant colonies in TA100 was increased dose-dependently from 30 µg/plate and the number of revertant colonies was approximately twice or more when compared with the negative control at 100 µg/plate (Table 5). There were no increases in the number of revertant colonies in TA98 at any dose level regardless of the presence or absence of S9 mix (Table 5). Specific mutagenicity calculated based on the data from TA100 with and without S9 mix was 1,590 and 1,910 revertants/mg/plate, respectively (Table 7). The positive controls induced a large number of revertant colonies in both strains with or without S9 mix (Table 5).

In vivo comet assays of 2C4MA and 2C4,5DFA using rats

Mortality and clinical signs

No deaths occurred in any group of either test compound throughout the experimental period in this study. At 1000 mg/kg of 2C4,5DFA, soiled perineal region by urine was observed on day 3. There were no findings in any of the other groups.

Percent tail DNA

The mean values of the % tail DNA for the liver and kidney are shown in Table 6, respectively.

Negative control

For the liver, the mean values of the % tail DNA in the negative control groups for 2C4MA and 2C4,5DFA were 1.29 and 1.26%, respectively, and there were no statistically significant differences in the mean values between these two negative control groups. For the kidney, the mean values of the % tail DNA in the negative control groups for 2C4MA and 2C4,5DFA were 1.87 and 1.58%, respectively, and there were no statistically significant differences in the mean values between these two negative control groups.

2C4MA

For the liver, the mean values of the % tail DNA at 100, 300 and 500 mg/kg were 1.61, 1.86 and 2.84%, respectively. There was a statistically significant increase ($p \leq 0.01$) in the % tail DNA at 500 mg/kg when compared with the matched negative control group (Table 6). For the kidney, the mean values of the % tail DNA at 100, 300 and 500 mg/kg were 1.73, 2.55 and 4.88%, respectively. There was a statistically significant increase ($p \leq 0.01$) in the % tail DNA at 500 mg/kg when compared with the matched negative control group (Table 6).

2C4,5DFA

For the liver, the mean values of the % tail DNA at 500 and 1000 mg/kg were 1.55 and 1.31%, respectively (Table 6). There were no statistically significant differences in the % tail DNA between the matched negative control and each of the treated groups (Table 6). For the kidney, the mean values of the % tail DNA at 500 and 1000 mg/kg were 1.69 and 3.08%, respectively (Table 6). There was a statistically significant increase ($p \leq 0.01$) in the % tail DNA at 1000 mg/kg when compared with the matched negative control group (Table 6).

EMS

For the liver, the mean value of the % tail DNA was 19.50% with a statistically significant increase ($p \leq 0.01$) when compared with the negative control group for 2C4,5DFA (Table 6). For the kidney, the mean value of the % tail DNA was 17.81% with a statistically significant increase ($p \leq 0.01$) when compared with the negative control group for 2C4,5DFA (Table 6).

Table 4. Bacterial reverse mutation test of 2-chloro-4,5-difluoroaniline with or without S9 mix.

Compound	Dose (μg/plate)	S9 mix	Number of revertant colonies per plate									
			Frameshift type						Base-pair substitution type			
			TA98						TA100			
DMSO	(0.1 mL)	+	28						98			
			32		[	30		]	100		[	99	]
2C4,5DFA	1	+	28						89			
			35		[	32		]	111		[	100	]
	3	+	31						99			
			39		[	35		]	105		[	102	]
	10	+	35						112			
			29		[	32		]	106		[	109	]
	30	+	30						106			
			36		[	33		]	130		[	118	]
	100	+	34						159			
			33		[	34		]	167		[	163	]
	300	+	33						124			
			37		[	35		]	144		[	134	]
	1000	+	35						163			
			33		[	34		]	144		[	154	]
	3000	+	0	*					0	*			
			1	*	[	1		]	1	*	[	1	]
	5000	+	0	*					0	*			
			0	*	[	0		]	0	*	[	0	]
2AA	0.5	+	243						--				
			238		[	241		]					
	1	+	--						666				
									667		[	667	]
DMSO	(0.1 mL)	–	18						97			
			20		[	19		]	105		[	101	]
2C4,5DFA	1	–	22						106			
			25		[	24		]	95		[	101	]
	3	–	21						91			
			17		[	19		]	97		[	94	]
	10	–	20						99			
			19		[	20		]	107		[	103	]
	30	–	16						97			
			23		[	20		]	109		[	103	]
	100	–	24						107			
			21		[	23		]	115		[	111	]
	300	–	19						99			
			23		[	21		]	88		[	94	]
	1000	–	23						88			
			21		[	22		]	73		[	81	]
	3000	–	3	*					1	*			
			1	*	[	2		]	2	*	[	2	]
	5000	–	1	*					3	*			
			1	*	[	1		]	2	*	[	3	]
AF-2	0.01	–	--						459				
									454		[	457	]
	0.1	–	666						--				
			649		[	658		]					

[ ]: The figures in the parentheses indicate the mean number of revertant colonies., --: Not treated, *: Growth inhibition (thin lawn).

Table 5. Bacterial reverse mutation test of 4-trifluoromethylaniline with or without S9 mix.

Compound	Dose (μg/plate)	S9 mix	Number of revertant colonies per plate									
			Frameshift type					Base-pair substitution type				
			TA98					TA100				
DMSO	(0.1 mL)	+	24					97				
			21		[	23	]	109		[	103	]
4TFMA	3	+	--					115				
								106		[	111	]
	10	+	21					109				
			27		[	24	]	117		[	113	]
	30	+	24					187				
			26		[	25	]	198		[	193	]
	100	+	27					267				
			23		[	25	]	256		[	262	] #
	300	+	35					481				
			33		[	34	]	476		[	479	] #
	1000	+	22	*				0	*			
			27	*	[	25	]	0	*	[	0	]
	3000	+	0	*				--				
			1	*	[	1	]					
2AA	0.5	+	262					--				
			250		[	256	]					
	1	+	--					632				
								641		[	637	]
DMSO	(0.1 mL)	–	20					123				
			21		[	21	]	127		[	125	]
4TFMA	3	–	--					113				
								114		[	114	]
	10	–	20					122				
			22		[	21	]	120		[	121	]
	30	–	22					184				
			20		[	21	]	158		[	171	]
	100	–	19					318				
			20		[	20	]	314		[	316	] #
	300	–	20					347	*			
			28		[	24	]	327	*	[	337	]
	1000	–	0	*				0	*			
			0	*	[	0	]	0	*	[	0	]
	3000	–	0	*				--				
			0	*	[	0	]					
AF-2	0.01	–	--					471				
								489		[	480	]
	0.1	–	554					--				
			562		[	558	]					

[ ]: The figures in the parentheses indicate the mean number of revertant colonies., --: Not treated, *: Growth inhibition (thin lawn), #: The results were judged as positive when the mean number of revertant colonies showed a dose-dependent increase and reached 2-fold over that of the negative control (DMSO).

DISCUSSION

In order to investigate relationships between the *in silico* analyses and *in vitro* and *in vivo* genotoxicity tests for aniline derivatives, AM1 values for the estimation of the stabilities of nitrenium ions were calculated for four aniline derivatives, 2C4MA, 4C2MA, 2C4,5DFA and 4TFMA and the AM1 values were compared with the results of Ames test and *in vivo* genotoxicity tests.

The AM1 values of 2C4MA, 4C2MA, 2C4,5DFA

Table 6. Percentage tail DNA of 2-chloro-4-methylaniline and 2-chloro-4,5-difluoroaniline in the liver and kidney of rats *in vivo* comet assay.

Compound	Dose (mg/kg/day)	Number of animals	% tail DNA [a]			
			Liver		Kidney	
			(Mean ± S.D.)		(Mean ± S.D.)	
Negative Control for 2C4MA (20%HCO-60/5%PG solution)	0	5	1.29 ± 0.42		1.87 ± 0.70	
2C4MA	100	5	1.61 ± 0.88		1.73 ± 0.40	
	300	5	1.86 ± 0.76		2.55 ± 0.48	
	500	5	2.84 ± 0.66	**	4.88 ± 1.13	**
Negative Control for 2C4,5DFA (0.5% MC aqueous solution)	0	5	1.26 ± 0.72		1.58 ± 0.59	
2C4,5DFA	500	5	1.55 ± 0.59		1.69 ± 0.20	
	1000	5	1.31 ± 0.26		3.08 ± 0.74	**
EMS	200	5	19.50 ± 3.86	**	17.81 ± 1.96	**

EMS as positive control was dissolved in water for injection., a: Calculated from 100 nuclei per animal, Significantly different from the matched negative control group (MiTOX Standard Pattern): ** $P < 0.01$

Table 7. Comparison of the specific mutagenicity and the stabilities of nitrenium ions between four aniline derivatives.

Compound	Specific mutagenicity (revertants/mg/plate)	Stabilities of nitrenium ions (AM1) (kcal/mol)
2C4MA	4,067 (TA100 with S9 mix)	-5.38
4C2MA	12,500 (TA100 with S9 mix)	-4.67
2C4,5DFA	Negative *	8.36
4TFMA	1,590 (TA100 with S9 mix) 1,910 (TA100 without S9 mix)	16.6

*: 2C4,5DFA was judged negative according to the evaluation criteria, however the number of revertant colonies in a TA100 strain with S9 mix tended to be increased.

and 4TFMA were -5.38, -4.67, 8.36 and 16.6 kcal/mol, respectively (Table 1). Aromatic amines including aniline derivatives are metabolized to hydroxyl amines through an N-hydroxylation reaction by cytochrome P450 and the hydroxyl amines lead to synthesis of nitrenium ions through activated esters. Ford *et al*. built a model that correlates the potential of mutagenicity in Ames tests to the stability of the nitrenium ion (Ford and Griffin, 1992; Ford and Herman, 1992). These authors employed the semi-empirical AM1 method to calculate the stability of the nitrenium ion relative to that of the baseline molecule, aniline. A negative AM1 value indicates that the nitrenium ion is more stable than that of aniline and the aromatic amine concerned is considered to be Ames positive. On the other hand, a positive AM1 value indicates that the aromatic amine concerned is Ames negative. Based on the AM1 values, the potential of mutagenicity of 2C4MA and 4C2MA was considered to be high (Positive results in the Ames tests) and that of 2C4,5DFA and 4TFMA was considered to be low (Negative results in the Ames tests).

In the bacterial reverse mutation tests (Ames tests), 2C4MA and 4C2MA induced reverse mutations in the two strains (TA98 and TA100) only with S9 mix, indicating that metabolites of these compounds cause mutagenicity. 2C4,5DFA was judged negative in the two strains (TA98 and TA100), however, the number of revertant colonies in TA100 with S9 mix tended to be increased dose-dependently, indicating that the metabolites of 2C4,5DFA have a

very weak mutagenic potential. 4TFMA induced reverse mutations in TA100 regardless of the presence or absence of S9 mix.

The specific mutagenicity was calculated based on the data from TA100 for the three aniline derivatives, 2C4MA, 4C2MA and 4TFMA, which gave positive results in Ames tests (Table 7). The specific mutagenicity of 2C4MA and 4C2MA was 4,067 and 12,500 revertants/mg/plate, respectively, with S9 mix indicating that the metabolites of these two aniline derivatives have a high mutagenic potential. The specific mutagenicity of 4TFMA was 1,590 revertants/mg/plate with S9 mix and 1,910 revertants/mg/plate without S9 mix. These values were similar between the condition with S9 mix and that without S9 mix, indicating that 4TFMA itself has a high mutagenic potential.

From the results of the *in silico* analyses and bacterial mutation tests (Ames tests) conducted for the four aniline derivatives, a good correlation was established between these two endpoints for 2C4MA and 4C2MA both of which had a negative AM1 value, indicating that the nitrenium ion of these two aniline derivatives affected DNA. The AM1 value of 2C4,5DFA was positive (8.36 kcal/mol) and the results of Ames test with S9 mix for this compound were equivocal.

Bentzien *et al*. reported that there were no misclassifications for compounds with AM1 values greater than 21.7 kcal/mol (Ames negative compounds) or smaller than -25.1 kcal/mol (Ames positive compounds) based on the correlation analyses between AM1 values and Ames tests conducted for large number of compounds. The AM1 value of 2C4,5DFA was in between these two cutoff values and the nitrenium ion may contribute to the equivocal results in the Ames tests. The AM1 value of 4TFMA (16.6 kcal/mol) was close to the cutoff value (21.7 kcal/mol) for Ames negative compounds and 4TFMA itself was considered to have mutagenicity based on the results of Ames test. Therefore, contribution of the nitrenium ion to the Ames positive results is doubtful for this compound.

In the present study, 2C4MA caused DNA damage in the liver and kidney in a comet assay with rats. 4C2MA has been reported to cause DNA damage in the liver and kidney in a comet assay and to have carcinogenicity (Sekihashi *et al*., 2002; Zimmer *et al*., 1980). From these, 2C4MA and 4C2MA have similar genotoxic profile based on the results of *in silico* analyses and *in vitro* and *in vivo* genotoxicity tests and 2C4MA is considered to have a carcinogenic potential as well as 4C2MA. For these two compounds, there were good correlations between the results of the *in silico* analyses, Ames tests and *in vivo* genotoxicity tests.

In the present study, 2C4,5DFA caused DNA damage in the kidney but not in the liver. Although the etiology of the absence of DNA damage in the liver is not known, this compound also has genotoxicity *in vivo* and is considered to have a potential for carcinogenicity. The results obtained in this compound give a good example to show that negative results in *in silico* analyses and Ames test do not necessarily lead to negative results in *in vivo* genotoxicity tests. This is also a good example to show that careful data analyses including dose-response relationship analyses in Ames tests is necessary even if the results of Ames tests were negative based on the standard criteria (number of revertant colonies is less than two-fold compared to the negative control).

4TFMA has been reported not to induce mutagenicity or clastogenicity in the liver in an *in vivo* UDS test using rats, in the bone marrow in an *in vivo* micronucleus test using rats and in the bone marrow in an *in vivo* chromosomal aberration test using hamsters (Pharmacology Review(s), Food and Drug Administration, Center for Drug Evaluation and Research, 2012, Application, Number: 202992Orig1s000) (Table 8).

The profile of *in vitro* mutagenicity (Ames test) of 4TFMA was different from that of other three compounds and 4TFMA itself is considered to induce bacterial reverse mutations. There are some possible explanations for the difference in the genotoxic response between bacteria and mammals. One possibility is that there are differences in the metabolism of 4TFMA between bacteria and mammals and that 4TFMA was metabolized into non-genotoxic metabolites more quickly in mammals than in bacteria. Another possibility is that there are differences in DNA repair ability between bacteria and mammals. It is well known that there are significant differences in DNA repair mechanisms and pathways between bacteria and mammalian cells (Hanawalt *et al*., 1979). The case of 4TFMA indicates an importance of considering the weight of evidence for Ames positive results in the evaluation of carcinogenic potential of aniline derivatives.

In conclusion, we set up the comparison of the stabilities of nitrenium ions estimated by *in silico* analyses, *in vitro* and *in vivo* genotoxicity tests for four aniline derivatives, 2C4MA, 4C2MA, 2C4,5DFA and 4TFMA in this study. The results of *in silico* analyses and *in vitro* and *in vivo* genotoxicity tests were consistent for aniline derivatives like 2C4MA and 4C2MA with strong mutagenicity but were not for those like 2C4,5DFA and 4TFMA with weak mutagenicity. Careful assessment for the risk of carcinogenicity is necessary for aniline derivatives with weak mutagenicity by combining *in silico* analyses and *in*

Table 8. Comparison of the results of *in vivo* genotoxicity tests between four aniline derivatives.

Compound	*In vivo* genotoxicity test	Dose level, route and target organ	Judgement
2C4MA	Comet Assay in Rats	500 mg/kg (male); p.o., Liver and Kidney	Positive
4C2MA	Comet Assay in Rats	500 mg/kg (male); p.o., Liver and Kidney	Positive
2C4,5DFA	Comet Assay in Rats	1000 mg/kg (male); p.o., Kidney	Positive
4TFMA	Unscheduled DNA Synthesis (UDS) Test in Rats	Up to 100 mg/kg ; p.o., Liver	Negative
	Micronucleus Test in Mice	Up to 300 mg/kg; i.m., Bone Marrow	Negative
	Chromosomal Aberration Test in Hamsters	Up to 75 mg/kg; p.o. Bone Marrow	Negative
	Carcinogenicity Test of Teriflunomide in Rats *	Up to 15 mg/kg/day; p.o., Plasma Concentration of 4-TFMA: Low Levels (ng/mL order)	Negative
	Carcinogenicity Test of Teriflunomide in Mice *	Up to 12 mg/kg/day; p.o., Plasma Concentration of 4-TFMA: Low Levels (ng/mL order)	Negative

*: Evaluation as a minor metabolite of teriflunomide

vitro and *in vivo* genotoxicity tests.

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