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Genotoxicity Study on Khal, a Halocidin Derivative, in Bacterial and Mammalian Cells

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Abstract

Khal was a synthetic congener of halocidin, a heterodimeric peptide consisting of 19 and 15 amino acid residues detected in *Halocynthia aurantium*. This compound was considered a candidate for the development of a novel peptide antibiotic. The genotoxicity of Khal was subjected to high throughput toxicity screening (HTTS) because they revealed strong antibacterial effects. Mouse lymphoma thymidine kinase ($tk^{+/-}$) gene assay (MOLY), single cell gel electrophoresis (Comet) assay and chromosomal aberration 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 Khal was determined the concentration of 25.51 μg/mL and 21.99 μg/mL with and without S-9, respectively. In the comet assay, Khal was not induced DNA damage in mouse lymphoma cell line. Also, the mutation frequencies in the Khal-treated cultures were similar to the vehicle controls. It is suggests that Khal is non-mutagenic in MOLY assay. And no clastogenicity was observed in Khal-treated Chinese hamster lung cells. The results of this battery of assays indicate that Khal has no genotoxic potential in bacterial or mammalian cell systems. Therefore, we suggest that Khal, as the optimal candidates with both no genotoxic potential and antibacterial effects must be chosen.

Keywords: Khal, Halocynthia aurantium, Genotoxicity, MOLY, Comet assay, Chromosome aberration, Ames reverse mutation assay, Antibacterial effects

In the last two decades, the incidence of human fungal infections has increased dramatically, in parallel with the wide spread of incurable infectious diseases associated with antibiotic-resistant bacteria. Fungal diseases have become a growing threat, especially in immunocompromised patients, for which few or no effective drugs are currently available[1]. Accordingly, a variety of studies have been conducted in an attempt to isolate natural antifungal substances with potential pharmaceutical utility, and to develop and design new synthetic or semi-synthetic drugs[2-4]. Antimicrobial peptides have recently become the focus of considerable interest as a candidate for a new type of antibiotic, due primarily to their potency against pathogenic microbes that are resistant to conventional antibiotics, as well as their broad-spectrum activity[5].

More than 880 antimicrobial peptides have been isolated from a wide variety of organisms, including vertebrates, invertebrates, and plants[6]. Among these peptides, the halocidin detected in *Halocynthia aurantium* appears to exhibit the potent antibacterial activity. It is a heterodimeric peptide consisting of two a-helical monomers with 18 and 15 amino acid residues, referred to as 18Hc and 15Hc, respectively. From the structure of 18Hc, several synthetic congeners have been designed and evaluated with regard to their antibacterial activities against a variety of antibiotic-resistant bacteria, under diverse conditions[7,8]. As a result of these trials, it was determined that Khal, a heterodimeric version of a synthetic peptide, considered of 19 and 15 amino acid residues linked by disulfide bond, exhibited profound antibacterial effects (data not shown). Therefore, it could consider that this compound is candidate for the development of a novel peptide antibiotic.

The purpose of this study was to demonstrate whether newly synthesized compound, Khal, with antibacterial effects has genotoxic properties. If this compound has not genotoxicity, it should be considered as potentially antibiotics 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 Khal such as bacterial reverse mutation assay[9-11], single cell gel electrophoresis (comet) assay[12-14], thymidine kinase gene forward mutation assay with mouse lym-

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

	−S-9		+S-9	
	Khal (μg/plate)	His+ revertants/plate (Mean±S.D.)	Khal (μg/plate)	His+ revertants/plate (Mean±S.D.)
TA98	28.4	15.7±2.3	56.8	26.3±2.5
	14.2	14.0±1.7	28.4	48.7±4.2
	7.1	14.7±1.2	14.2	82.0±9.9
	3.6	30.0±1.7	7.1	77.3±2.5
	1.8	30.3±5.0	3.6	69.7±5.8
	0.9	34.3±3.8	1.8	71.0±4.4
	NC (SDW)	31.0±9.2	NC (SDW)	77.3±5.0
	PC (2NF)	538.0±59.6	PC (2AA0.5)	1,850.0±213.0
TA100	28.4	146.0±31.0	56.8	211.7±15.9
	14.2	174.0±5.4	28.4	304.3±23.0
	7.1	186.0±15.6	14.2	297.0±26.0
	3.6	203.0±2.1	7.1	276.0±23.1
	1.8	261.0±9.0	3.6	306.3±28.1
	0.9	252.0±7.4	1.8	296.7±9.5
	NC (SDW)	244.0±18.9	NC (SDW)	288.7±11.2
	PC (SA)	1,487.0±137.0	PC (2AA1)	2,669.0±200.0

SA: Sodium Azide, 2-NF: 2-Nitrofluorene, 2-AA: 2-Aminoanthracene

phoma cells and chromosomal aberration assay.

Assessment of Mutation Induction by Khal 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[11]. The mutagenic potential of Khal 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 Khal, 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, Khal was cytotoxic at doses above 28.4 μg/plate and 56.8 μg/plate in the absence and presence of S9 mixture, respectively, 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 Khal used. These results suggest that Khal is not mutagenic in *S. typhimurium* TA98 and TA100 strains both in the presence and absence of metabolic activation in this assay.

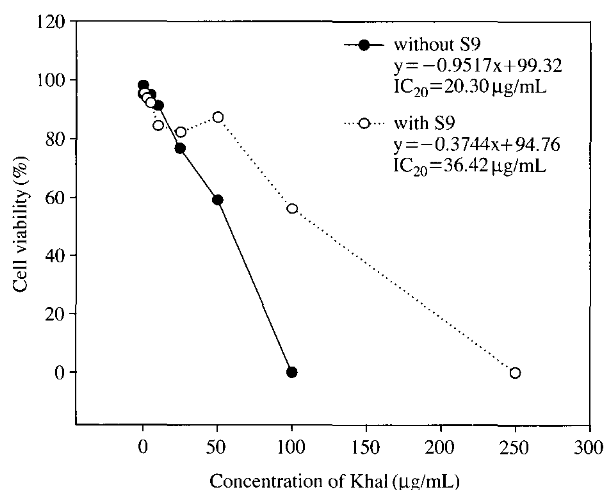

Fig. 1. Cytotoxicity of Khal in L5178Y mouse lymphoma cell line in the presence and absence of S-9 metabolic activation system.

Cytotoxicity of Khal in L5178Y Cells and CHL Cells

Cytotoxicity of L5178Y cells and CHL cells following exposure to a range of concentrations of Khal was determined by trypan blue dye exclusion assay. The survival percentage relative to solvent control (medium) was determined as a percentage of the number of cells survived after treatment with or without metabolic activation system. Khal was decreased the via-

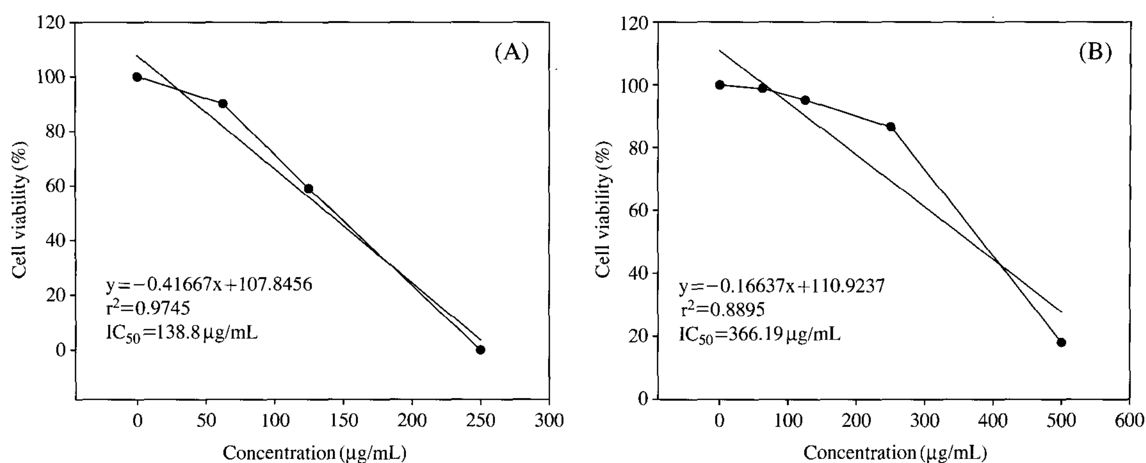

Fig. 2. Cell viability of Khal in the absence (A) and presence (B) of S-9 metabolic activation system in CHL cells using trypan blue exclusion assay.

bility of the cells studied proportionally to the concentration in both cell lines. And based on results of cytotoxicity assay, 20% inhibitory concentration (IC_{20}) on L5178Y cell growth of each compound was determined as 20.30 μg/mL and 36.42 μg/mL in the absence and presence of S-9 metabolic activation system, respectively (Fig. 1). These concentrations were considered to be in the acceptable range for conducting the Comet assay. Also, the 50% cell growth inhibition concentrations (IC_{50}) of Khal on CHL fibroblast cells were determined as 138.8 μg/mL and 366.2 μg/mL in the absence and presence of S-9 metabolic activation system, respectively (Fig. 2). These concentrations were considered to be in the acceptable range for conducting the chromosomal aberration assay.

Screening of DNA Damage with Khal Using the Single Cell Gel Electrophoresis (Comet) Assay

We also investigated whether Khal 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[15] introduced microelectrophoretic technique, Singh *et al.*[12] 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 Washington D.C. on March, 1999 by Environmental Mutagen Society supported with OECD. Our laboratory[14,16] also involved in this harmonization and published as preliminary form for OECD guideline with Tice *et al*[17].

The results of the comet assay are shown in Fig. 3. 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, respectively. In this assay, the DNA damaging effect of Khal was assessed at concentrations from 36.42 to 9.10 μg/mL in presence of S-9 metabolic activation systems (+S-9) and from 20.30 to 5.08 μg/mL in -S-9. According to the analysis of variance (ANOVA), there were no significant differences between the Khal-treated cells and solvent controls in the absence and presence of S-9 mixture, suggesting that Khal was not induced DNA damages under this experimental condition used in this assay.

Detection of Gross Genetic Alteration on Khal Using L5178Y *thymidine kinase* (*tk*)$^{+/-}$ −3.7.2C Mouse Lymphoma Assay (MOLY)

Next, we investigated whether Khal 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

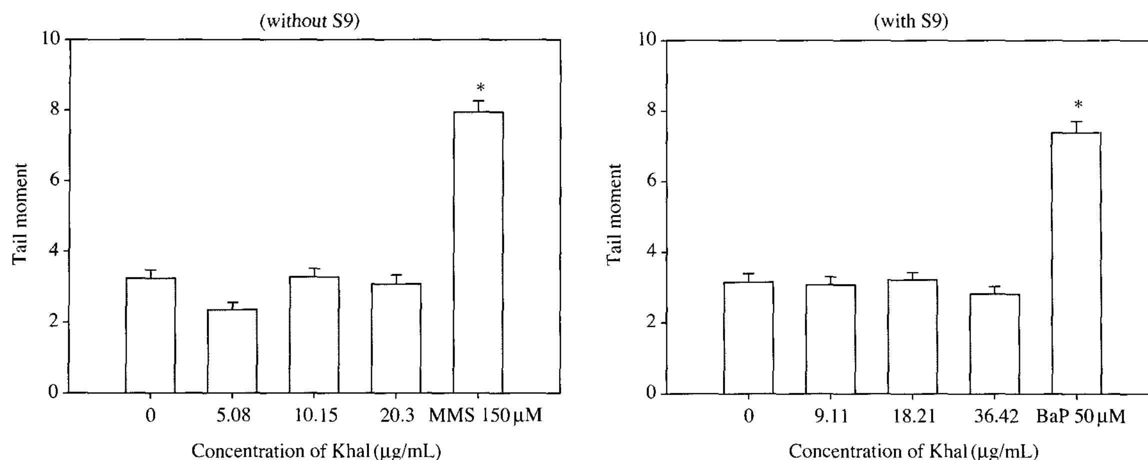

Fig. 3. Tail moment of Khal in L5178Y mouse lymphoma cell line assessed by the comet assay. L5178Y cells were treated with indicated concentrations of Khal in the absence and presence S-9 metabolic activation system. Values are mean±S.D. (n=4). Positive controls were MMS (150 µM) in the absence and BaP (50 µM) in the presence of S-9 metabolic activation system, respectively.

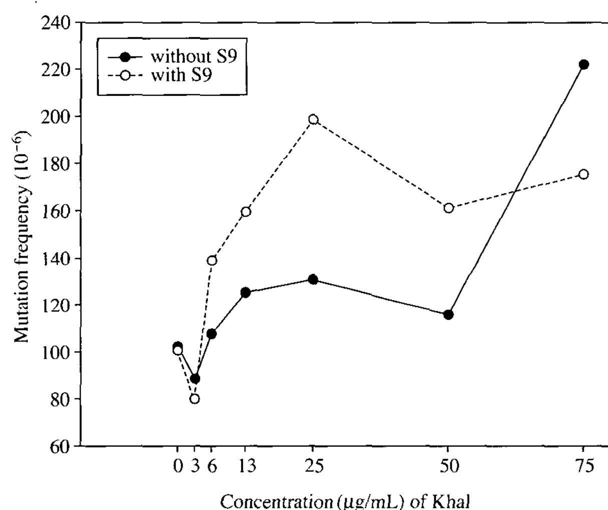

Fig. 4. Mutation frequency of Khal in L5178Y $tk^{+/-}$ mouse lymphoma cells. Total *tk* mutation frequency is displayed. Results are taken from one representative experiment.

a more complex eukaryotic system for more sensitive detection of mutagens[18]. The *tk* mutant frequencies (including the small and large colony *tk* mutant frequencies) from one representative experiment with Khal are displayed in Fig. 4. 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 Khal with and without S-9 dose not induced a mutation frequency over twice the background. It is suggests that Khal is non-mutagenic in MOLY assay.

Assessment of Clastogenicity of Khal in the CHL Cells Using Chromosomal Aberration Assay

The types and frequencies of chromosomal aberrations seen in treated and control cultures for 6 h of Khal treatment are listed in Table 2. The solvent (medium)-treated control was revealed only 0-0.5% of spontaneous chromosomal aberrations in 200 metaphase cells. Cyclophosphamide (3 µg/mL) used as an indirect-acting mutagen that requires metabolic activation and mitomycin C (0.3 µg/mL) as a direct-acting mutagen, induced remarkable chromosomal aberrations (about 20-30%) in CHL fibroblasts. Low frequencies of breaks and fragments were seen in Khal-treated cultures and solvent controls, both with and without S-9 mixture. No statistically significant increases in the mean percentage of aberrant cells in the types of aberrations noted between Khal-treated cultures and solvent-control were seen both with and without S-9 mixture (Table 2). From these results, 6 h treatment of Khal was not revealed clastogenicity both in the presence and absence of S-9 mixture in this assay.

Discussion

In the present study, a preliminary profile of the genotoxic potential of Khal was obtained using three *in vitro* screening tests: single cell gel electrophoresis

Table 2. Results of chromosome aberration assay following Khal treatment in CHL cells.

Treatment				Chromosome aberrations / 200 cells									
			S-9 Mix	Chromatid type		Chromosome type		Total aberration	Extra aberration				
Compound	Conc. (μg/mL)	hr		Br	Ex	Br	Ex	(%)	ctg	csg	poly	endo	nor
Medium	–	6	+	0	0	0	0	0	1	0	0	0	199
CP	3.0	6	+	18	34	2	2	28.0	3	0	0	0	145
Khal	366.2	6	+	1	0	1	0	1.0	2	0	0	4	192
	183.1	6	+	0	0	0	0	0	0	0	0	0	200
	91.6	6	+	0	0	0	0	0	1	0	0	0	199
Medium	–	6	–	1	0	0	0	0.5	1	0	0	0	198
MMC	0.1	6	–	11	42	0	0	26.0	6	0	0	0	148
Khal	138.8	6	–	2	0	0	0	1.0	1	1	0	1	196
	69.4	6	–	1	0	0	0	0.5	1	0	0	0	198
	34.7	6	–	0	0	0	0	0	0	0	0	0	200

Conc.: concentration, Br: breakage, Ex: exchange, ctg: chromatid gap, csg: chromosome gap, poly: polyploid, endo: endoreduplicate, nor: normal, MMC: mitomycin C, CP: cyclophosphamide

(Comet) assay, a mammalian cell mutation screening ment of clastogenicity using chromosomal aberration assay and bacterial mutation screening test (Ames assay). The results of the present study show that the genotoxic activity of Khal, a synthetic peptide congener of halocidin, could have been easily detected if these simplified, inexpensive, short-term in vitro screening tests had been used during antibacterial compound development, prior to distribution of it to general population. The results of this battery of assays indicate that Khal has no genotoxic potential in bacterial or mammalian cell systems. Therefore, we suggest that Khal, as the optimal candidates with both no genotoxic potential and antibacterial effects must be chosen.

Methods

Khal was synthesized and donated by Dr. Lee in Department of Life Science, Hoseo University, Korea. Stock solution of this compound was prepared freshly in medium before use. Eagles minimum essential medium (EMEM), RPMI-1640, 0.25% trypsin-EDTA, trypan blue, colcemid, fetal bovine serum (FBS) 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[9,11]. The S-9 fraction prepared was stored immediately at −80°C before use.

Cell Lines and Culture

A clonal sub-line of a Chinese hamster lung (CHL) fibroblast cells was obtained from the National Institute of Health Sciences, Tokyo, Japan. The karyotype of CHL cells consisted of 25 chromosomes. The cells had been maintained by 3-4 day passages and grown in a monolayer with EMEM supplemented with 10% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin. 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.

Ames Salmonella Bacterial Mutagenicity Assay

This test performed essentially as described by Ames *et al.*[9,10]. 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 an *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 $\pm$ 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, 1×10^5 CHL cells or 1×10^6 L5178Y cells were treated to various concentrations of Khal in 12-well plate in the absence and presence S-9 metabolic activation system for 6 h or 2 h, respectively. After the staining of 0.4% trypan blue, 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.*[12,13] with minor modifications[14,17]. For the comet assay, 8×10^5 cells were seeded into 12 wells plate (Falcon 3043) and then treated with Khal. At all doses of Khal 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 Khal for 2 h, cells were centrifuged for 3 min at $\times$ 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) 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/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. Khal at each concentration was 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 chemical. 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*[19]. 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[20].

In vitro Chromosomal Aberrations Assay in CHL Cells

The clastogenicity of Khal was evaluated for their ability to induce chromosomal aberrations in CHL cells. The experiment was performed as described by OECD[21] and Ishidate and Odashima[22] with some minor modifications[23-29], which are briefly summarized as follows. Concentration selection for this assay was based on cytotoxicity. Three different doses, including the IC_{50} value as a maximum dose, were prepared and separately added to 3-day-old cultures (approximately 10^5 cells/60 mm dish). In the absence and in the presence of S-9 mixture, cultures were treated for 6 h with Khal and then maintained for 18 h in the fresh medium to adjust a time equivalent to about 1.5 normal cell cycle lengths. Cyclophosphamide (CP) and mitomycin C (MMC) were used as a positive control in combination with or without S-9 mixture, respectively. After 22 h incubation, the treatment was followed by addition of medium containing colcemid at a concentration of 0.2 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 20 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 Axioscope microscope (Karl Zeiss, FRG). The classification of aberration types referred to JEMS-MMS[30]. 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 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[31] 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.

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