MOLECULAR & CELLULAR TOXICOLOGY, Vol. 3, No. 1, 53-59, March 2007

Genotoxicity Study of Dimethyl Isophthalate in Bacterial and Mammalian Cell System

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Accepted 8 March 2007

Abstract

This study was conducted to evaluate the mutagenic potential of dimethyl isophthalate (DMIP) using Ames bacterial reverse mutation test, chromosomal aberration test and mouse lymphoma $tk^{+/-}$ gene assay. As results, in Ames bacterial reversion assay, DMIP was tested up to the concentration of 5,000 μg/plate and did not induce mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, and *Escherichia coli* WP2*uvr*A with or without metabolic activation (S9 mix). Using cytotoxicity test, the maximal doses of DMIP for chromosomal aberration assay were determined at 1,250 μg/mL, which was a minimum precipitation concentration (IC_{50}>1,940 μg/mL or 10 mM) and at 155 μg/mL (IC_{50}: 155 μg/mL) in the presence and the absence, respectively, of S9 mix. DMIP in the presence of S9 mix induced statistically significant ($P<0.001$) increases in the number of cells with chromosome aberrations at the dose levels of over 250 μg/mL, when compared with the negative control. However, DMIP in the absence of S9 mix did not caused significant induction in chromosomal aberrant cells. In MLA, DMIP at the dose range of 242.5-1,940 μg/mL in the presence of S9 mix induced statistically significant increases in mutation frequencies related to small colony growth, whereas any significant mutation frequency was not observed in absence of S9 mix. From these results, it is conclusively suggested that dimethyl isophthalate may be a clastogen rather than a point mutagen.

Keywords: Dimethyl isophthalate, Genotoxicity, Bacterial reverse mutation test, Chromosomal aberration test, Mouse lymphoma $tk^{+/-}$ gene assay

Dimethyl isophthalate (DMIP) is widely used in the production of sodium dimethyl isophthalate-5-sulfonate (SIPM) for enhancing the chromaticity of polyethylene terephthalate (PET). The major sources releasing large amounts of DMIP into the environment are the wastewater generated from the production of dimethyl terephthalate (DMTP), which contain high concentrations of DMIP at 35-40% as the major by-product of DMTP manufacturing processes (Fajardo *et al.*, 1997)[1]. In Korea, 971 tons of DMIP was imported as a polymer modifier and as a chemical intermediate in 2002[2].

The impact of these chemicals on the environment and their toxicity to living organism are of great concern today. DMTP is suspected of causing cancer and chronic kidney inflammation[3]. Several studies conducted in rats, rabbits, and mice have all indicated that dimethyl terephthalate (DMTP) is absorbed readily from the digestive tract and eliminated rapidly in the urine within 48-hours. Most of the absorbed DMTP is metabolized to terephthalic acid (TPA) via ester hydrolysis. Because DMIP and DMTP are close structural analogues, it is anticipated that DMIP is absorbed readily from the digestive tract, most of the absorbed DMIP is metabolized to isophthalic acid (IPA) via ester hydrolysis, and IPA is eliminated rapidly in the urine[4].

However, so far only limited information is available on the identification of transformation intermediates and characterization of biochemical pathway of DMIP prior to the formation of isophthalic acid (IPA)[5]. Especially, *in vitro/in vivo* genetic toxicity, toxicokinetics, reproductive toxicity of DMIP have not been thoroughly studied.

The objectives of this study were conducted to evaluate the mutagenic potential of DMIP using Ames bacterial reverse mutation test, chromosomal aberration test, mouse lymphoma $tk^{+/-}$ gene assay (MLA).

Bacterial Reverse Mutation Test on DMIP

For the dose range-finding, three tester strains of bacteria were exposed to 1.2-5,000 μg/plate of DMIP (Figure 1) in the absence and the presence of S9 mix. The exposure of DMIP at 5,000 μg/plate caused 50% decreases in the number of revertant colonies of *Sal-*

monella typhimurium TA100, compared with that of DMSO exposure (negative control). Thus, the main mutation study was performed using the exposing-dose ranges of 156.3-5,000 μg/plate in five bacterial strains (*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2*uvr*A) in the absence and the presence of S9 mix. The results of the mutation test are presented as a mean and standard deviation of triplicate in the revertant colonies (Table 1). The frequency of revertant colonies for any of the bacterial strains, with any dose of DMIP, either with or without metabolic activation, did not increase concentration-related, which was representing non-mutagenic.

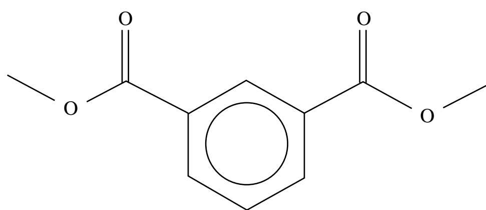

Figure 1. Chemical structure of dimethyl isophthalate.

The frequency of revertant colonies for the negative control in Table 1 was considered to be acceptable and all of the positive controls induced marked increases in the frequency of revertant colonies confirming the activity of S9 mix and the sensitivity of the bacterial strains. For the study validation, the master strains were checked for characteristics, spontaneous reversion rate representing amino acid requirement, ampicillin resistance for R-factor, and sensitivity to crystal violet for *rfa* membrane mutation and to UV for DNA repair. These data are not given in this report.

Chromosome Aberration Assay on DMIP

In order to evaluate the concentration of 50% cell growth inhibition (IC_{50}), cytotoxicity test was performed using MTT assay and the result was shown that IC_{50} values were 155 μg/mL and over 1,940 μg/mL (or 10 mM) in the absence and the presence, respectively, of S9 mix (Figure 2). Considering IC_{50} values and precipitation of DMIP, the dose levels of the exposure in chromosome aberration test were determined as 38.8, 77.5, 155 μg/mL without metabolic activation and 312.5, 625, 1,250 μg/mL with

Table 1. Summary of revertant colony numbers obtained per plate with or without S9 mix in bacterial reverse mutation test.

S9 mix	Test substance	Dose (μg/plate)	Number of revertant colonies/plate				
			Base replacement type			Frame shift type	
			TA100	TA1535	WP2*uvr*A	TA98	TA1537
−	DMSO		53.7±12.1	33.7±2.9	58.7±2.3	35.3±3.2	13.3±1.5
	DMIP	156.3	54.7±2.1	31.3±1.5	57.7±2.1	35.0±3.5	13.3±1.5
		312.5	50.0±7.8	34.0±4.0	52.3±3.8	34.0±3.6	13.7±2.5
		625	26.0±9.8	35.0±7.2	57.0±10.8	27.3±5.8	8.0±2.6
		1250	17.3±2.1	16.0±1.7	36.7±9.1	23.0±1.7	2.3±0.6
		2500	20.0±6.1	17.0±2.6	29.7±5.5	22.3±2.3	2.7±2.1
		5000	25.7±5.9	16.0±1.7	28.0±7.0	34.0±5.3	4.0±1.7
	SA	1	680.7±18.6	−	−	−	−
	SA	1	−	576.0±24.2	−	−	−
	AF-2	0.01	−	−	128.0±7.0	−	−
	2-NF	1	−	−	−	243.0±7.9	−
	9-AA	80	−	−	−	−	436.3±41.7
+	DMSO		170.3±16.2	16.7±1.5	59.3±5.0	54.0±7.0	12.0±1.7
	DMIP	156.3	173.0±18.7	9.0±2.6	57.3±5.5	40.0±1.7	8.0±1.0
		312.5	183.0±14.1	14.7±1.2	59.7±9.1	58.7±8.5	12.7±3.2
		625	172.7±24.8	14.3±4.5	50.3±8.6	50.3±3.2	10.0±2.6
		1250	146.3±14.2	12.3±3.2	40.7±7.8	51.7±9.1	11.0±2.6
		2500	118.3±55.0	12.7±0.6	27.7±6.8	50.3±1.5	9.7±1.2
		5000	80.0±12.8	15.3±1.5	29.7±3.8	52.3±6.8	8.7±2.3
	2-AA	1	422.0±10.6	−	−	−	−
		2	−	107.7±3.1	−	−	−
		20	−	−	356.7±32.1	−	−
		0.5	−	−	−	233.3±28.6	−
		2	−	−	−	−	110.7±4.5

Data are presented as mean±SD (N=3), DMSO: Dimethyl sulfoxide (100 μL), SA: sodium azide, AF-2: 2-aminofluorene, 2-NF: 2-nitrofluorene, 9-AA: 9-aminoacridine, 2-AA: 2-aminoanthracene.

metabolic activation. The test results of short term (6 hours) and long term (24 hours) exposures, and confirmation experiment (6 hour exposure) are shown in Table 2. In the presence of S9 mix, DMIP induced statistically significant ($P < 0.001$) increases in the number of cells with chromosome aberrations at all dose levels, 312.5, 625, 1,250 μg/mL, when compared with the negative vehicle (DMSO). In the absence of S9 mix, DMIP caused no statistically significant ($P > 0.05$) increases in the number of chromosomal aberrant cells at any dose level and for any exposure time, when compared with the vehicle. In the confirmation test of a short term exposure in the presence of S9 mix, DMIP at the concentrations, 125, 250, 500 μg/mL induced statistically significant dose-related increases; 3.0, 10.0, and 15.0%, respectively in the percentage of aberrant cells.

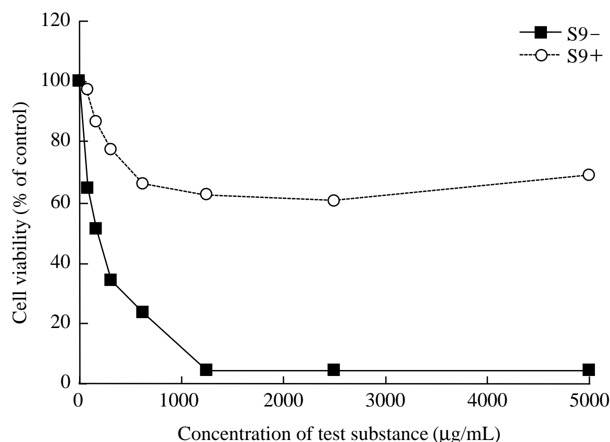

Figure 2. Effect of DMIP on the viability of CHL cells for a 6 hour exposure with S9 mix or for a 24 hour exposure without S9 mix.

Positive control compounds, mitomycin C and cyclophosphamide monohydrate, caused a statistically significant ($P < 0.001$) increases in the number of cells with chromosome aberrations, representing that the test conditions were adequate and that the metabolic activation (S9 mix) functioned properly.

Mouse Lymphoma Assay on DMIP

Using MLA, genotoxic potential of DMIP in the absence and the presence of S9 mix was assessed up to the concentration of 1,940 μg/mL 0 mM), which was the dose of over 20% relative survival (% RS) of L5178Y cells for a 3 hour exposure (Figure 3). Figure 4 showed the results from a 3 hour exposure in the absence and the presence of S9 mix and a 24 hour

Table 2. Metaphase analysis of chromosome aberrations in CHL cells exposed to DMIP with or without S9 mix.

S9 mix	Time (hour)	Concentration (μg/mL)	ctb	cte	csb	cse	Aberration excluding gap (%)	ctg	csg	endo	Aberration including gap (%)	Normal cells (No.)
+	6	NC	1	1	0	0	1.0	2	0	0	2.0	196
		312.5	7	14	0	0	8.5*	6	0	0	11.5	177
		625.0	7	29	3	1	17.5*	7	1	1	20.0	160
		1250.0	52	64	20	4	67.0*	9	3	3	68.0	64
		CP	17	23	3	1	21.5*	11	4	4	28.5	143
−	6	NC	1	1	0	0	1.0	3	1	1	3.0	194
		38.8	0	0	0	0	0.0	3	0	0	1.5	197
		77.5	2	1	0	0	1.5	5	2	2	5.0	190
		155.0	2	0	0	0	1.0	3	1	1	3.0	194
		MMC	20	37	4	1	29.0*	8	3	3	33.5	133
−	24	NC	2	1	0	0	1.5	2	0	0	2.5	195
		38.8	1	1	0	0	1.0	2	1	1	2.5	195
		77.5	0	0	0	0	0.0	2	1	1	1.5	197
		155.0	1	0	0	0	0.5	1	2	2	2.0	196
		MMC	25	38	4	0	31.5*	8	4	4	37.0	126
+	6[a]	NC	1	1	0	0	1.0	1	1	0	2.0	196
		125	2	4	0	0	3.0	7	1	0	7.0	186
		250	6	17	0	0	10.5*	7	1	2	14.5	171
		500	8	21	1	2	15.0*	6	2	0	18.5	162
		CP	11	29	1	1	18.5*	6	3	0	21.5	157

*: $P < 0.001$, Otherwise: $P > 0.05$ comparison with negative control by Fisher's exact test.
NC: DMSO (1%), CP: Cyclophosphamide monohydrate (10 μg/mL), MMC: Mitomycin C (1 μg/mL), ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange, ctg: chromatid gap, csg: chromosome gap, [a]: the results from a 6 hour confirmation test.

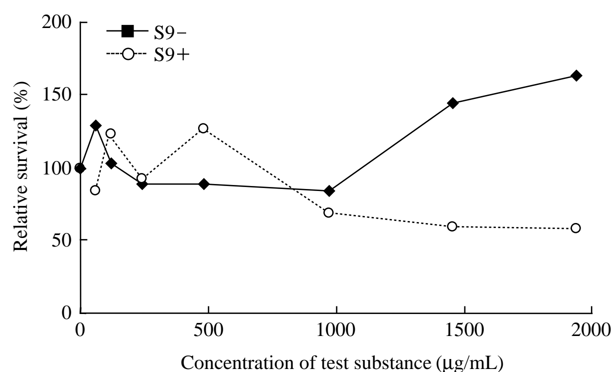

Figure 3. Relative survival (RS) rate of L5178Y cells exposed to DMIP for 3 hours with or without S9 mix.

Table 3. Plate counts and mutation frequencies for large and small colonies.

Test substance	Dose (μg/mL)	S9−, 3 h		S9+, 3 h		S9−, 24 h	
		SC	LC	SC	LC	SC	LC
DMSO		18.5	25	14.0	2.0	12.0	0.5
DMIP	121.3	−	−	−	−	13.0	3.0
	242.5	18.5	1.5	15.0	2.5	12.5	1.0
	485	16.5	2.5	22.5	3.0	21.5	0.5
	970	17.0	2.5	29.5	2.0	−	−
	1455	22.0	2.0	33.5	6.0	−	−
	1940	20.0	2.5	42.5	2.5	−	−
MMS	10	47.0	9.5	−	−	81.0	16.5
CP	3	−		67.0	9.5	−	−

Data are presented as means (N=2).
SC: the number of small colonies, LC: the number of large colonies.

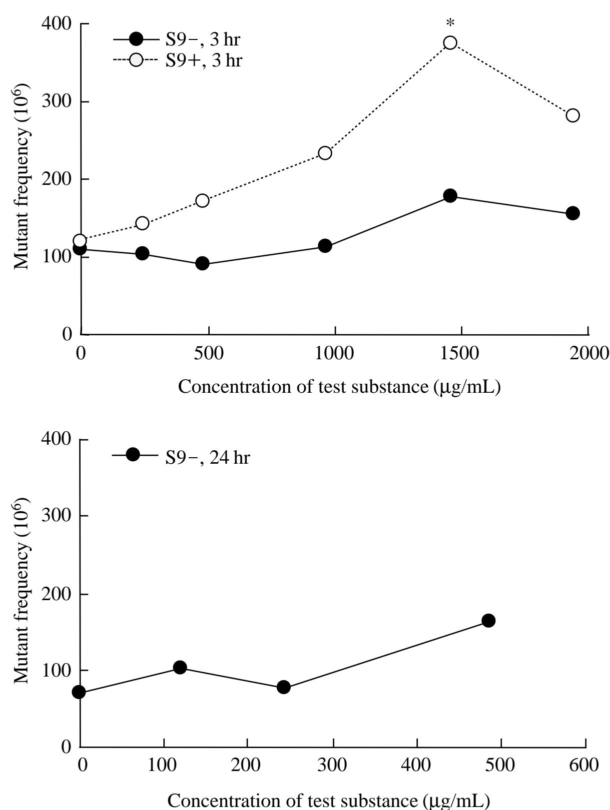

Figure 4. Mutation frequencies by DMIP in L5178Y cells in a 3 hour exposure with or without S9 mix and in a 24 hour exposure without S9 mix. *: Statistically significant ($P<0.05$).

continuous exposure in the absence of S9 mix. DMIP did not induce significant changes in MFs and a dose response for short term and continuous exposures in the absence of S9 mix. The mutant colonies couldn't be counted in a 24 hour exposure of DMIP over 970 μg/mL, since L5178Y cells was not survived well. However, DMIP caused a significant induction and a dose response in MFs in the presence of S9 mix. Those inductions in MFs were related to the increases in small colonies rather than large colonies of L5178Y mutant cells on the well plate (Table 3).

The adequacy of the experimental conditions for detection of induced mutation was confirmed by employing positive control chemicals, MMS and CP without and with, respectively, S9 mix. Background mutation frequencies were within the historical control range, and positive controls gave marked increases in MFs, meeting assay acceptance criteria.

Discussion

We investigated the genotoxic potential of DMIP using three different assay systems, AMES, CA and MLA. AMES has been known to detect mutagenic potential in a prokaryotic system and CA to detect clastogenic potential in an eukaryotic system, while MLA to detect point mutation as well as small deletion of chromosome in an eukaryotic system.

Under the present experimental conditions, DMIP in the absence and the presence of S9 mix did not induced a point mutation in bacterial systems. Chromosome aberration assay using CHL cells demonstrated that the treatment of DMIP without metabolic activation did not cause induction in chromosomal aberrant cells, while the metabolism of DMIP with metabolic activation caused significant induction in the frequency of chromosomal aberration. MLA assay showed that DMIP induced the mutation frequency only in the presence of S9 mix, which related to the increases in small mutation colonies but not in large colonies.

A striking feature of the *tk* mutant colonies recovered in the MLA is the presence of two size classes of mutants. Immediately following their isolation, the cells in the large colonies grow at a normal rate, while cells in the small colonies grow slowly. The relative frequency of the two colony classes is mutagen dependent. Generally, clastogens induce more small colony mutants while point mutagens induce more large colony mutants[6-8]. Thus, it is suggested that DMIP may be a clastogen rather than a point mutagen.

From the several reports about the toxicity of DMIP, it was anticipated that DMIP may not cause genetic damages[4]. However, the present study showed clastogenic potential of DMIP in eukaryotic cells was different in the absence and the presence of S9 mix. DMIP as a parent compound was more cytotoxic but its metabolites were clastogenic at high concentrations. From the studies about degradation mechanisms by microorganisms, it has been demonstrated that DMIP and DMTP are transformed to corresponding monoesters first (MMIP and MMTP) and then to isophthalic acid (IPA) and teraphthalic acid (TPA), which are converted to protocatechuic acid (PCA) before cleavage of the aromatic ring[5,9,10]. Recent reports have suggested the phthalates and their degradation products, the so-called monophthalates, play an important role in the development of allergic respiratory diseases. With respect to the toxicological effects, the monophthalate is considered to be more potent than its parent compound[11,12]. The fate of DMIP *in vivo* system has been largely neglected, but anticipated to be similar in microorganisms[4]. Thus, positive result of the present study could be expected and further researches about t *in vivo* experiment or metabolites of DMIP are recommended.

Methods

Chemicals and Reagents

Dimethyl isophthalate (DMIP) was purchased from Fluka Company. Sodium azide (SA), 2-nitrofluorene (2-NF), 9-aminoacridine (9-AA), 2-aminoanthracene (2-AA), methyl methanesulfonate (MMS), cyclophosphamide monohydrate (CP) from Sigma-Aldrich Co., 2-aminofluorene (AF-2) from Wako Co., and mitomycin C from Amresco Inc. were obtained. Eagles minimum essential medium (EMEM), RPMI-1640, Colcemid, fetal bovine serum were purchased from Invitrogen corporation. The S9 fraction, a postmitochondrial supernatant was purchased from MOLTOX™ (Molecular Toxicology, Inc.).

Ames *Salmonella* Bacterial Mutagenicity Assay

This test performed essentially as described by Ames *et al.* and OECD guideline[13-16]. The dose range for test substance was determined by performing a toxicity assay using strain *Salmonella typhimurium* TA98, TA100 and *Escherichia coli* WP2*uvr*A and half-log dose intervals of DMIP up to 5,000 μg/plate. Spontaneous revertant numbers were counted and plotted against the dose of DMIP to produce a survival curve for the *his*+ genotype. The mutagenicity assay was performed by mixing one of the tester strains which was cultured overnight, with DMIP in the presence and in the absence of S9 mixture condition, sodium phosphate buffer added instead of S9 mix both in negative and positive control in test tube. Then, incubating the mixture in shaking incubator for 30 min at 37°C and after incubation, the mixture mixed with top agar containing a minimal amount of histidine or tryptophan and then poured onto the surface of a *r*-ray sterile Petri dish (Falcon, USA) containing 30 mL of solidified bottom agar. The finished plates were incubated for 48 h at 37°C, and revertant colonies were counted later. Negative control plates containing no added test substance but positive control plates containing appropriate amounts of chemicals known to be active were included with each tester strain. All platings were done in triplicate, and the results were tabulated as the mean ± standard deviation for each condition.

In Vitro Chromosomal Aberrations Assay in CHL Cells

The clastogenicity of DMIP was evaluated for their ability to induce chromosomal aberrations in CHL cells. The experiment was performed as described by OECD guideline[17] and Ishidate and Odashima[18] with some minor modifications[19,20], which are briefly summarized as follows. Concentration selection for this assay was based on solubility (testing was performed up toprecipitating concentrations, 1,250 μg/mL), and determination of cytotoxicity. Three different doses, including the IC_{50} value as a maximal dose, were prepared and separately added to 3-day-old cultures (approximately 10^6 cells/60 mm dish). In the absence and in the presence of S9 mix, cultures were treated for 6 h with DMIP and then maintained for 18 h in the fresh medium to adjust a time equivalent to about 1.5 normalcellcycle lengths. Cyclophosphamide monohydrate (CP) and Mitomycin C (MMC) were used as positive controls in combination with or without S9 mix, respectively. After 22 h incubation, the treatment was followed by addition of medium containing Colcemid at a concentration of 0.25 μg/mL. Then, 2 h

further incubated in the presence of Colcemid, metaphase cells were harvested by trypsinization and centrifugation. The cells were swollen by adding with hypotonic (0.075 M) KCl solution for 30 min at 37°C, and washed three times in ice-cold fixative (methanol : glacial acetic acid=3 : 1). After centrifugation, the fixative was removed, and cell pellet suspensions were prepared by pipetting gently. A few drop of cell pellet suspension were dropped onto pre-cleaned glass microscope slides, and dried in the air. Slides were stained with 5% Giemsa buffered solution at pH 6.8 for scoring of chromosome aberrations. The number of cells with chromosomal aberrations was recorded on 200 well-spread metaphase cells at the magnification of 1,000 with Inverted microscope (Nikon, Optiphot-2). The classification of aberration types referred to JEMS-MMS[21]. Breaks less than the width of a chromatid were designated as gaps in our criteria, and it was not included as chromosomal aberration. The incidence of polyploid and endoreduplicated cells was also recorded when these events were observed. Solvent-treated cells served as negative controls in this experiment. CHL cells usually have less than 3.0% cells with spontaneous chromosome aberrations. Aberration frequencies, defined as aberrations observed divided by number of cells counted, were analyzed using Fishers exact test[22] with Dunnetts adjustment and compared with results from the solvent controls. Therefore, data from count up well-spread 200 metaphase cells were expressed as percentages, and then dose-dependent responses and the statistical significance in *P*-value will be considered as positive results.

L5178Y *tk*$^{+/-}$-3.7.2C Mouse Lymphoma Assay (MLA)

This test was performed as described by Ryu, *et al*.[23]. To prepare working stocks for gene mutation experiments, cultures were purged of *tk*$^{-/-}$ mutants by exposure for 1 day to THMG medium (culture medium containing 3 μg/mL thymidine, 5 μg/mL hypoxanthine, 0.1 μg/mL methotrexate and 7.5 μg/mL glycine) and then the cells were transferred to THG medium (THMG but without methotrexate) for 2 days. The purged cultures were checked for low background *tk*$^{-/-}$ mutants and stored in liquid nitrogen. Each experiment started with working stock. The cells were usually used on day 3 or 4 after thawing and during logarithmic growth. S9 fraction (post-mitochondrial supernatant of Arochlor-1254 induced rat liver) for exogenous metabolic activation was purchased from MOLTOX (Lot No. 1987, Molecular Toxicology Inc.). S9 mix was prepared just prior to use by combining 4 mL S9 with 2 mL each 180 mg/mL glucose-6-phosphate, 25 mg/mL NADP and 150 mM KCl. The concentration of S9 mix was 5% during treatment and the final concentration of S9 was 2%. For treatment, cells were centrifuged and suspended at a concentration of 0.5×10^6 cells in 10 mL of medium in 15 mL polystyrene tubes. DMIP was tested with and without S9 mix. DMIP at each concentration were added and these tubes were gassed with 5% CO_2 in air and sealed. The cell culture tubes were placed on a roller drum and incubated at 37°C for 3 h. At the end of the treatment period, the cell cultures were centrifuged and washed twice with fresh medium and resuspended in fresh medium. We conducted preliminary experiments to determine the solubility and cytotoxicity of DMIPs. Cytotoxicity was determined by relative survival (RS) and relative total growth (RTG) following 3 h treatments at concentrations up to 1,940 μg/mL 0 mM), usually regardless of solubility. The recommended highest concentration was one with a 10-20% RS and/or RTG. Mutant selection was performed using the modified microwell version of the assay as described by Clements *et al*.[24]. Simply, the treated cells in medium containing 3 μg TFT/mL for selection or without TFT for cloning efficiency were distributed at 200 μL/well into 96-well flat-bottom microtiter plates. For mutant selection, two plates were seeded with ~2,000 cells/well. For cloning efficiency, two plates were seeded with ~1 cell/well. All plates were incubated in 5% CO_2 in air in a humidified incubator at 37°C. After 11-13 days incubation, clones were counted and the colony size distribution was determined. Mutant frequencies were calculated using a statistical package (MutantTM; UKEMS, York, UK) in accordance with the UKEMS guidelines[25].

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