MOLECULAR & CELLULAR TOXICOLOGY, Vol. 3, No. 3, 172-176, September 2007

Evaluation of the Genetic Toxicity of Synthetic Chemical (XVIII)-*in vitro* Mouse Lymphoma Assay and *in vivo* Supravital Micronucleus Assay with Butylated Hydroxytoluene (BHT)

Youn-Jung Kim[1] **& Jae-Chun Ryu**[1]

[1]Cellular and Molecular Toxicology Laboratory,
Korea Institute of Science and Technology, PO Box 131,
Cheongryang, Seoul 130-650, Korea
Correspondence and requests for materials should be addressed to J. C. Ryu (ryujc@kist.re.kr)

Accepted 14 August 2007

Abstract

Butylated hydroxytoluene (BHT) is widely used antioxidant food additives. It has been extensively studied for potential toxicities. BHT appears adverse effects in liver and thyroid. In this study, we evaluated the genetic toxicity of BHT with more advanced methods, *in vitro* mouse lymphoma assay $tk^{+/-}$ gene assay (MLA) and *in vivo* mouse supravital micronucleus (MN) assay. BHT did not appear the significantly results in the absence and presence of metabolic activation system with MLA. Also, *in vivo* testing of BHT yielded negative results with supravital MN assay. These results suggest that BHT itself was not generally considered genotoxic.

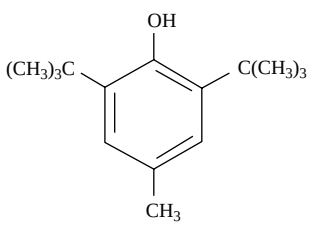
Keywords: Butylated hydroxytoluene, *in vitro* Mouse Lymphoma Assay, *in vivo* supravital micronucleus assay, Genotoxicity

Butylated hydroxytoluene (BHT) (CAS No. 128-37-0) is a substituted toluene conforming generally to the formula in Figure 1. BHT is used as an antioxidant which finds many applications in a wide variety of industries. It is used to preserve and stabilize the freshness, nutritive value, flavour and colour of foods and animal feed products for many years[1]. It protects these materials from oxidation during prolonged storage. BHT can also improve the stability of pharmaceuticals, fat-soluble vitamins and cosmetics[2]. The service life of rubber, elastomers and plastics is increased by the addition of BHT[3], and from such use BHT may be present as an indirect food additive. Approximately 40 countries reportedly permit the use of BHT as a direct or indirect food additive[4]. The US Food and Drug Administration (FDA) currently permit BHT as food additives.

On chronic oral exposure of rats, liver and thyroid are the main targets. Doses above 25 mg/kg bw/day BHT resulted in thyroid hyperactivity, enlargement of the liver, induction of several liver enzymes. 25 mg/kg bw/day BHT can be considered as NOAEL for chronic exposure. The haemorrhagic effects of high repeated doses of BHT seen in certain strains of mice and rats, but not in other species, may be related to its ability to interact with prothrombin and vitamin K.

The evaluation of BHT concluded that there was limited evidence for carcinogenicity in experimental animals, and also no data for humans[5]. Several reports discuss chronic carcinogenicity bioassays of BHT in rodents[5-7]. These studies do not provide convincing evidence that BHT has carcinogenic activity in either mice or rats. Several reports indicated neoplasia promoting activity when given after an initiating carcinogen for mouse lung[8] and colon[9], and rat liver[10] and urinary bladder[11]. Consistent with these observations, BHT inhibits intercellular molecular transfer[12], a property of neoplasm-promoting agents[13].

BHT did not cause DNA damage in *Bacillus subtilis*[14] or mutation in *Salmonella typhimurium*[12,15]. It did not induce chromosomal aberrations in plants[16] or mutation and chromosomal aberrations in *Drosophila melanogaster*[17]. In one study, it was reported to be mutagenic to cultured Chinese hamster V79 cells in the presence of an exogenous metabolic system[18]. Binding of BHT to the DNA of liver of rats exposed *in vivo* has been reported[19], but no adduct was identified. Moreover, BHT was negative for DNA repair in isolated hepatocytes[12]. BHT did not induce micronuclei in bone marrow or dominant lethal mutations in mice[20,21]. BHT also exhibited no evidence of muta-



BHT

Figure 1. Chemical structure of BHT.

Table 1. Toxicity and mutagenicity of BHT in L5178Y $tk^{+/-}$ mouse lymphoma cells for 3 h treatment.

3 h									
	−S-9					+S-9			
Treatment (μg/mL)	%RS	RTG	Mutation frequency ($\times 10^{-6}$)		Treatment (μg/mL)	%RS	RTG	Mutation frequency ($\times 10^{-6}$)	
0	100.00	1.00	208.54		0	100.00	1.00	85.59	
2.5	69.62	0.80	254.75	NS	2.5	170.80	0.56	140.48	NS
5.0	92.78	0.93	198.80	NS	5.0	266.44	0.77	85.27	NS
10.0	70.62	0.87	101.58	NS	10.0	278.77	0.58	100.42	NS
20.0	68.64	0.94	204.55	NS	20.0	165.98	0.45	99.40	NS
40.0	54.60	0.21	265.05	NS	40.0	135.23	0.47	80.10	NS
Linear trend			NS		Linear trend			NS	
MMS					CP				
10	84.99	0.38	1422.79	*	3	86.88	0.67	247.67	*

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

genicity in the Salmonella/microsome mutagenesis assay or the adult rat liver epithelial cell/hypoxanthine ± guanine phosphoribosyl transferase mutagenicity assay[12].

In this study, we evaluated the genetic toxicity of BHT with more advanced methods not mentioned above, *in vitro* mouse lymphoma assay $tk^{+/-}$ gene assay (MLA) and *in vivo* mouse supravital micronucleus (MN) assay.

BHT did not Induced Mutation in MLA Assay

The genotoxic potentials of BHT were assessed with various concentrations in the absence and presence of S-9 activation, respectively using MLA. Table 1 summarizes the results of the MLA after treatment of L5178Y cells with BHT at different concentrations for 3 h. 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.

BHT was slightly more toxic without S-9 than with S-9. Dose-related increases in toxicity were observed under BHT treatment without S-9, with a pronounced drop in the RS and RTG. The limit of 0.1 RTG is widely accepted as the maximum level of cytotoxicity for mutagenicity evaluation to avoid biologically irrelevant effects that might occur in severely stressed cells (e.g., treatments resulting in cytotoxicity $>90\%$)[22]. Therefore 40 μg/mL was evaluated as the highest concentration for mutagenicity. Background mutant frequencies (MF) (208.54×10^{-6} and 85.59×10^{-6}) were within the historical control range, and positive controls gave large dose-dependent increases in MF, meeting assay acceptance criteria. Treatment of cells with BHT for 3 h did not lead to a significant increase of MF at all concentrations in the absence and presence of S-9 (Table 1).

Table 2. Toxicity and mutagenicity of BHT in L5178Y $tk^{+/-}$ mouse lymphoma cells for 24 h treatment.

24 h				
	−S-9			
Treatment (μg/mL)	%RS	RTG	Mutation frequency ($\times 10^{-6}$)	
0	100.00	1.00	95.58	
6.25	107.59	0.93	104.28	NS
12.50	94.40	0.87	100.53	NS
25.00	97.15	0.55	162.32	NS
50.00	76.29	0.32	105.93	NS
Linear trend			NS	
MMS				
10	52.13	0.43	1405.42	*

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

Following the ICH recommendation, the MLA was conducted using a 24 h treatment (without S-9) in situations where the short treatment (3 h) was negative[23]. Also, the maximum concentration was changed to 50.0 μg/mL, so that RTG at this concentration for 24 h was 0.32 (Table 2). Because ssignificant increases in MF were not observed in 24 h experiment like 3 h, the results of the mutagenesis experiments testing BHT were evaluated as negative.

BHT did not Induced Micronucleus Formation in Peripheral Blood from Mice Exposed Intraperitoneally

BHT also subjected to acridine orange micronucle-

Table 3. Frequencies of MNRETs in peripheral blood of ICR mice after a single intraperitoneal (i.p.) administration of BHT.

Chemical	Route of administration	Dose (mg/kg)	No. of mouse	Sampling time (h)	%MNRET[a] (Mean ± S.D.)	*P* value
Solvent[b] control	i.p.	−	6	48	0.22 ± 0.11	
MMC	i.p.	1	6	48	3.44 ± 0.91	< 0.05
BHT	i.p.	69.0	6	36	0.19 ± 0.10	> 0.05
		69.0	6	48	0.33 ± 0.14	> 0.05
		69.0	6	60	0.23 ± 0.06	> 0.05
		34.5	6	48	0.26 ± 0.13	> 0.05
		17.3	6	48	0.21 ± 0.12	> 0.05

[a]2000 Reticulocytes were counted in each mouse; [b]Solvent control group was injected i.p. with corn oil.
MNRET: micronucleated reticulocyte; MMC: mitomycin C

us (MN) assay with mouse peripheral reticulocytes. The 50% lethal dose (LD_{50}) on intraperitoneal (i.p.) injection in male ICR mice was reported as 138.0 mg/kg. To determine the optimal sampling time, a single half dose of LD_{50}, 69.0 mg/kg of BHT was injected into mice, and blood samples collected at 12 h intervals from 36 to 60 h. Maximum frequency of micronucleated reticulocytes (MNRETs) was observed at 48 h after treatment (Table 3). The frequency of MNRETs induced 48 h after i.p. injection at a single dose of 17.3, 34.5 and 69.0 mg/kg of BHT was not dose-dependently increased (Table 3).

Discussion

In this study, BHT did not induce the base-pair as well as frameshift mutations or small deletions in MLA and also it was not appeared the clastogenic potential in *in vivo* supravital micronucleus (MN) assay. These data were consistent with data in review of Bomhard, Bremmer, and Herbold[24]. They concluded that BHT was not "a relevant mutagenic/genotoxic risk to man." BHT was nonmutagenic in various bacterial system and mammalian cells with or without S-9. Also, BHT was typically nonclastogenic in mammalian and plant cells. However, BHT was mutagenic only at cytotoxic doses in Chinese hamster V79 fibroblasts and DNA damage occurred after addition of S-9 rat liver homogenate during rec-assays using *Bacillus subtilis*. These positive data may be due to be performed in stressed conditions. Consequently, our data and numerous studies suggest that BHT itself was not generally considered genotoxic.

Methods

Materials

Butylated hydroxytoluene (BHT: CAS No. 128-37-0) was purchased from Junsei Chem. (Japan). 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® (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[25]. The S-9 fraction prepared was stored immediately at −80°C before use.

Cell Line 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 (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 Arochlor1254-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. BHT was tested with and without S-9 mixture. BHT 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 or 24 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 BHT. Cytotoxicity was determined by RS and 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.*[26]. Simply, the treated cells in medium containing 3 μg/mL TFT 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. MF were calculated using a statistical package (Mutant™: UKEMS, UK) in accordance with the UKEMS guidelines[27].

In vivo Supravital Micronucleus Assay in Mice

Outbred mice of strain ICR, 7-8 weeks old, were used in this study. The mice were allowed an adaptation period of about 1 week, then randomized and subjected to the study. The six animals were housed for each group. The test article was applied orally in three doses in volumes of 10 mL/kg. The micronucleus test with mouse peripheral blood reticulocytes using acridine orange (AO) supravital staining method was performed essentially as described by Hayashi *et al.*[28]. To determine the clastogenicity, BHT dissolved in corn oil was administrated intraperitoneally (i.p.) at either 17.3, 34.5, or 69.0 mg/kg. A single dose of MMC at 1 mg/kg was injected i.p. as a positive control, and corn oil was administrated i.p. with 0.1 mL/kg as a solvent control. Peripheral bloods were collected from mouse tail vein at 12 h intervals from 36 h to 60 h after administration. The 10 μL of 1 mg/mL AO dissolved in distilled water was placed on a glass slide pre-heated at about 70˚C, spread out, and dried at room temperature. This glass slides were stored in a dark and dry location at room temperature until used. Peripheral blood was taken by piercing a tail blood vessel. Five μL of blood was obtained directly without anticoagulant from a tail, and placed on an AO-coated glass slide. Glass slide was covered with coverslip, and allowed to be supravitally stained. To score and data analysis, two thousand reticulocytes (RETs) of type I, II, and III per animal were observed[30] and RETs with micronucleated reticulocytes (MNRETs) were recorded under the fluorescent microscopy which had the combination of a blue excitation and a yellow to orange barrier filter. The data was analyzed by pair-wise test for statistical differences between the control and specific treatment groups.

References

1. JECFA. Toxicological evaluation of certain food additives and contaminants in food. Who Food Additives Series. No. 35, pp. 3-86. Joint FAO/WHO Expert Committee on Food Additives. Geneva (1996).
2. FDA. Number of brand name products in each product code, cosmetic product formulation data. In Division of Cosmetics Technology. pp. 33-34. Food and Drug Administration, Washington, DC (1981).
3. Sherwin-Williams. BHT: The versatile antioxidant for today and tomorrow (Bull. A Ox12). Cleveland, OH (1992).
4. ILSI. Butylated Hydroxytoluene (BHT). A Monograph. International Life Sciences Institute, Washington, DC (1984).
5. IARC. IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Vol. 40, Some Naturally Occurring and Synthetic Food Components, Furocoumarins and Ultraviolet Radiation. Butylated hydroxytoluene (BHT). pp. 161-206. International Agency for Research on Cancer, Lyon (1986).
6. Kahl, R. Synthetic antioxidants: biochemical actions and interference with radiation, toxic compounds, chemical mutagens and chemical carcinogens. *Toxicology* **33**:185-228 (l984).
7. WHO. Toxicological evaluation of certain food additives and contaminants. *In Food Additives Series*, **21**. World Health Organization Cambridge (1987).
8. Witschi, H., Williamson, D. & Lock, S. Enhancement of urethan tumorigenesis in mouse lung by butylated hydroxytoluene. *J Nation Can Inst* **58**:301-305 (1977).
9. Lindenschrnidt, R. C. *et al*. The effects of dietary butylated hydroxytoluene on liver and colon tumor development in mice. *Toxicology* **38**:151-160 (1986).
10. Maeura, Y. & Williams, G. M. Enhancing effect of butylated hydroxytoluene on the development of liver altered foci and neoplasms induced by N-2-fluoremyl -acetamide in rats. *Food Cosmet Toxicol* **22**:191-198 (1984).

11. Imaida, K. *et al*. Promoting activities of butylated hydroxyanisole, butylated hydroxytoluene and sodium -1-ascordsate on forestomach and urinary bladder carcinogenesis initiated with the methylnitrosourea in F344 male rats. *Gann* **75**:769-775 (1984).
12. Williams, G. M., McQueen, C. A. & Tong, C. Toxicity studies of butylated hydroxyanisole and butylated hydroxytoluene. I. Genetic and cellular effects. *Food Chem Toxicol* **28**:793-798 (1990).
13. Yamasaki, H. Role of disrupted gap junctional intercellular communication in detection and characterization of carcinogens. *Mutat Res* **365**:91-105 (1996).
14. Kinae, N. *et al*. Studies on the toxicity of pulp and paper mill effluents -1. Mutagenicity of the sediment samples derived from Kraft paper mills. *Water Res* **15**:17-24 (1981).
15. Brusick, D. Genotoxicity of phenotic antioxidants. *Toxicol Indust Health* **9**:223-230 (1993).
16. Alekperov, U. K., Abutalybov, M. G. & Bagirova, A. D. Ionol modification of natural and induced chromosome aberrations in Crepis capillaris L. *Doklady Biological Sciences* **220**:31-32 (1975).
17. Prasad, O. & Kamra, O. P. Radiosensitization of Drosophila sperm by commonly used food additives-butylated hydroxyanisole and butylated hydroxytoluene. *Inter J Rad Biol* **25**:67-72 (1974).
18. Paschin, Y. V. & Bahitova, L. M. Inhibition of the mutagenicity of benzol a pyrene in the V79/HGPRT system by bioantioxidants. *Mutat Res* **137**:57-59 (1984).
19. Nakagawa, Y., Hiraga, K. & Suga, T. Biological fate of butylated hydroxytoluene (BHT) binding of BHT of nucleic acid *in vivo. Biochem Pharmacol* **29**:1304-1306 (1980).
20. Bruce, W. R. & Heddle, J. A. The mutagenic activity of 61 agents as determined by the micronucleus, Salmonella and sperm abnormality assays. *Canad J Genetic Cytol* **21**:319-333 (1979).
21. Epstein, S. S. *et al*. Detection of chemical mutagens by the dominant lethal assay in the mouse. *Toxicol Appl Pharmacol* **23**:288-325 (1972).
22. Clive, D. *et al*. Consensus agreement regarding protocol issues discussed during the mouse lymphoma workshop: Portland, Oregon, May 7, 1994. *Environ Mol Mutagen* **25**:165-168 (1995).
23. Moore, M. *et al*. Mouse lymphoma thymidine kinase gene mutation assay: meeting of the International Workshop on Genotoxicity Testing, San Francisco, 2005, recommendations for 24-h treatment. *Mutat Res* **627**:36-40 (2007).
24. Bomhard, E. M., Bremmer, J. N. & Herbold. B. A. Review of the mutagenicity/genotoxicity of butylated hydroxytoluene. *Mutat Res* **277**:187-200 (1992).
25. Ames, B. N. *et al*. Carcinogens are mutagens : a simple test system combining liver homogenates for activation and bacteria for detection. *Proc Natl Acad Sci USA* **70**:2281-2285 (1973).
26. Clements, J. Gene mutation assays in mammalian cells, In O'Hare, S & Atterwill, C. K. (Ed.), *Methods in Molecular Biology*, Vol. 43, *in vitro* Toxicity Testing Protocols. Humana Press Inc. Totowa, NJ. 277-286 (1990).
27. Robinson, W. D. *et al*. Statistical evaluation of bacterial/mammalian fluctuation tests, in *Statistical Evaluation of Mutagenicity Test Data* (Kirkland, D. J., ed.). Cambridge University Press. Cambridge, UK, 102-140 (1990).
28. Hayashi, M. *et al*. The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. *Mutat Res* **245**:245-249 (1990).