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Forward Gene Mutation Assay of Seven Benzophenone-type UV Filters using L5178Y Mouse Lymphoma Cell

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Abstract

The effects of high energy short wave solar radiation on human skin have received much publicity as the major cause of accelerated skin ageing and of skin cancers. To meet public demand, the cosmetic industry has developed sun protection factor products, which contain a variety of so-called "UV filters", among others benzophenone (BP) and its metabolites are the widely used UV filters. UV filters are also used to prevent UV light from damaging scents and colors in a variety of cosmetics products and to protect of plastic products against light-induced degradation. There are many variants of BP in use. In this respect, to regulate and to evaluate the hazardous effect of BP-type UV filters will be important to environment and human health. The genotoxicity of 7 BP-type UV filters was evaluated in L5178Y ($tk^{+/-}$) mouse lymphoma cells *in vitro*. BP, benzhydrol, 4-hydroxybenzophenone 2-hydroxy-4-methoxybenzophenone and 2, 4-dihydroxybenzophenone did not induce significant mutation frequencies both in the presence and absence of metabolic activation system. 2, 2′-Dihydroxy-4-methoxybenzophenone appeared the positive results at the highest dose, *i.e.* 120.4 μg/mL only in the absence of metabolic activation system. And also, 2, 3, 4-trihydroxybenzophenone revealed a significant increase of mutation frequencies in the range of 138.1-207.2 μg/mL in the absence of metabolic activation system and 118.3-354.8 μg/mL in the presence of metabolic activation system. Through the results of MLA with 7 BP-type UV filters in L5178Y cells *in vitro*, we may provide the important clues on the genotoxic potentials of these BP-type UV filters.

Keywords: Mutation frequency, *In vitro* mouse lymphoma assay, Thymidine kinase gene, Benzophenone-type UV filter

Benzophenone (BP)-type UV filters are used primarily as photoinitiators, fragrance enhancers, and ultraviolet curing agents and occasionally, as flavor ingredients[1-4]. They are also used in the manufacture of insecticides, agricultural chemicals and pharmaceuticals and as additives for plastics, coatings and adhesives[5-7]. In addition, 2-hydroxy-4-methoxybenzophenone (HMB) and 2, 2′-dihydroxy-4-methoxybenzophenone (DHMB) are especially added to cosmetic products such as sunscreen lotions, moisturizers, creams, hair sprays, shampoo, lipsticks, hair dyes and photo-affinity labeling to reduce premature aging of the skin by protecting it against photodegradation[8-10]. Some of the physico-chemical properties of BP-type UV filters are given in Table 1.

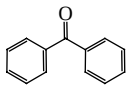
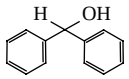
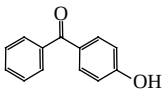
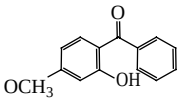
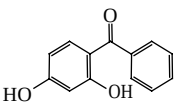
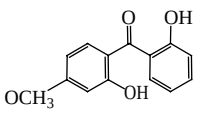
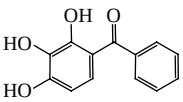
Currently, with the increased use of sunscreens, questions should be raised concerning the environmental impact of sunscreen ingredients. UV filters applied to the skin may contact with moisture when released from the skin during swimming or bathing[11]. In addition, indirect input (e.g., rubber off with towels, washed off during showering, etc.) through wastewater treatment plants is possible. Although the amounts of UV filters used are small compared to those of many other chemicals used everyday (e.g., soaps and detergents), the environmental consequences may still be significant, due to the possible direct input of surface waters.

Furthermore, BP has been listed among "chemicals suspected of having endocrine disrupting effects" by the World Wildlife Fund and the Japanese Environment Agency[12]. In the previous studies, BP-type UV filters have been seen to exert a uterotrophic effect *in vivo*, to stimulate cell proliferation of MCF-7 breast cancer cells and to increase the secretion of tumor marker pS2 *in vitro*[13].

Therefore, the approved UV filters and their maximum allowed concentrations in commercial products have been legislated by various regulatory authorities in Europe[14], the USA[15] and Japan[16]. The maximum authorized concentration of HMB in sunscreens is 10% in EU, 6% in USA and 5% in Japan. In Korea, according to the Korea Food and Drug Administration (KFDA) the maximum authorized level of HMB and DHMB is 5 and 3%, respectively.

Moreover, BP-type UV filters have been reported to have potential genotoxic effects. Several assay systems having rapidity and reliability have been introduced

Table 1. Structure and some physico-chemical properties of the test compounds.

Compound	Chemical structure	Formula	Molecular mass	CAS number	b.p./m.p. (°C)	Log K_{ow}[a]
Benzophenone (BP)		$C_{13}H_{10}O$	182.22	119-61-9	305/49	3.38
Benzhydrol (BH)		$C_{13}H_{12}O$	184.24	91-01-0	297-299/65-69	2.71
4-Hydroxybenzophenone (HBP)		$C_{13}H_{10}O_2$	198.22	1137-42-4	150-160/132-135	3.07
2-Hydroxy-4-methoxy benzophenone (HMB)		$C_{14}H_{12}O_3$	228.24	131-57-7	150-160/66	3.52
2, 4-Dihydroxy benzophenone (DHB)		$C_{13}H_{10}O_3$	214.22	131-56-6	194/144-145	2.96
2, 2′-Dihydroxy-4-methoxy benzophenone (DHMB)		$C_{14}H_{12}O_4$	244.24	131-53-3	170-175/68	3.82
2, 3, 4-Trihydroxy benzophenone (THB)		$C_{13}H_{10}O_4$	230.22	1143-72-2	-/140-142	–

[a]K_{ow}: Octanol-water partition coefficient

to evaluate the genotoxicity of chemicals, 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. The parent compound, BP, was negative in *Salmonella* mutagenesis assay[17] and in the *Escherichia coli* pol A assay[18], but HMB was mutagenic in *Salmonella*[19] and induced sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells[20]. DHMB also was positive in *Salmonella* mutagenesis assay[20].

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 to detect a broad spectrum of mutational events[21]. 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[22]. 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[22,23]. 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

Table 2. Cytotoxicity of 7 benzophenone-type UV filters in L5178Y mouse lymphoma cell line in the presence and absence of S-9 metabolic activation system. IC_{80} (80% inhibition concentration of cell growth) was calculated by the results of cell viability (unit=μg/mL).

	BP	BH	HBP	HMB	DHMB	DHB	THB
−S-9	142.8	331.5	356.9	176.6	160.5	262.1	276.2
+S-9	141.7	255.3	293.2	216.1	153.2	252.3	473.0

BP: Benzophenone; BH: Benzhydrol; 4 HBP: 4-Hydroxybenzophenone; HMB: 2-Hydroxy-4-methoxybenzophenone; DHMB: 2, 2′-Dihydroxy-4-methoxybenzophenone; DHB: 2, 4-Dihydroxybenzophenone; THB: 2, 3, 4-Trihydroxybenzophenone; MMS: methylmethanesulfonate; CP: cyclophosphamide

detectable chromosome aberrations such as translocations. In the past decade, our laboratory had also been evaluated genotoxicity assay, especially MLA[24-28].

Therefore, to detect the different response of BP-type UV filters in terms of their genotoxic effect, we examined the mutagenic profiles of commonly used and harmful environmental chemicals, seven BP-type UV filters, using MLA in L5178Y cells.

Cytotoxicity of Seven Benzophenone-type UV Filters

To determine the optimal concentration, cytotoxicity of each compound was assessed by exposure to 0-5,000 μg/mL with 2-fold serial dilution for 2 hours using trypan blue exclusion assay. All measurements of cytotoxicity were performed in the presence and absence of S-9 metabolic activation systems. Based on results of cytotoxicity assay, 80% inhibitory concentration (IC_{80}) of each compound was calculated and used as maximum concentration (Table 2).

L5178Y $tk^{+/-}$-3.7.2C Mouse Lymphoma Assay (MLA) on Benzophenone-type UV Filters

The genotoxic potentials of the seven BP-type UV filters were assessed with various concentrations in the absence and presence of S-9 activation, respectively using MLA. Table 3 and 4 summarizes the results of the MLA after treatment of L5178Y cells with BP, benzhydrol (BH), 4-hydroxybenzophenone (HBP), HMB, DHMB, 2, 4-dihydroxybenzophenone (DHB) and 2, 3, 4-trihydroxybenzophenone (THB) 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 (MFs) were within the historical control range and positive controls gave large dose-dependent increases in MFs, meeting assay acceptance criteria. The *tk* mutant frequencies (including the small and large colony *tk* mutant frequencies) of the seven BP-type UV filters are displayed in Figure 1.

BP and BH did not induce significant changes in MFs and dose response in the absence and the presence of S-9 (Table 3 and Figure 1(A)-(B)). HBP was not observed significant changes in MFs and dose response in the presence of S-9 and in the absence of S-9, no significant increase of MF was induced, although dose related response was observed (Table 3 and Figure 1(C)).

HMB and DHB did not appear significant changes in MFs and dose related response in the absence and the presence of S-9 (Table 3 and Figure 1(D)-(E)). Treatment of cells with DHMB for 3 h led to a clearly increased MF at the highest concentrations in the absence of S-9. DHMB at 120.4 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 (Table 3 and Figure 1(F)). THB was observed dose-related and significant increases in MFs at concentration $>$138.1 μg/mL in the absence of S-9 and at concentration $>$118.3 μg/mL in the presence of S-9. The significant increases of MFs for THB caused 12.4-fold in the absence of S-9 and 5.4-fold in the presence of S-9, as compared to the negative control cultures at the highest evaluated concentration (*i.e.*, 207.2 μg/mL in the absence of S-9 and 354.8 μg/mL presence of S-9) (Table 3 and Figure 1(G)).

Discussion

We investigated whether these seven BP-type UV filters 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 mutagens[29].

The metabolic pathway of BP primary undergoes reduction of the keto group to BH[30]. HBP is formed probably via aromatic hydroxylation which is typical of the cytochrome P-450 enzyme system, at a *para*-position of BP[31]. Under the experimental conditions used, BP and its two metabolites, BH and HBP were considered to be negative in this *in vitro* MLA. There is limited evidence for BP having either genotoxic or carcinogenic activity. BP was not mutagenic in the standard Ames test using TA98, TA100, TA1535, or TA1537 strains of *Salmonella typhimurium*[17], or in

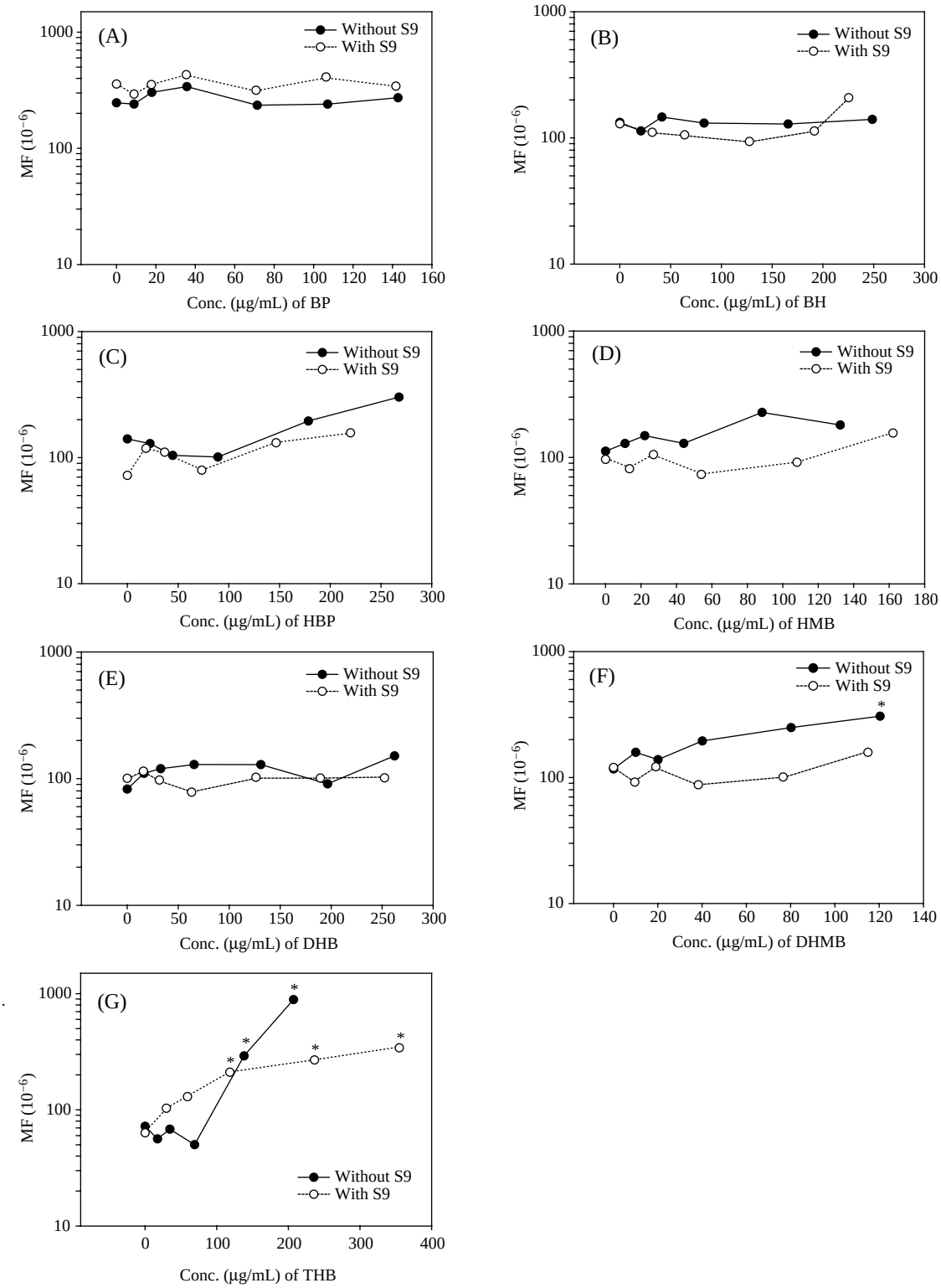


Figure 1. Mutation frequencies (MF) by seven benzophenone-type UV filters in L5178Y cells in the absence and presence of S-9 metabolic activation system (3 h treatment). Total *tk* mutation frequency is displayed. Results are taken from one representative experiment. *Statistically significant ($P < 0.05$). (A) BP: Benzophenone, (B) BH: Benzhydrol, (C) 4 HBP: 4-Hydroxybenzophenone, (D) HMB: 2-Hydroxy-4-methoxybenzophenone, (E) DHB: 2, 4-Dihydroxybenzophenone, (F) DHMB: 2, 2′-Dihydroxy-4-methoxybenzophenone, (G) THB: 2, 3, 4-Trihydroxybenzophenone.

Table 3. Toxicity and mutagenicity of seven benzophenone-type UV filters in L5178Y ($tk^{+/-}$) mouse lymphoma cells.

Treatment (μg/mL)		−S-9		Treatment (μg/mL)		+S-9	
		%RS	RTG			%RS	RTG
BP	0	100.00	1.00	BP	0	100.00	1.00
	8.9	91.84	0.80		8.9	125.62	1.20
	17.9	98.59	0.59		17.7	125.62	1.16
	35.7	100.00	0.78		35.4	110.43	0.97
	71.4	101.43	0.95		70.8	157.41	1.42
	107.1	120.67	1.09		106.3	110.43	0.89
	142.8	120.67	1.02		141.7	139.24	1.13
MMS	10	118.90	1.36	CP	3	112.01	1.09
BH	0	100.00	1.00	BH	0	100.00	1.00
	20.7	135.57	0.93		31.9	50.00	0.91
	41.4	148.43	0.86		63.8	54.82	1.09
	82.9	166.91	0.79		127.6	23.53	0.80
	165.7	198.15	1.14		191.5	20.75	0.53
	248.6	27.75	0.32		225.3	11.91	1.39
MMS	10	187.20	1.57	CP	3	37.44	0.06
HBP	0	100.00	1.00	HBP	0	100.00	1.00
	22.3	89.08	1.01		18.3	103.11	0.82
	44.6	101.48	0.79		36.7	79.11	0.88
	89.2	81.79	0.95		73.3	92.78	1.22
	178.4	104.53	1.03		146.6	100.00	0.75
	267.7	45.42	0.35		219.9	81.40	0.75
MMS	10	98.55	1.89	CP	3	88.78	1.23
HMB	0	100.00	1.00	HMB	0	100.00	1.00
	11.0	140.88	1.30		13.5	139.27	1.43
	22.1	118.54	1.05		27.0	130.66	1.20
	44.1	149.47	1.48		54.0	132.74	1.22
	88.3	112.01	0.85		108.0	112.30	1.22
	132.4	47.85	0.35		162.1	30.92	0.12
MMS	10	161.25	1.74	CP	3	119.18	1.14
DHB	0	100.00	1.00	DHB	0	100.00	1.00
	16.4	83.66	0.80		15.8	78.06	0.98
	32.8	77.55	1.00		31.5	82.65	0.74
	65.5	78.72	1.12		63.1	106.32	0.91
	131.0	78.72	0.96		126.2	81.47	1.12
	196.5	84.97	1.11		189.3	68.71	1.17
	262.1	70.99	0.76		252.3	79.18	1.40
MMS	10	95.06	1.04	CP	3	90.13	0.96
DHMB	0	100.00	1.00	DHMB	0	100.00	1.00
	10.0	113.79	0.95		9.6	60.55	0.93
	20.1	97.20	1.10		19.1	74.97	1.25
	40.1	152.37	1.53		38.3	78.31	1.39
	80.2	107.40	0.91		76.6	92.37	1.21
	120.4	49.06	0.23		114.9	54.04	0.58
MMS	10	108.96	1.35	CP	3	72.84	1.02
THB	0	100.00	1.00	THB	0	100.00	1.00
	17.3	101.56	1.11		29.6	81.25	0.84
	34.5	101.56	1.02		59.1	64.67	0.65
	69.1	89.98	0.69		118.3	33.25	0.33
	138.1	36.58	0.17		236.5	31.75	0.16
	207.2	19.53	0.02		354.8	17.96	0.06
MMS	10	91.32	1.24	CP	3	69.62	0.73

BP: Benzophenone; BH: Benzhydrol; 4 HBP: 4-Hydroxybenzophenone; HMB: 2-Hydroxy-4-methoxybenzophenone; DHMB: 2, 2′-Dihydroxy-4-methoxybenzophenone; DHB: 2, 4-Dihydroxybenzophenone; THB: 2, 3, 4-Trihydroxybenzophenone; MMS: methylmethanesulfonate; CP: cyclophosphamide

the *Escherichia coli* pol A assay[18], with or without S9 metabolic activation. It did not induce micronuclei in bone marrow erythrocytes of male mice. However, HBP increased dose-dependent MF in the absence of S-9, though the changes of MF were not statistically significant. Recently, BP and its metabolically related BH and HBP induced *umu* gene expression after metabolic activation by human P450s[32]. Therefore, a further attempt may be made to evaluate the genotoxic property of these compounds through *in vivo* test.

HMB was enzymatically converted to at least three metabolites. DHB is a major intermediate formed by *O*-demethylation of the parent compound, which in turn is converted to THB by aromatic hydroxylation[33]. HMB is also converted to DHMB by aromatic hydroxylation[33]. In our study, HMB and its major metabolite, DHB were considered to be negative in this *in vitro* MLA. The genotoxicity of HMB was reported previously that this chemical was not mutagenic in *Salmonella* with metabolic activation, and induced sisterchromatid exchanges and chromosomal aberrations in CHO cells in the presence of a metabolic activation system[34]. There was no increase in the frequency of micronucleated erythrocytes in the blood of mice receiving HMB[34]. On the other hand, the parent compound, HMB, was previously found to be non -mutagenic in *Salmonella* mutagenesis assay, but DHMB was mutagenic[35], and the similar data were found in our study. The results obtained from our study showed that two metabolites of HMB, DHMB and THB revealed concentration-related mutagenic effect. To make up of the weak points of the *in vitro* effects induced by HMB and its metabolites, furthermore they will be needed to confirm *in vivo* genotoxicity assay.

In summary, BP and its metabolites, BH and HBP did not induce significant mutation frequencies both in the presence and absence of metabolic activation system. HMB and its metabolite, DHB also appeared the negative results in the presence and absence of metabolic activation system. Among the metabolites of HMB, DHMB revealed a significant increase of mutation frequencies only in the absence of metabolic activation system and THB appeared the positive results in the presence and absence of metabolic activation system. Through the results of MLA with seven BP-type UV filters in L5178Y cells *in vitro*, we may provide the important clues on the genotoxic potentials of these BP-type UV filters.

Methods

Materials

BP was obtained from Sigma (St. Louis, MO, USA). BH, HBP, HMB, DHB, DHMB and THB were purchased from Aldrich (Milwaukee, WI, USA or St. Louis, MO, USA). Stock solutions of seven BP-type UV filters were prepared immediately in dimethylsulfoxide (DMSO) before use. The final concentration of DMSO used in the medium was below 1%. 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[36,37]. 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.

Cytotoxicity Test

Cytotoxicity of cells was checked by the trypan blue exclusion assay. For the determination of cell cytotoxicity, about 10^6 cells were treated for 2 hours with the chemicals. After the staining of 0.4% trypan blue (Life Technologies, MD, USA.), the total number of cells and the number of unstained cells were counted in four of the major sections of a hemocytometer. The average number of cells per section was calculated. Cell viability of treated chemicals was related to controls that were treated with the solvent. All experiments were duplicated in an independent test.

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

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. Seven BP-type UV filters 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. Mutant selection was performed using the modified microwell version of the assay as described by Clements *et al.*[38]. 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[39].

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