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Genotoxicity on 21α- and β-methylmelianodiol, a Component of *Poncirus trifoliata*, in Bacterial and Mammalian Cells

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Abstract

21α- and β-Methylmelianodiol were isolated as the inhibitor of IL-5 bioactivity from *Poncirus tripoliata*. To develope as an anti-septic drug, the genotoxicity of 21α- and β-methylmelianodiol was subjected to high throughput toxicity screening (HTTS) because they revealed strong IL-5 inhibitory activity and limitation of quantity. Mouse lymphoma thymidine kinase ($tk^{+/-}$) gene assay (MOLY), single cell gel electrophoresis (Comet) assay in mammalian cells and Ames reverse mutation assay in bacterial system were used as simplified, inexpensive, short-term *in vitro* screening tests in our laboratory. These compounds are not mutagenic in *S. typhimurium* TA98 and TA100 strains both in the presence and absence of metabolic activation. Before performing the comet assay, IC_{20} of 21α-methylmelianodiol was determined the concentration of 25.51 μg/mL and 21.99 μg/mL with and without S-9, respectively. Also 21β-methylmelianodiol was determined the concentration of 24.15 μg/mL and 22.46 μg/mL with and without S-9, respectively. In the comet assay, DNA damage was not observed both 21α-methylmelianodiol and 21β-methylmelianodiol in mouse lymphoma cell line. Also, the mutant frequencies in the treated cultures were similar to the vehicle controls, and none of 21α- and β-methylmelianodiol with and without S-9 doses induced a mutant frequency over twice the background. It is suggests that 21α- and β-methylmelianodiol are non-mutagenic in MOLY assay. The results of this battery of assays indicate that 21α- and β-methylmelianodiol have no genotoxic potential in bacterial or mammalian cell systems. Therefore, we suggest that 21α- and β-methylmelianodiol, as the optimal candidates with both no genotoxic potential and IL-5 inhibitory effects must be chosen.

Keywords: 21α- and β-Methylmelianodiol, *Poncirus tripoliata*, genotoxicity, MOLY, comet assay, Ames reverse mutation assay, IL-5 inhibitory effects

The immature fruits of *Poncirus trifoliata* L. (*Rutaceae*) or *Poncirus fructus* (PF), well known as 'Jisil' in Korea, have been used in folk medicine as a remedy for inflammation, digestive ulcers, gastritis and dysentery, and still occupies an important place in traditional Oriental medicine. Recently, anti-inflammatory, anti-helicobacter pylori activity and anti-anaphylactic activities of PF were reported[1,2], and PF has been used for the treatment of various cancers in clinics, however, no reports on the anti-cancer activities of PF have been found.

The incubation of HL-60 cells with PF leads to an activation of the caspase-3 and the induction of apoptosis[3]. Through this finding, Yi *et al.* suggested that PF could be a candidate as an anti-leukemic agent through apoptosis of cancer cells[3]. In other study, it reported that PF inhibited mast cell-mediated anaphylactic reactions[4]. Also, it was reported that PF was able to inhibit TNF-α secretion by Stem cell factor (SCF)-untreated control level. PF also inhibited IL-6 secretion from SCF-treated rat peritoneal mast cells (RPMCs). The results obtained in this study provide evidence that PF may contribute to the prevention or treatment of various inflammatory diseases because these cytokines play an important role in initiating inflammation.

To date, many anti-inflammatory drugs have been developed and applied by physicians. Interfering with the action of IL-5 represents one of the new immunomodulatory therapeutic strategies in the treatment of allergic diseases including bronchial asthma. Compared to established immunosuppressive agents like corticosteroids, a major advantage of this strategy is the specificity of reducing eosinophilic inflammation, thus possibly acting nearly without side effects. However small organic compounds to inhibit IL-5 activity have been rarely found. Recently, much effort has been directed toward the search for compounds or herbs that influence IL-5 inhibitory effects and their mechanism of action.

Hereupon, we isolated some compounds from fruits of PF and assessed IL-5 inhibitory activity of these. Especially, 21α- and β-methylmelianodiol appeared the strongly IL-5 inhibition (data not shown). Therefore, we could consider that these compounds are candidates of anti-inflammatory drugs, isolated from PF.

The purpose of this study was to demonstrate whether newly isolated compounds, 21α- and β-methylmelianodiol, with anti-inflammatory effects have genotoxic properties. If something among these derivatives has not genotoxicity, it should be considered as potentially ideal drug candidate through further investigations for their safety. In this study, *in vitro* assay have been performed to determine these cytotoxic and genotoxic potentials. We adopted various methods to assess the genotoxicity of 21α- and β-methylmelianodiol such as bacterial reverse mutation assay[5-7], single cell gel electrophoresis (comet) assay[8-10] and thymidine kinase gene forward mutation assay with mouse lymphoma cells.

Assessment of Mutation Induction by 21α- and β-methylmelianodiol in the Bacterial System Using Ames Reverse Mutation Assay

For the bacterial mutation screening, only two tester strains of *Salmonella typhimurium* (TA98 and TA100) were used because these two strains detect the vast majority of bacterial mutagens in our experience[7]. The mutagenic potential of 21α- and β-methylmelianodiol was investigated in the *S. typhimurium* microsomal activation assay. This assay detects materials that cause specific point mutations such as base-pair substitution and frameshift mutation in a bacterial model. The genotoxic evaluations were performed with 21α- and β-methylmelianodiol, in different *S. typhimurium* strains (TA98 and TA100), in the presence and in the absence of S-9 mixture. Positive controls specific to each of the two tester strains resulted in the expected increases in the number of histidine revertants. In observation of the background lawns of treated bacteria, 21α- and β-methylmelianodiol were not cytotoxic at doses 5,000 μg/plate in the absence and presence of S-9 mixture, and so, we determined this dose as optimal maximum concentrations of these compounds for this assay. As shown in Table 1, no significant increase of revertants in two strains at all concentrations of 21α- and β-methylmelianodiol used. These results suggest that 21α- and β-methylmelianodiol is not mutagenic in *S. typhimurium* TA98 and TA100 strains both in the presence and absence of metabolic activation in this assay.

Cytotoxicity of 21α- and β-methylmelianodiol

Relative survival of L5178Y cells following exposure to a range of concentrations of 21α- and β-

Table 1. Mutagenicity of 21α- and β-methylmelianodiol in *Salmonella typhimurium* TA 98 and TA100 in the presence and absence of S-9 metabolic activation system

S-9 mix	Compound dose (μg/plate)	His^+ revertants/plate (Mean±S.D.)			
		21α-methylmelianodiol		21β-methylmelianodiol	
		TA 98	TA 100	TA 98	TA 100
−	DMSO	23±3	165.3±22	42±4	210±21
−	79	26±3	191±4	-	-
−	157	24±8	175±6	41±6	191±7
−	313	43±2	190±4	37±10	202±4
−	625	14±7	190±3	42±4	199±12
−	1,250	19±3	196±3	35±4	241±28
−	2,500	17±4	193±11	35±3	190±22
−	5,000	12±6	179±14	41±4	223±6
−	SA 1	-	857±68	-	495±10
−	2-NF 0.2	331±80	-	374±65	-
+	DMSO	45±8	194±3	46±6	231±10
+	79	49±5	202±6	-	-
+	157	53±2	203±12	43±9	229±16
+	313	47±6	209±3	44±4	209±22
+	625	43±11	200±8	55±5	227±12
+	1,250	41±13	196±19	53±3	201±24
+	2,500	40±3	191±24	33±6	187±0
+	5,000	29±1	219±12	43±9	206±20
+	2-AA 0.5	3,464±56	-	1,394±212	-
+	2-AA 1	-	1,372±303	-	2,944±100

DMSO: dimethyl sulfoxide, SA: Sodium Azide, 2-NF: 2-Nitrofluorene, 2-AA: 2-Aminoanthracene

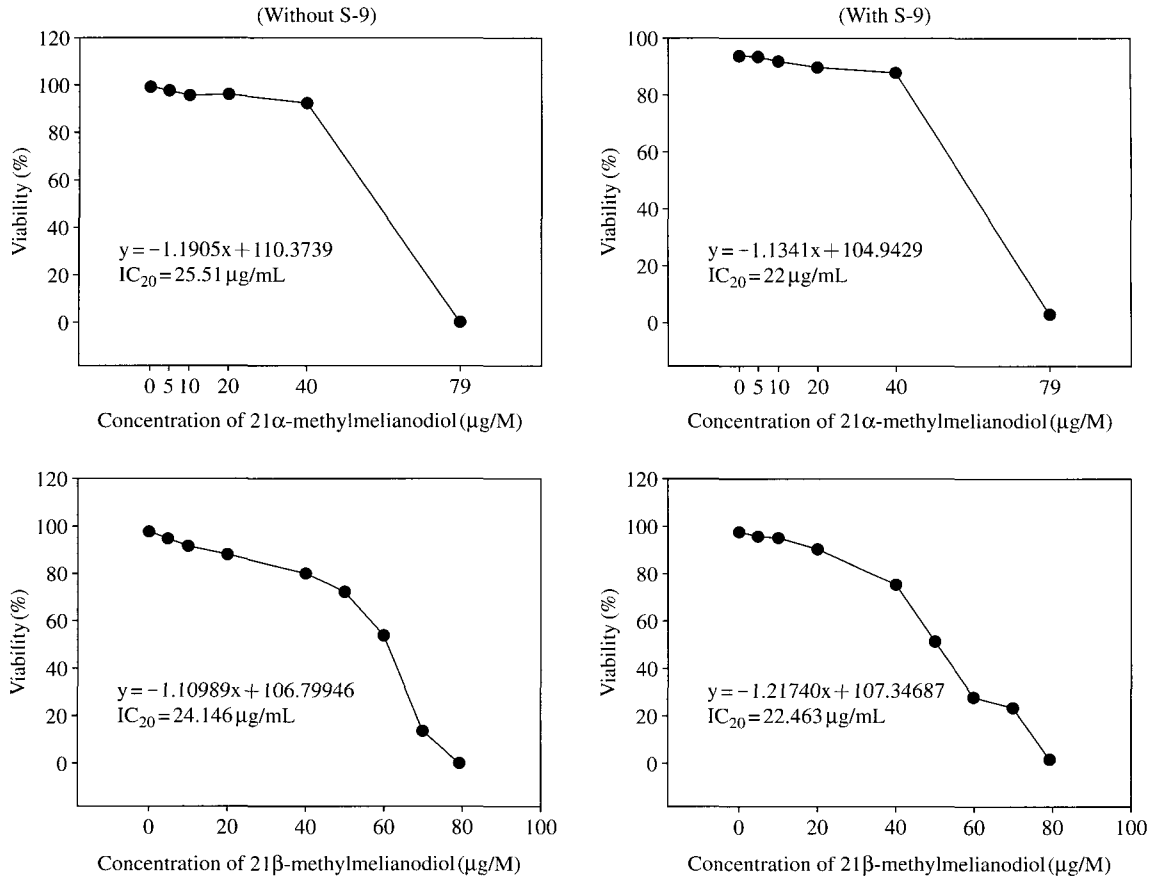

Fig. 1. Cytotoxicity of 21α- and β-methylmelianodiol in L5178Y mouse lymphoma cell line in the presence and absence of S-9 metabolic activation system.

methylmelianodiol was determined by trypan blue dye exclusion assay. The survival percentage relative to solvent control (DMSO) was determined as a percentage of the number of cells survived after treatment with or without metabolic activation system. Based on results of cytotoxicity assay 20% inhibitory concentration (IC_{20}) of each compound was calculated. IC_{20} of 21α-methylmelianodiol was 25.51 μg/mL and 22.00 μg/mL in the absence and presence of metabolic activation system, respectively. In the case of 21β-methylmelianodiol, IC_{20} in the L5178Y cells was 24.15 μg/mL and 22.46 μg/mL in the absence and presence of metabolic activation system, respectively (Fig. 1). These concentrations were considered to be in the acceptable range for conducting the Comet assay[11].

Screening of DNA damage with 21α- and β-methylmelianodiol using the Single Cell Gel Electrophoresis (Comet) Assay

We also investigated whether 21α- and β-methylmelianodiol could induce subtle DNA damages at concentrations resulting in no obvious cytotoxic effects. As one of the mechanisms of carcinogenicity, induction of DNA damage was ascertained by a comet assay, which is widely used for the detection and measurement of DNA strand breaks. Since Ostling and Johanson[12] introduced microelectrophoretic technique, Singh *et al.*[8] have modified and improved the microgel electrophoresis technique to evaluate DNA damage in single cells under alkaline conditions. The single cell gel electrophoresis (SCGE, comet, microgel electrophoresis) assay is rapid, sensitive, visual and simple technique to quantify DNA strand breaks in individual cells. The intensity and the length of comet images were expressed in terms of the tail moment. However, some variations could be occurred in procedures, laboratories's conditions and kind of cells used. Hence, to overcome and to harmonize these matters in comet assay, International Workshop on Genotoxicity Test Procedure (IWGTP) was held with several topics including comet assay at Was-

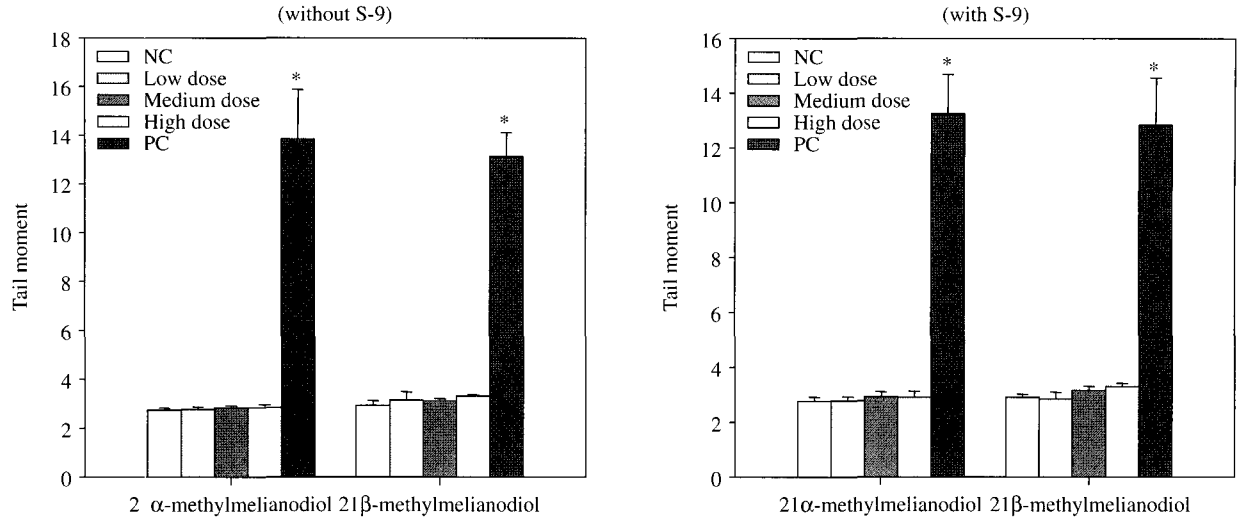

Fig. 2. Tail moment of 21α- and β-methylmelianodiol in L5178Y mouse lymphoma cell line assessed by the comet assay. L5178Y cells were treated with indicated concentrations of 21α- and β-methylmelianodiol in the absence and presence S-9 metabolic activation system. Values are mean ± S.D. (n = 4). Positive controls were MMS (150 μM) in the absence and BaP (50 μM) in the presence of S-9 metabolic activation system, respectively. NC: DMSO, PC: +S9 (BaP-50), −S9 (MMS-150), 21α-methylmelianodiol Low: +S-9 (5.5 μg/mL), −S-9 (6.4 μg/mL), Medium: +S-9 (11.0 μg/mL), −S-9 (12.8 μg/mL), High: +S-9 (22.0 μg/mL), −S-9 (25.5 μg/mL), 21β-methylmelianodiol Low: +S-9 (5.6 μg/mL), −S-9 (6.0 μg/mL), Medium: +S-9 (11.2 μg/mL), −S-9 (12.1 μg/mL), High: +S-9 (22.5 μg/mL), −S-9 (24.1 μg/mL).

hington D.C. on March, 1999 by Environmental Mutagen Society supported with OECD. Our laboratory[10,13] also involved in this harmonization and published as preliminary form for OECD guideline with Tice *et al.*[11].

The results of the comet assay are shown in Fig. 2. The response of the positive control (150 μM MMS and 50 μM BaP) was significantly greater ($p < 0.001$) than solvent control in conditions without or with S-9 metabolic activation system (S-9), respectively. In this assay, the DNA damaging effect of 21α-methylmelianodiol was assessed at concentrations from 22.0 to 5.5 μg/mL in presence of S-9 metabolic activation systems (+S-9) and from 25.5 to 6.4 μg/mL in S-9. 21α-methylmelianodiol was not induced DNA damage both in +S-9 and in −S-9 (Fig. 2). Also, 21β-methylmelianodiol was assessed at the concentration from 22.5 to 5.6 μg/mL in +S-9 and from 24.2 to 6.0 μg/mL in −S-9. 21β-methylmelianodiol was not induced significant DNA strand breaks in the +S-9 and −S-9, respectively. According to the analysis of variance (ANOVA), there were no significant differences between the 21α- and β-methylmelianodiol treated cells and solvent controls in the absence and presence of S-9 mixture, suggesting that 21α- and β-methylmelianodiol was not induced DNA damages under this experimental condition used in this assay.

Detection of Gross Genetic Alteration on 21α- and β-methylmelianodiol Using L5178Y *thymidine kinase* (*tk*)$^{+/-}$-3.7.2C mouse lymphoma assay (MOLY)

Next, we investigated whether derivatives induce the base-pair as well as frameshift mutations or small deletions in L5178Y cells using MOLY assay. The MOLY assay detects a broader range of mutations in a more complex eukaryotic system for more sensitive detection of mutagens[14]. The *tk* mutant frequencies (including the small and large colony *tk* mutant frequencies) from one representative experiment with 21α- and β-methylmelianodiol are displayed in Fig. 3. Background mutant frequencies were within the historical control range, and positive controls gave large dose-dependent increases in mutant frequencies, meeting assay acceptance criteria. The mutant frequencies in the treated cultures were similar to the vehicle controls, and none of 21α- and β-methylmelianodiol with and without S-9 doses induced a mutant frequency over twice the background. It is suggests that 21α- and β-methylmelianodiol are non-mutagenic in MOLY assay.

Conclusion

In the present study, a preliminary profile of the

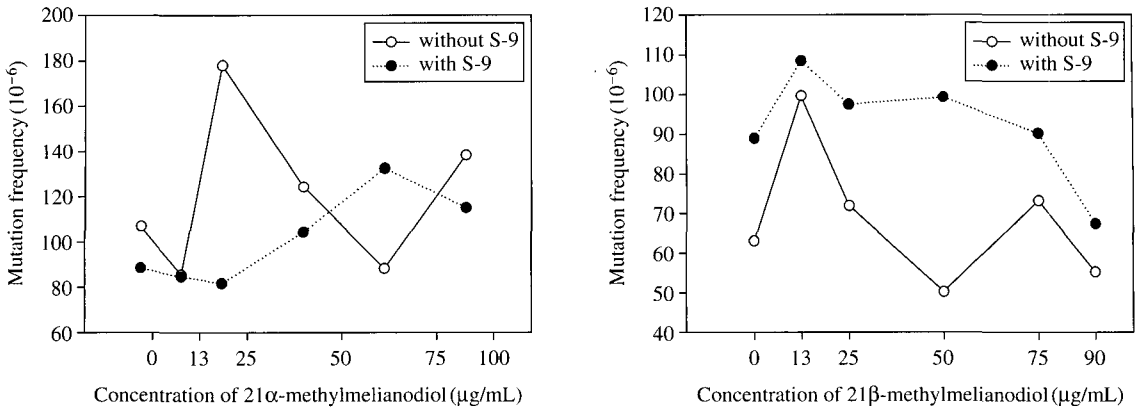

Fig. 3. Mutation frequency of 21α- and β-methylmelianodiol in L5178Y $tk^{+/-}$ mouse lymphoma cells. Total *tk* mutant frequency is displayed. Results are taken from one representative experiment.

genotoxic potential of 21α- and β-methylmelianodiol was obtained using three *in vitro* screening tests: single cell gel electrophoresis (Comet) assay, a mammalian cell mutation screening test using L5178Y mouse lymphoma cells and bacterial mutation screening test (screening Ames assay). The results of the present study show that the genotoxic activity of 21α- and β-methylmelianodiol, a component of *Poncirus trifoliate*, could have been easily detected if these simplified, inexpensive, short-term *in vitro* screening tests had been used during anti-inflammatory drug development, prior to distribution of it to general population. The results of this battery of assays indicate that 21α- and β-methylmelianodiol have no genotoxic potential in bacterial or mammalian cell systems. Therefore, we suggest that 21α- and β-methylmelianodiol, as the optimal candidates with both no genotoxic potential and IL-5 inhibitory effects must be chosen.

Methods

Materials

21α- and β-methylmelianodiol were isolated from *Poncirus trifoliate* by Dr. Lee from youngnam University. Stock solution of these compounds was prepared freshly in dimethylsulfoxide (DMSO) before use. RPMI-1640, trypan blue and horse serum were the products of GIBCO®(California, USA). Low melting agarose was a product of Amresco (Solon, OH, 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[5,7]. The S-9 fraction prepared was stored immediately at −80°C before use.

Cell lines and Culture

The L5178Y $(tk)^{+/-}$ -3.7.2C mouse lymphoma cell line 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.

Ames *Salmonella* Bacterial Mutagenicity Assay

This test performed essentially as described by Ames *et al.*[5,6]. Media and positive control chemicals were obtained from commercial sources and were the purest grades available. The dose range for test chemical was determined by performing a toxicity assay using strain *Salmonella typhimurium* TA 100 and half-log dose intervals of the test substance up to 5 mg/plate. Strain TA 100 was chosen as the representative tester strain because of its high spontaneous reversion rate. Spontaneous revertant numbers were counted and plotted against the dose of the test chemical 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 the test substance in the presence and in the absence of S-9 mixture condition, sodium phosphate buffer added instead of S-9 mixture both in negative and positive control in test tube. Then, incubating the mixture in water bath for 30 min at 37°C and after incubation, the mixture mixed with top agar containing a minimal amount of histidine and then poured onto the surface of a *r*-ray sterile Petri dish (Falcon, USA) containing 25 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 chemical but positive control plates containing appropriate amounts of chemicals known to be active were included with each tester strain (Table 1). All platings were done in triplicate, and the results were tabulated as the mean ± standard deviation for each condition. A response was considered to be positive in our criteria if there was a dose-dependent increase in revertants per plate resulting in at least a doubling of the background reversion rate for strains TA 98 or TA 100.

Cytotoxicity (Cell Growth Inhibition)

Cytotoxicity of cells was checked by the trypan blue exclusion assay. For the determination of cell cytotoxicity, 1×10^6 L5178Y cells were treated to various concentrations of 21α- and β-methylmelianodiol in 12-well plate in the absence and presence S-9 metabolic activation system for 2 h. After the staining of 0.4% trypan blue (Life Technologies, MD, U.S.A), the total number of cells and the number of unstained cells were counted in five of the major sections of a hemocytometer, and then average number of cells per section was calculated. Cell viability of treated chemical was related to controls that were treated with the solvent. All experiments were repeated twice in an independent experiment.

Single Cell Gel Electrophoresis (Comet) assay

DNA damages were detected by the alkaline version of standard comet assay described by Singh *et al.*[8,9] with minor modifications[10,11]. For the comet assay, 8×10^5 cells were seeded into 12 wells plate (Falcon 3043) and then treated with 21α- and β-methylmelianodiol. At all doses of 21α- and β-methylmelianodiol used in the experiment, the cell viability exceeded 80%. In the experiments, parallel cultures were performed and benzo[a]pyrene (BaP) and methyl methanesulfonate (MMS) were used as a positive control in the presence or absence of S-9 mixture, respectively. After treatment with derivatives for 2 h, cells were centrifuged for 3 min at × 100 g (about 1,200 rpm), and gently resuspended with PBS and 100 μL of the cell suspension was immediately used for the test. Cells were mixed with 0.1 mL of 1% low melting point agarose (LMPA, Gibco BRL, Life Technologies, Inc., MD, USA) and added to fully frosted slide (Fisher Scientific, PA, USA), had been covered with a bottom layer of 100 μL of 1% normal melting agarose (Amresco, OH, USA). The cell suspension was immediately covered with cover glass and the slides were then kept at 4°C for 5 min to allow solidification. After removing the cover glass gently, the slides were covered with a third layer of 100 μL of 0.5% LMPA by using a cover glass and then the slide were kept again at 4°C for 5 min. The cells embedded in the agarose on slides were lysed for 1.5 h in reaction mixture of 2.5 M NaCl, 0.1 M Na_2-EDTA, 10 mM Tris-HCl (pH 10), 1% Triton X-100 at 4°C. Slides were then placed in 0.3 M NaOH containing 1 mM Na_2-EDTA (approximately pH 13) for 20 min to unwind DNA before electrophoresis. Electrophoresis was conducted at 25 V (about 1 V/cm across the gels) and approximately 300 mA for 20 min at 4°C. All of the steps described above were conducted under yellow light or in the dark to prevent additional DNA damage. After the electrophoresis, the slides were washed gently to remove alkali and detergents that would interfere with ethidium bromide staining, by placing the slides vertically in glass jar containing 0.4 M Tris (pH 7.5) three times for 10 min. The slides were stained by 50 μL of ethidium bromide in distilled water solution on each slide, and then covering the slide with a cover glass. Image of 100 randomly selected cells (50 cells from each of two replicate slides) was analysed each sample. All experiments were repeated in an independent test. Measurement was made by image analysis with Komet 3.1 (Kinetic Imaging Limited, Liverpool, UK) system, determining the mean tail moment (percentage of DNA in the tail times tail length) of the 50 cells. Differences between the control and the other values were tested for significance using one way of analysis of variance (ANOVA).

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/mLglycine) 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. 21α- and β-methylmelianodiol 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.*[15]. 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 (Mutant TM; UKEMS, York, UK) in accordance with the UKEMS guidelines[16].

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