Evaluation of the Genetic Toxicity of Synthetic Chemicals (XVI) - *in vitro* Mouse Lymphoma Assay with 3 chemicals -

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Abstract

The detection of many synthetic chemicals used in industry that may pose a genetic hazard in our environment is of great concern at present. Since these substances are not limited to the original products, and enter the environment, they have become widespread environmental pollutants, thus leading to a variety of chemicals that possibly threaten the public health. In this respect, to regulate and to evaluate the chemical hazard will be important to environment and human health. The genotoxicity of 3 synthetic chemicals was evaluated in L5178Y $tk^{+/-}$ mouse lymphoma cells *in vitro*. 9H-carbazole (CAS No. 86-74-8) did not induce signifi-cant mutation frequencies both in the presence and absence of metabolic activation system. 1, 3-Dichloro-2-propanol (CAS No. 96-23-1) revealed a significant increase of mutation frequencies in the range of 625-373 µg/mL in the absence of metabolic activation system and 157-79 µg/mL in the presence of metabolic activation system. And also, fenpropathrin (CAS No. 64257-84-7) appeared the positive results only in the absence of metabolic activation system. Through the results of MLA tk assay with 3 synthetic chemicals in L5178Y cells *in vitro*, we may provide the important clues on the genotoxic potentials of these 3 chemicals.

Keywords: Mutation Frequency, *in vitro* Mouse Lymphoma Assay, Thymidine Kinase, 9H-carbazole, 1, 3-Dichloro-2-propanol, Fenpropathrin

Despite the many toxicological researches on synthetic chemicals, there are few reports on the genotoxicity of some chemicals especially well used in chemical reaction processes in industry. In this re-spect, administrative authority has great concern to regulate and to evaluate the chemical hazards, and conducted the toxicity evaluations of synthetic chemicals. Our laboratory had also been involved in toxicity evaluation, especially in genotoxicity[1-6]. Several assay systems having rapidity and reliability have been introduced for this purpose, such as reversion test with bacterial gene mutation[7,8], chromosomal aberration assay with mammalian cells[9], mouse lymphoma $tk^{+/-}$ gene assay (MLA) with L5178Y $tk^{+/-}$ mouse lymphoma cells[10-13], micronucleus assay with rodents. These assay systems are now well used to evaluate the genotoxicity of chemicals and also frequently adopted as methods for an index of genotoxicity in worldwide. And also, it was well applied as a screening probe for the detection of possible carcinogenic substances in our environment.

Among these various assay systems, MLA using the thymidine kinase (tk) locus has been widely used to detect the ability of chemicals to induce genetic damage in cultured mammalian cells. A large body of information demonstrates the capability of the MLA tk assay to detect a broad spectrum of mutational events[14]. Therefore, the International Committee for Harmonization (ICH), in selecting a recommended test battery, discussed the MLA and the *in vitro* mammalian cytogenetic assays as possible alternatives. The cells used for the assay are mouse lymphoma cells (L5178Y $tk^{+/-}$ 3.7.2C), heterozygous at the thymidine kinase locus (*Tk1*) on chromosome 11. Inactivating the tk^+ allele (this functional allele is also referred to as $Tk1^b$, on chromosome 11b) induces trifluorothymidine (TFT) resistance, and $tk^{-/-}$ mutants can be selected for in a background of $tk^{+/-}$ non-mutant cells. Mutant colonies have a bimodal size distribution, with so-called large colonies growing at the rate of $tk^{+/-}$ cells and small colonies growing at a slower rate[15]. Early cytogenetic studies demonstrated that small colony mutants are often associated with chromosome aberrations involving chromosome 11 whereas large colony mutants are often cytogenetically normal[15,16]. Both large and small colony mutants are represented in spontaneous and induced mutants, and the proportion of small colony mutants is mutagen dependent. Extensive molecular and cytogenetic analysis has shown that mouse lymphoma cells can detect a variety of mutations, including point mutations and small mutations within *Tk1*, losses of

Tk1^b (the functional allele), larger deletions including *Tk1^b* and cytogenetically detectable chromosome aberrations such as translocations.

In this study, we examined the genotoxicity of commonly used and harmful environmental chemicals, 9H-carbazole, 1, 3-dichloro-2-propanol (1, 3-DCP) and fenpropathrin, using MLA mentioned above.

9H-Carbazole (dibenzopyrrole diphenylenimine, CAS no. 86-74-8), a group of polynuclear aromatic compounds, is used in manufacture of dyes, reagents, explosives, insecticide and lubricants, as color inhibitors in detergents, etc. 9H-Carbazole and several of its derivatives has been detected as a major component of cigarette smoke condensate[17,18]. Therefore, humans are always unavoidably exposed to this compound in various situations. 9H-Carbazole has induced carcinomas in liver and fore-stomach of mice by administration in diet, by skin application and by subcutaneous injection[19].
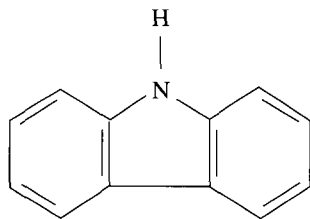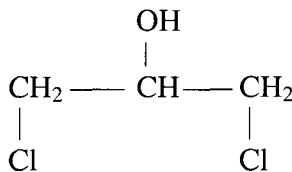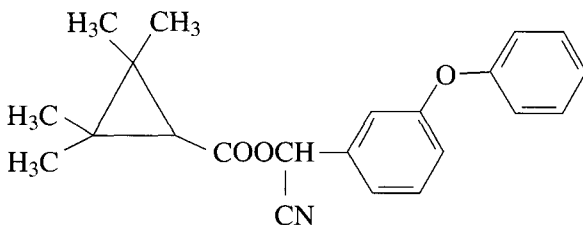
Dichloropropanols are a family of chlorinated compounds used in industries such as hard resin production, chlorination of water or fabrication of paper[20]. 1, 3-DCP (CAS no. 96-23-1), notably, is also a metabolite of the flame retardant substance Tris (1, 3-dichloro-2-propyl phosphate) used in clothing[21] and is an impurity of the pesticide 1, 3-dichlropropene[22]. 1, 3-DCP can also be found when vegetable proteins are hydrolysed. 1, 3-DCP could be detected in food products such as seasonings, which enhanced meaty flavours and soy sauce[23]. 1, 3-DCP has recently been associated with fulminant hepatitis in two workers cleaning a tank which had contained epichlorohydrin[24]. Other toxic effects of 1, 3-DCP in humans include irritation of the mucous membranes, eyes and skin, as well as nausea and vomiting[25]. It was also reported that 1, 3-DCP was carcinogenic in rats[26] and this was confirmed *in vitro* by Piasecki *et al.* (1990)[23].

In recent years, fenpropathrin (CAS no. 64257-84-7), a new synthetic analogue of pyrethroid insecticides, was imported in large amounts and widely used in China, especially in the south of the country, to control insect pests in staple crops such as cereals, potatoes, tobacco, cotton, and fruit. The contamination of surface waters in agricultural areas by synthetic pyrethroids through overspray or drift is of concern, because these compounds have been found to be extremely toxic to aquatic organisms[27].

In this study, we aim to elucidate the mutagenic profiles of these 3 synthetic chemicals, 9H-carbazole, 1, 3-DCP and fenpropathrin, used in chemical process with MLA using L5178Y cells.

Fig. 1. Chemical structures of the test compounds.

L5178Y *tk^{+/-}*-3.7.2C Mouse Lymphoma Assay (MLA) on 9H-carbazole, 1, 3-dichloro-2-propanol and Fenpropathrin

The genotoxic potentials of 9H-carbazole, 1, 3-DCP and fenpropathrin (Fig. 1) were assessed with various concentrations in the absence and presence of S-9 activation, respectively using MLA. Table 1, 2 and 3 summarizes the results of the MLA after treatment of L5178Y cells with 9H-carbazole, 1, 3-DCP and fenpropathrin at different concentrations. DMSO was used as the negative control. The adequacy of the experimental conditions for detection of induced mutation was confirmed by employing positive control chemicals, methylmethanesulfonate (MMS) and cyclophosphamide (CP) for assays in the absence and presence of S-9, respectively. Background mutation frequencies (MF) $(44.66 \times 10^{-6}$ and $93.68 \times 10^{-6})$ were within the historical control range, and positive controls gave large dose-dependent increases in MF, meeting assay acceptance criteria.

9H-Carbazole did not induce significant changes in

Table 1. Toxicity and mutagenicity of 9H-carbazole in L5178Y $tk^{+/-}$ mouse lymphoma cells.

Treatment (μg/mL)	−S-9				Treatment (μg/mL)	+S-9			
	%RS	RTG	Mutation Frequency (×10⁻⁶)			%RS	RTG	Mutation Frequency (×10⁻⁶)	
0	100.00	1.00	67.99		0	100.00	1.00	93.68	
20	107.74	1.24	52.90	NS	0.156	94.88	0.81	70.37	NS
39	81.75	1.14	34.19	NS	0.313	83.21	0.54	91.54	NS
78	97.82	1.03	74.11	NS	0.625	57.88	0.34	98.81	NS
157	86.53	0.73	90.78	NS	1.250	28.87	0.05	136.55	NS
Linear trend			NS		Linear trend			*	
MMS					CP				
10	87.78	0.88	604.51	*	3	75.84	0.39	367.63	*

NS: Not significant; MMS: methylmethanesulfonate; CP: cyclophosphamide

Table 2. Toxicity and mutagenicity of 1, 3-dichloro-2-propanol in L5178Y $tk^{+/-}$ mouse lymphoma cells.

Treatment (μg/mL)	−S-9				Treatment (μg/mL)	+S-9			
	%RS	RTG	Mutation Frequency (×10⁻⁶)			%RS	RTG	Mutation Frequency (×10⁻⁶)	
0	100.00	1.00	59.83		0	100.00	1.00	44.66	
78	100.00	1.85	52.90	NS	20	125.45	0.95	73.66	NS
157	92.03	1.59	73.95	NS	39	129.09	0.85	57.13	NS
313	55.00	1.10	150.87	*	79	97.76	0.70	114.33	*
625	40.60	0.59	310.27	*	157	68.93	0.23	211.81	*
Linear trend			***		Linear trend			***	
MMS					CP				
10	90.56	1.20	375.20	*	3	72.00	0.70	281.25	*

NS: Not significant; *, **, ***: Significant at 5%, 1% and 0.1% level, respectively; MMS: methylmethanesulfonate; CP: cyclophosphamide

Table 3. Toxicity and mutagenicity of fenpropathrin in L5178Y $tk^{+/-}$ mouse lymphoma cells.

Treatment (μg/mL)	−S-9				Treatment (μg/mL)	+S-9			
	%RS	RTG	Mutation Frequency (×10⁻⁶)			%RS	RTG	Mutation Frequency (×10⁻⁶)	
0	100.00	1.00	59.76		0	100.00	1.00	48.37	
313	94.29	0.29	70.17	NS	0.156	109.39	0.99	51.46	NS
625	85.86	0.23	55.54	NS	0.313	94.05	0.28	49.41	NS
1250	55.91	0.01	163.30	*	0.625	135.86	0.19	49.16	NS
2500	81.68	0.03	152.96	*	1.250	119.42	0.05	77.60	NS
Linear trend			*		Linear trend			NS	
MMS					CP				
10	78.27	0.63	316.23	*	3	55.71	0.62	370.51	*

NS: Not significant; *: Significant at 5%, 1% and 0.1% level, respectively; MMS: methylmethanesulfonate; CP: cyclophosphamide

MFs and dose response in the absence of S-9, and in the presence of S-9, no significant increase of MF was observed, although dose response was observed (Table 1 and Fig. 2).

1, 3-DCP induced dose-related and significant increases in MFs up to 5.2-fold in the absence of S-9 and 4.7-fold in the presence of S-9 (Table 1 and Fig. 3), as compared to the negative control cultures at the highest evaluated concentration (i.e., 625 μg/mL in the absence of S-9 and 157 μg/mL presence of S-9).

Treatment of cells with fenpropathrin for 3 h led to a clearly increased MF at concentrations > 1,250
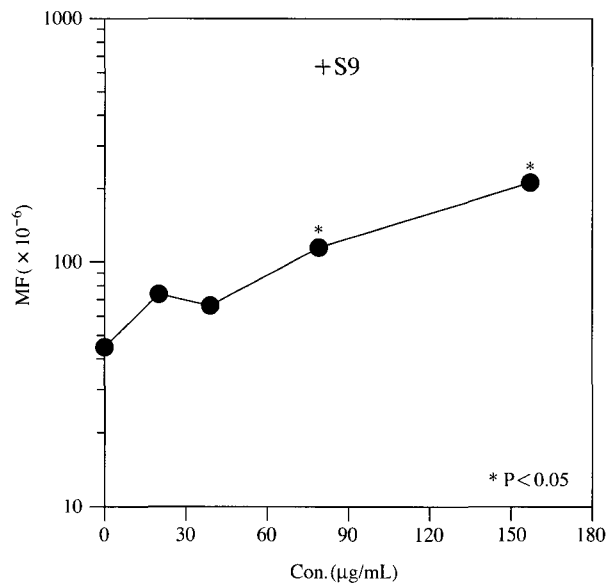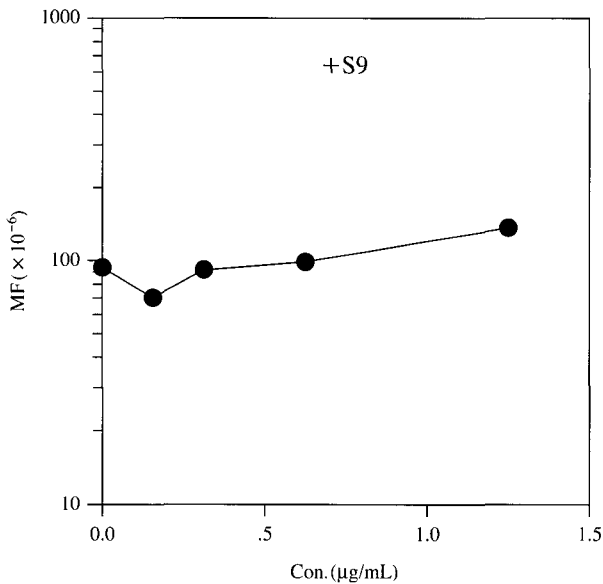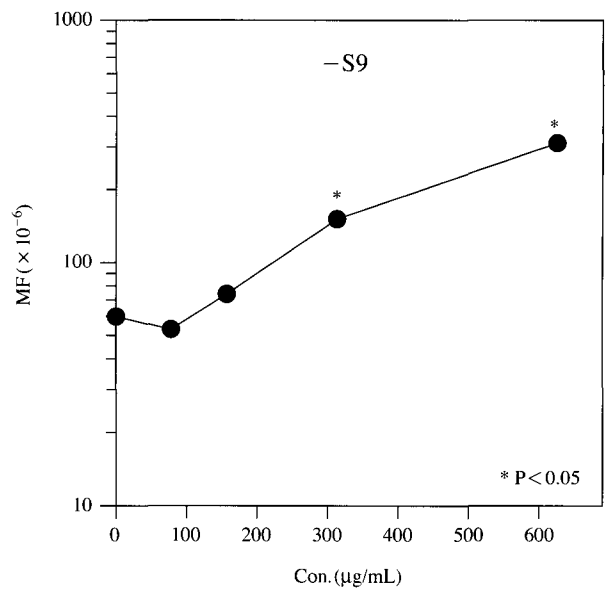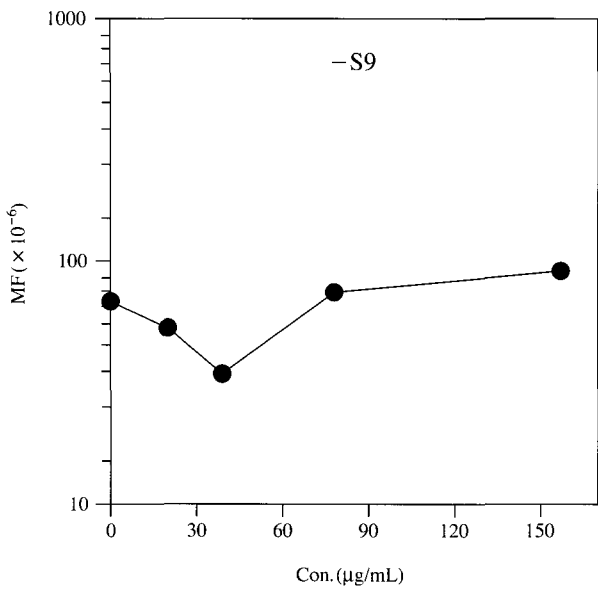
Fig. 2. Mutation frequencies by 9H-carbazole in L5178Y cells in the absence and presence of S-9 metabolic activation system (3 h treatment). Total *tk* mutation frequency is displayed. Results are taken from one representative experiment.

Fig. 3. Mutation frequencies by 1, 3-dichloro-2-propanol in L5178Y cells in the absence and presence of S-9 metabolic activation system (3 h treatment). Total *tk* mutation frequency is displayed. Results are taken from one representative experiment. *statistically significant ($P < 0.05$).

µg/mL in the absence of S-9 (Table 3 and Fig. 4). Fenpropathrin at 2,500 µg/mL caused a 2.6-fold increase in the spontaneous MF. A concentration-related mutagenic effect was measured in the absence of S-9 system. However, in the presence of S-9, it did not appear significant increase of MFs at all concentrations.

Discussion

We investigated whether these 3 chemicals induce the base-pair as well as frameshift mutations or small deletions in L5178Y cells using MLA. The MLA detects a broader range of mutations in a more complex eukaryotic system for more sensitive detection of
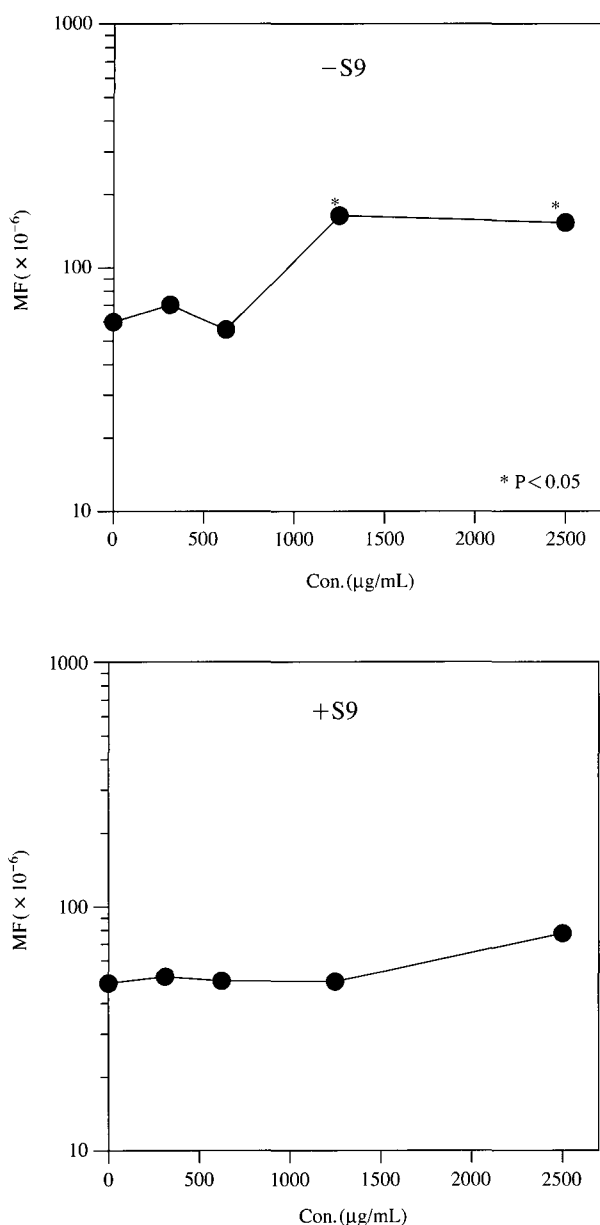
Fig. 4. Mutation frequencies by fenpropathrin in L5178Y cells in the absence and presence of S-9 metabolic activation system (3 h treatment). Total *tk* mutation frequency is displayed. Results are taken from one representative experiment. *statistically significant ($P<0.05$).

mutagens.

Under the experimental conditions used, 9H-carbazole was considered to be negative in this *in vitro* MLA study. There is limited evidence for carbazole having either genotoxic or carcinogenic activity. This chemical has been found non-mutagenic in *Salmonella*[17], Chinese hamster ovary cells[28], or UDS

primary rat hepatocytes[29]. On the other hand, most of its derivatives have been shown to be genotoxic in different test systems[30-32]. Therefore, a further attempt may be made to evaluate the genotoxic property of this compound through *in vivo* test.

1, 3-DCP was considered to be positive in this *in vitro* MLA study. The genotoxicity of 1, 3-DCP was reported previously that this chemical caused base substitutions in *Salmonella typhimurium* TA1535 both with and without metabolic activation[33]. Its genotoxic mechanisms are, however, not yet entirely understood. Recently, some researcher suggested that positive genotoxicity results in *in vitro* testing of vicinal chloroalcohols such as 1, 3-DCP are due to directly acting genotoxic intermediates arising from a chemical reaction with the culture medium rather than from enzymatic biotransformation[34,35].

A concentration-related mutagenic effect of fenpropathrin was measured in the absence of S-9 system. Fenpropathrin was previously found to be non-genotoxic in a bacterial reverse mutation assay (Ames test) and an *in vitro* chromosomal aberration assay using Chinese hamster ovary cells[1]. Also, this chemical slightly increased the number of micronuclei and micronucleated cells in white blood lymphocyte cultures[36]. The positive mutagenic response by fenpropathrin in the MLA led us to further investigate its mode of action.

In summary, 9H-carbazole did not induce significant mutation frequencies both in the presence and absence of metabolic activation system. 1, 3-DCP revealed a significant increase of mutation frequencies in the absence and presence of metabolic activation system. And also, fenpropathrin appeared the positive results only in the absence of metabolic activation system. Through the results of MLA *tk* assay with 3 synthetic chemicals in L5178Y cells *in vitro*, we may provide the important clues on the genotoxic potentials of these 3 chemicals.

Methods

Materials

Stock solutions of used chemicals were prepared freshly in medium before use. RPMI-1640, pluronic solution, antibiotics and horse serum were the products of GIBCO® (California, USA). All other chemicals used were of analytical grade or the highest grade available. The preparation of rat liver S-9 fraction for metabolic activation system was previously reported[15,16]. The S-9 fraction prepared was stored immediately at −80°C before use.

Cell Lines and Culture

The mouse lymphoma L5178Y cell line ($tk^{+/-}$ 3.7.2c) was cultivated in 90% RPMI-1640 with 1 mM sodium pyruvate, 0.1% pluronic supplemented with 10% heat-inactivated horse serum and antibiotics. These cells were maintained at 37°C in humidified 5% CO_2 atmosphere.

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

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. A single lot of post-mitochondrial supernatant fractions of rat liver homogenates (S-9) for exogenous metabolic activation had been made from the liver of phenobarbital- and 5, 6-benzoflavone-pretreated Sprague Dawley rats. S-9 mixture was prepared just prior to use by combining 4 mL S-9 with 2 mL each 180 mg/mL glucose-6-phosphate, 25 mg/mL NADP and 150 mM KCl. The concentration of S-9 mixture was 5% during treatment and the final concentration of S-9 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. All chemicals were tested with and without S-9 mixture. Three chemicals 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 the test chemicals. Cytotoxicity was determined by relative survival (RS) and relative total growth (RTG) following 3 h treatments at concentrations up to 5 mg/mL, 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.*[37]. 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[38].

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