AATEX 3, 1-8 (1995)

Developmental Toxicity of Butylated hydroxytoluene using Rat and Human Embryonic Cell Assays

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Summary

Butylated hydroxytoluene (BHT, 3, 5-di-tert-butyl-4-hydroxytoluene) and 4-hydroxy-4-methyl-2, 6-di-t-butylcyclohexa-2, 5-dienone (BHTOH) were assayed using cell culture methods for assessing potential teratogenicity. In the rat embryonic cell differentiation assay, BHT and BHTOH showed similar inhibitory effects on the differentiation of both midbrain (MB) and limb bud (LB) cells. From the dose-response curve, the concentrations of BHT and BHTOH that inhibited the production of differentiated foci by 50% (IC_{50}) in MB cells were 254 and 245 μM, respectively. The inhibitory action of BHT on the human embryonic palatal mesenchymal (HEPM) cell growth was increased in the presence of S-9 mix prepared using livers from untreated f344 rats and from those treated with phenobarbital and 5, 6-benzoflavone (PB-BF). In the HEPM cell growth assay, hamster and mouse PB-BF-induced S-9 were also effective in causing the metabolic activation of BHT. *In vivo/in vitro* methods for determing teratogenicity were investigated using a rat embryonic cell differentiation assay. BHT was orally administered to pregnant rats at a dose of 1000 mg//kg on day 11 of gestation. Embryonic MB and LB cells were then prepared from day-12 embryos, and cultured. The differentiated foci slightly reduced to 92–94% of the control values in MB and LB cultures. It was assumed that BHT up to 1000 mg/kg as a single oral dose was not harmful to the rat embryos during organogenesis under our experimental conditions.

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Introduction

Butylated hydroxytoluene (BHT, 3, 5-di-*tert*-butyl-4-hydroxytoluene) is used extensively as an antixidant in food for human consumption and in rubber materials. Available evidence in rats and humans suggests only a low retention of BHT in the adipose tissue of both species and no evidence of any progressive accumulation during continuous feeding[1-3].

Clegg (1965)[4] reported the lack of any teratogenic effect of BHT in rats and mice from the results of experiments involving (1) daily administration at a dose of 750 mg/kg for 32–46 days prior to mating and up to day 18 of pregnancy to mice, (2) single administration at 1000 mg/kg on a specific day of pregnancy to mice, (3) daily administration at 750 mg/kg for 70 days before pairing, continuing through pregnancy to rats, and (4) single administration at 1000 mg/kg on day 9, 11 or 13 of pregnancy to rats.

However, BHT has been shown to induce reversible mixed function oxidases and liver enlargement in rats[5] and peliosis, hepatocellular vacuolation, degeneration and nec-

was then flooded (0.5 ml/well) with culture medium containing the test substance or vehicle only as a control. The adhering cells formed separate micromass islands, and were cultured for 5 days at 37°C in 5% CO_2/95% air. Test chemicals BHT and BHTOH were dissolved in DMSO. The final concentration of DMSO in the medium did not exceed 0.1% (v/v).

In the cell differentiation assay, the cultures, after fixation, were stained wit alcian blue (LB cultures) or haematoxilin (MB cultures), as described previously [11]. The number of individual foci was counted under a dissecting microscope (SZH, Olympus Co., Tokyo). Five to ten replicate wells were prepared for each assay. Control values for the number of differentiated foci/cell island were 242±22 (mean+SD) and 152±18 in LB and MB cultures, respectively. The concentration at which each compound inhibited the formation of differentiated foci by 50% of the control values (IC_{50}) was estimated by interpolation from each plotted concentration responses[11].

HEPM cell culture and assay.

HEPM cells used in this study were kindly provided by Dr. T. Yoneda, Osaka University. The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, streptomycin (50 μg/ml) and penicillin (50 U/ml) in an incubator under a humidified atmosphere of 5% CO_2 and 95% air, as described in our previous report[13].

The solutions of BHT and BHTOH were prepared in sterile DMSO, and subsequent dilutions for incubation were made with DMEM-FCS medium. Initially, 0.1 ml aliquots of DMEM-FCS medium were placed into each well of a plastic microtiter plate, then 0.1 ml of medium containing the test compound (containing <0.1 μl DMSO) was added to the first well, and 0.1 ml of the medium plus test substance was removed from the first well and added to the second well. Similarly, 0.1 ml of the medium was removed from the second well into the next, and thus a decreasing stepwise gradient of the chemicals was prepared by continuing the serial dilutions. After medium was discarded, attached cells were fixed with methanol then stained with Giemsa. Cell numbers were counted using a colony counter (New Brunswick Scientific Co., Edison, NJ, USA). Net growth was calculated by subtracting the cell number at 24 hr from the final number.

We also used the HEPM cell growth assay to investigate the effects of metabolic activation of BHT. To do this, hepatic 9000-g supernatant fractions (S-9) from untreated F344 rats and from those which had been treated with phenobarbital (sequential doses of 30+60+60+60 mg/kg, ip) and 5, 6-benzoflavone (80 mg/kg, ip) (PB-BF-treated rats), were obtained from Kikkoman Co., Chiba, Japan. Experiments were also carried out with S-9 mix prepared from the liver of Balb/c mice and Golden hamsters that had been treated with PB-BF. S-9 and cofactors in 20 μl of HEPES-buffered saline at pH 7.4 were added to fresh medium along with the test chemicals to final concentrations as follows: 1.2 mg of S-9 protein/ml, 0.58 mM NADP, 0.74 mM glucose-6-phosphate, and 0.83 mM $MgCl_2$ in HEPES buffer (0.67 mM) at pH 7.4. After incubation for 6 hr at 37°C, the reaction mixture was removed, fresh growth medium was added, and the cultures were incubated for a further 72 hr with no further change of medium.

Results

Figure 1 shows the concentration-related changes in the number of differentiated foci of rat embryo MB cells after exposure to BHT or BHTOH. These two compounds had similar inhibitory potentials on the MB cells. Figure 2 shows the concentration-related changes in the number o differentiated foci of rat embryo LB cells after exposure to BHT or BHTOH. BHTOH showed weaker inhibition than BHT

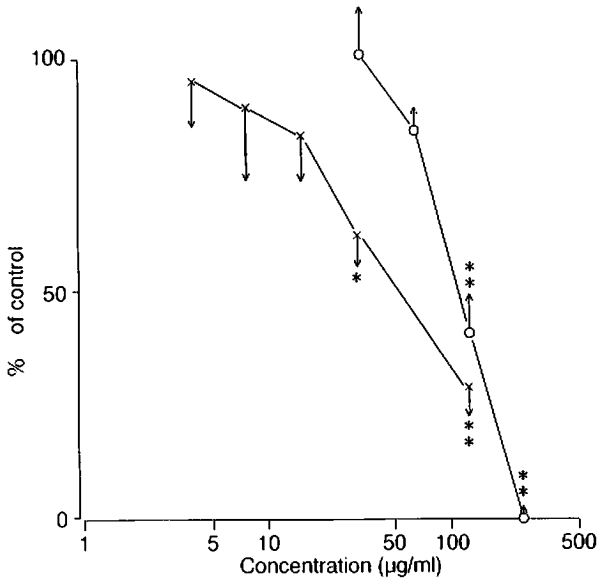

Fig. 3. Effects of BHT on cell growth in the HEPM assay (6 hr) in the presence (×) or absence (○) of S9 mix prepared using the livers of F344 rats treated with phenobarbital and 5, 6-benzoflavone. Significant inhibition of cell growth, in comparison with controls, is indicated (*P<0.01; **P<0.001; Student's *t*-test). Bars indicate S.D.

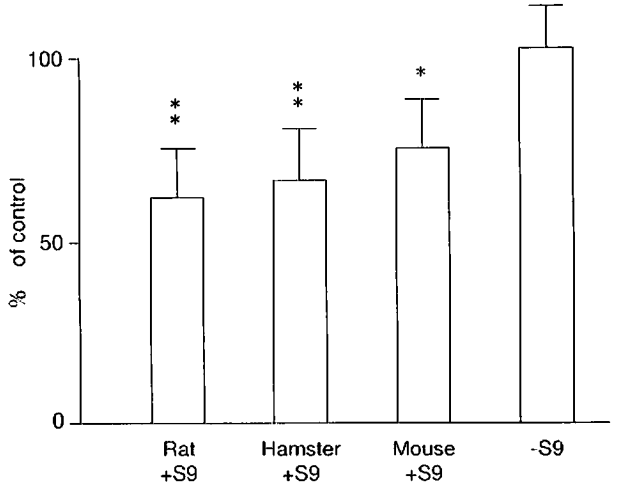

Fig. 4. Comparison of the effects of S9 mixes prepared using livers from rats, hamsters and mice that had been treated with phenobarbital and 5, 6-benzoflavone on the activity of BHT in the HEPM cell growth assay. BHT was tested at a concentration of 0.14 mM in cell culture medium, and significantly greater (Student's *t*-test) inhibition of cell growth was observed in the presence than in the absence of any S9 mix (*P<0.05; **P<0.01). Bars indicate S.D.

Discussion

Flint (1987)[19] suggested that as a general rule, IC_{50} values >50 μg/ml are indicative of teratogenic hazard. The IC_{50} values of BHT and BHTOH were <50 μg/ml in MB and LB cell differentiation assays (Figs. 1&2), and therefore, according to Flint's designation, BHT and BHTOH are unlikely to present a teratogenic hazard.

The metabolism of BHT has been investigated extensively in rabbits and rats[1,20], mice and humans[21]. The principal metabolic pathways of BHT in all species studied to date involve oxidation of the *para*-methyl and of one or both of the *tert*-butyl substituents. Oxidation of the methyl group is catalyzed by the microsomal enzyme BHT-oxidase[22], and several derivatives including the quinone-methide, 2, 6-di-*tert*-butyl-4-hydroxymethylphenol and BHTOH have been identified in rat liver.

Data further suggest that both the route and rate of metabolism are independent of dose over the range 0.5–500 mg/kg. The potential for BHT to accumulate in the tissues in rats is low and the retained material is rapidly eliminated when animals are returned to a control diet[1].

In our experiments, BHT was converted to more toxic compounds when the metabolic activation system was incorporated into the HEPM cell-growth assay (Fig. 3). Our results showed that S-9 mixes prepared from PB-BF-treated rats had a tendency to have higher activity than those prepared from untreated control rats (Table 1). Thus, BHT may be converted to more toxic compounds by the enzymes induced by both PB and BF, which are known to induce different forms of metabolizing enzymes.

S-9 mixes from rats, hamsters and mice were compared in the HEPM assay and no significant differences were observed between the three species (Fig. 4).

In vivo/*in vitro* test[15] was carried out at a dose of 1000 mg BHT/kg body weight administered orally on day 11 of gestation. The uteri were removed 16 hr later, and MB and LB cells were prepared from the embryos and cultured. In the cell differentiation assay, there were no differences between the BHT-treated and its control vehicle only (corn oil) groups. However, there were significant differences between the ETU-treated group and its control (distilled water) group. The dose of 100 mg/kg of ETU is clearly teratogenic in the pregnant rats[18], and the dose of 1000 mg/kg of BHT is equal to half of the LD50 value in rats[23].

After a single oral dose of ^{14}C-BHT, 80–90% of the administered radioactivity was excreted within 4 days in rats[1]. It was assumed that most of the BHT was readily detoxified by onjugation *in vivo*.

In conclusion, BHT up to 1000 mg/kg as a single oral dose under our experimental conditions was not harmful to rat embryos during organogenesis.

Received: July 5, 1994; Accepted: August 19, 1994

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