Bull. Environ. Contam. Toxicol. (2000) 64:66-73
© 2000 Springer-Verlag New York Inc.

Bulletin of Environmental Contamination and Toxicology

Micronucleus Test of Herbicide Terbutol and Its Metabolites in Cultured Chinese Hamster Lung Cells and Male CD-1 Mice

T. Suzuki,[1] Y. Nakagawa,[2] S. Tayama,[2] K. Yaguchi,[1] S. Suzuki,[1] T. Suga[3]

[1] Tama Branch Laboratory, Tokyo Metropolitan Research Laboratory of Public Health, 3-16-25, Shibazaki-cho, Tachikawa, Tokyo 190-0023, Japan
[2] Tokyo Metropolitan Research Laboratory of Public Health, 3-24-l) Hyakunin-cho, Shinjuku, Tokyo 169-0073, Japan
[3] Department of Clinical Biochemistry, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0355, Japan

Received: 25 August 1999/Accepted: 14 November 1999

Terbtuol, 2,6-di-*tert*-butyl-4-methylphenyl *N*-methylcarbamate, belongs to the phenylcarbamate herbicides and has been classified as a mitotic disrupter herbicide. The compound has been observed to disrupt mitotic microtuble organizing centers in plants, resulting in multipolar spindles and bifurcated pharagmoplasts (Hoffman and Vaugh 1994). Terbutol has been used at golf courses in Japan to control goose or crab grass. The herbicide is degraded by oxidation of 4-methyl group of benzene ring, oxidative demethylation at N-methyl group, and hydrolysis of carbamate linkage in turf grass soil at golf courses (Suzuki et al. 1996). The metabolites appeared in golf course drainage and groundwater at ppb or ppt levels and were more persistent than other pesticides used at golf courses (Suzuki *et al.* 1995a). Since there is little information on mutagenicity of terbutol and its metabolites in mammalian cells, this study was undertaken to investigate its effect on micronuclei in cultured Chinese hamster lung cells (CHL/IU) and micronucleated reticulocytes in male CD-l mice.

MATERIALS AND METHODS

Terbutol had a purity of > 99% and was obtained from GL Science (Tokyo, Japan). 2,6-Di-*tert*-butyl-4-hydroxymethylphenyl *N*-methylcarbamate (4-hydroxymethyl-terbutol), 2,6-di-*tert*-butyl-4-formylphenyl *N*-methylcarbamate (4-formyl-terbutol), 2,6-di-*tert*- butyl-4-carboxyphenyl *N*-methylcarbamate (4-carboxy-terbutol), 2,6-di-*tert*-butyl-4-methylphenyl carbamate *(N*-demethyl-terbutol), and

Correspondence to: T. Suzuki

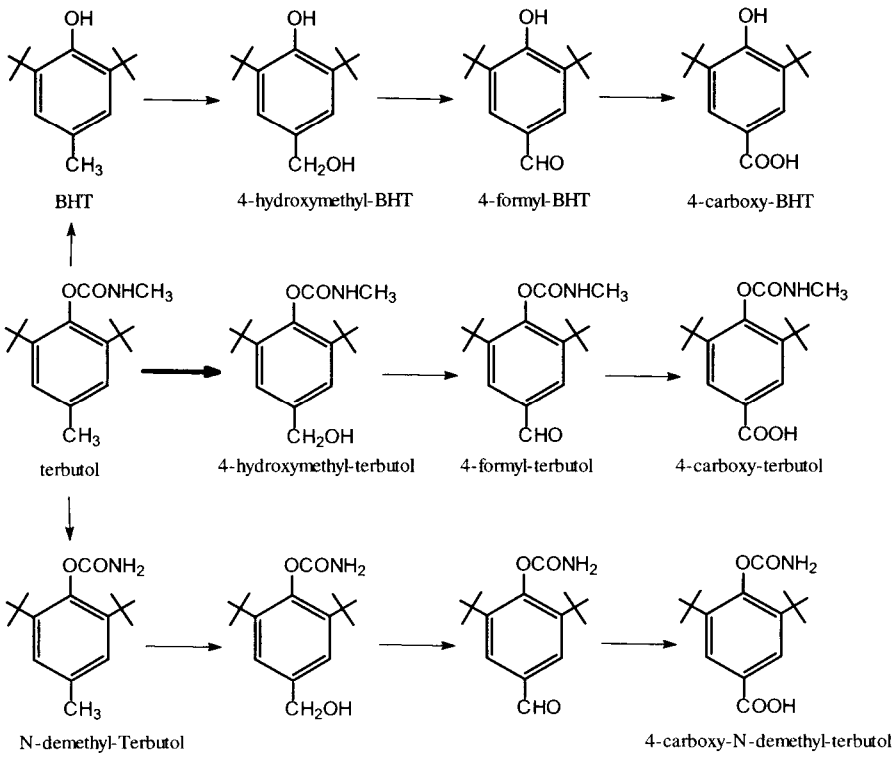

Figure 1. Proposed metabolic pathway of terbutol on isolated rat hepatocytes.

2,6-di-*tert*-butyl-4-carboxyphenyl carbamate (4-carboxy-*N*-demethyl-terbutol) were prepared using previous methods (Suzuki et al. 1995a), and these compounds were checked for purity (> 97%) using HPLC. 2,6-Di-*tert*-butyl-4-fromylphenol (4-formyl-BHT) was obtained from Tokyo Chemical Industry (Tokyo, Japan). These chemical structures are shown in Figure 1 (Suzuki et al. 1995b). Mitomycin C, corn oil, acridine orange, and dimethyl sulfoxide were obtained from Wako Pure Chemical (Osaka, Japan).

Cells lined from female Chinese hamster lung (CHL/IU) were obtained from Dainippon Seiyaku (Osaka, Japan) and were cultured in Eagle's minimum essential medium supplemented with 10 % heat-inactivated calf serum at 37 °C in an atmosphere of 5 % CO_2. The modal chromosome number was 25 and the doubling time under our experimental conditions was about 16 - 17 hr.

Approximately 1.0 x10^5cells diluted in 5 mL of culture medium were seeded in tissue culture dishes (50 mm i.d x 15 mm, NUNCRON ®, Nunc, Denmark). After about 24 hr, the compounds dissolved in DMSO were added to the medium, of which final concentration were 0.5 %, and incubated for 48 hr. Slide preparation and scoring of the micronuclei were performed according to Matsuoka *et al.* (1993). Cells were harvested with trypsin containing EDTA. After the hypotonic treatment with 0.075 M KCl solution for 10 min at room temperature, cells were fixed with cooled acetic acid : methanol (1:3, v/v) > 2 times. Finally, cells were suspended in methanol containing 1 % acetic acid, and the suspension was dropped with Pasteur pipette on a clean glass slide and air-dried. After staining with 40 µg/mL acridine orange solution, slides were observed by fluorescence microscopy using blue excitation at 1000 x magnification. The > 1000 cells were scored and categorized as follows; (A) normal cells, (B) cells with micronuclei, (C) polynuclear cells, and (D) mitotic cells (Matsuoka *et al.* 1993).

Male CD-l mice (4 weeks old) were purchased from Charles River Japan Inc. (Yokohama, Japan) and were housed at 25 ± 1 °C and relative humidity of 50 to 60 %. They were given commercial solid food CE-2 (Clea Japan Inc., Tokyo, Japan) and were given tap water ad libitum prior to experiments. At the age of 8 weeks, three 30-g mice were assigned at random to the different groups and were orally administered by gavage terbutol and its metabolites suspended in corn oil. Mitomycin C dissolved in phosphate-buffered saline was used for a positive control and administered intraperitoneally at a dose of 1 mg/kg. Micronucleated reticulocyte assay was performed according to the method of Hayashi *et al* (1990). From each of 3 mice in a group, about 5 µL of peripheral blood was collected from the tail vessel at 24-hr intervals from 24 to 72 hr after treatment and was placed on an acridine orange-coated glass slide. The number of micronucleated reticulocytes per 1000 reticulocytes was counted by fluorescent microscopy

The frequency of miclonuclei and micronucleated reticulocytes was analyzed using a binomial distribution, $\alpha = 0.05$. For those cases showing positive results, a one-tailed trend test combined with a one-tailed pairwise comparison of each dose

against the control was performed (Hayashi 1991).

RESULTS AND DISCUSSION

Incubation of cells with terbutol (27.7 to 277 µg/ml) caused a concentration-dependent induction (1.9 to 9.0 %) of micronuclei (Table 1). At concentrations of 29.3 and 146.5 µg/ml of 4-hydroxymethyl-terbutol, the mean frequencies of micronuclei were 1.3 and 1.7 %, respectively. In 4-formyl-terbutol-treated groups at doses of 2.9, 14.6, and 29.1 µg/ml, means micronuclei ranged from 1.7 - 6.8 %. 4-Carboxy-terbutol induced significant increases of micronuclei at doses of 30.7, 153.5, and 307 µg/ml. For *N*-demethyl-terbutol, mean frequencies were 1.1 - 1.6 %; significant at dose of 52.6 µg/ml. 4-Carboxy-*N*-demethyl-terbutol induced significant micronuclei at doses of 29.3, 146.5, and 293 µg/ml. These values for terbutol and its metabolites were relatively low compared to the micronuclei frequency induced by the positive control, mitomycin C. The micronuclei frequency in solvent control (DMSO) was 0.9 %. Among the test compounds, 4-formyl-terbutol induced micronuclei at the lowest concentrations. The frequencies of polynuclear cells induced by terbutol, 4-formyl-terbutol and *N*-demethyl-terbutol ranged from 2.2 - 14.3 %, 1.0 - 4.6 %, and 1.0 - 2.4 %, respectively (Table 1). For the other compounds, the frequencies of polynuclear cells were 0.5 - 2.4 %. The frequencies of mitotic cells were slightly increased by terbutol or its metabolites. For *N*-demethyl-terbutol, the frequencies of mitotic cells were decreased at higher concentrations.

Frequencies of micronucleated reticulocytes in the control group were below 0.3 %, and those of the positive control (mitomycin C) were significantly elevated at 48 hr (Table 2). The mean frequencies of micronucleated reticulocytes in 4-formyl-terbutol-treated groups ranged from 0.6 - 1.0 %. There was a significant dose-dependent increase of micronucleated reticulocytes frequencies from 50 to 1000 mg/kg at 48 hr. For the other compounds, there was no significant change at the dose of 1000 mg/kg. For the ratio of reticulocytes to 1000 normochromatic erythrocytes, control and MMC-treated mice were from 4.0 to 4.2% and from 2.1 to 2.5 %, respectively. Terbutol and its metabolites did not affected the frequency of

Table 1. Frequency of micronuclei induced in CHL/IU cells treated with terbubl and its metabolites for 48 hr.

compound	concentration	micronuclei cells[a]		polynuclear cells[b]	mitotic cells[b]	cell number[c]
	(μg/mL)	$\bar{X}$±SE	*P* value[#]	($\bar{X}$±SE)	($\bar{X}$±SE)	(x10^6 cells)
control (DMSO)	0.5%	9 ± 1	-	3 ± 1	12 ± 1	2.4 ±0.3
mitomycin C	0.05	377 ± 20 *	-	41 ± 2	27 ± 7	1.5 ±0.3
terbutol	27.7	19 ± 4*		22 ± 6	22 ± 5	2.2 ±0.4
	138.5	74 ± 14 *	<0.001	104 ± 9	17 ± 2	1.8 ±0.3
	277.0	90 ± 14 *		143 ± 24	25 ± 5	1.2 ±0.2
4-hydroxymethyl-terbutol	14.7	13 ± 1		5 ± 3	17 ± 3	2.2 ±0.4
	29.3	15 ± 3*	0.005	8 ± 2	17 ± 2	2.1 ±0.5
	146.5	17 ± 2*		14 ± 4	14 ± 7	1.3 ±0.5
4-formyl-terbutol	2.9	17 ± 3*		10 ± 1	16 ± 2	2.1 ±0.4
	14.6	25 ± 10*	<0.001	12 ± 5	19 ± 6	1.9 ±0.8
	29.1	68 ± 36 *		46 ± 34	21 ± 3	1.6 ±0.8
4-carboxy-terbutol	30.7	16 ± 2*		6 ± 2	17 ± 5	2.0 ±0.2
	153.5	15 ± 3*	0.002	8 ± 2	21 ± 3	2.0 ±0.1
	307.0	20 ± 1*		11 ± 3	18 ± 4	2.1 ±0.2
N-demethyl-terbutol	13.2	12 ± 1		10 ± 1	23 ± 2	2.3 ±0.2
	26.3	11 ± 1	0.012	13 ± 6	16 ± 2	2.0 ±0.5
	52.6	16 ± 2*		24 ± 2	8 ± 2	1.3 ±0.4
4-carboxy-*N*-demethyl-terbutol	29.3	12 ± 4		6 ± 3	21 ± 3	2.2 ±0.1
	146.5	22 ± 9*	<0.001	10 ± 4	21 ± 4	2.2 ±0.1
	293.0	29 ± 11*		22 ± 10	24 ± 5	2.0 ±0.4

[a] mean (± SEM) frequencies per 1000 cells in three separate experiments.

[b] number (± SEM) of polynuclear and mitotic cells appearing in the same microscopic field when scoring 1000 cells of normal and micronuclei cells.

[c] mean (± SEM) cell number of CHL/IU cells after 48 hr in three separate experiments.

* significantly different from concurrent control data at $\alpha = 0.05$ on binominal distribution.

[#] *p* value determined by one-tail trend test based on pooled control data.

reticulocytes (data not shown). Bone marrow toxicity did not appear by a single oral administration of terbutol and its metabolites at the dose of 1000 mg/kg.

The results obtained in this experiment indicate that terbutol and its metabolites cause induction of miclonuclei in CHL/IU cells. The order of micronuclei induction potency is 4-formyl-terbutol > terbutol > the others. In the present study, cytochalasin B which was applied to the *in-vitro* microlucei test to induce binucleate cells was not applied because it is better to eliminate co-treatment with other bioactive chemicals in the evaluation of chemical clastogenicity (Lindholm *et al.* 1991, Matsuoka *et al.* 1993). Treatment period of

Table 2. Frequency of micronucleated reticulocytes in peripheral blood of mice treated with terbutol and its metabolites.

compound [a]	dose (mg/kg)	micronucleated reticulocytes [b] 24hr [c]	48hr [c]	72hr [c]
Control (corn oil)	-	2.5 ± 0.8	2.5 ± 0.8	2.3 ± 0.6
mitomycin C	1	13.0 ± 2.6 *	26.2 ± 7.4 *	10.4 ± 3.9 *
terbutol	1000	1.3 ± 0.5	3.0 ± 1.0	1.3 ± 0.5
4-hydroxymethyl-terbutol	1000	3.5 ± 1.1	3.2 ± 1.1	3.2 ± 0.5
4-formyl-terbutol	1000	4.3 ± 0.6	9.5 ± 0.5 *	6.2 ± 0.7 *
	500	4.4 ± 1.1	10.3 ± 0.6 *	4.5 ± 0.6
	200	7.0 ± 1.5 *	7.0 ± 1.4 *	2.0 ± 0.8
	100	7.0 ± 1.2 *	8.1 ± 2.3 *	4.2 ± 1.1
	50	4.6 ± 1.5	6.0 ± 1.2 *	2.7 ± 1.5
	10	4.2 ± 0.8	3.9 ± 0.9	2.6 ± 1.0
p value [#]		0.015	<0.001	0.021
4-carboxy-terbutol	1000	1.9 ± 0.1	2.3 ± 1.5	3.2 ± 1.9
N-demethyl-terbutol	1000	3.5 ± 1.2	3.7 ± 0.9	3.2 ± 0.6
4-carboxy-*N*-demethyl-terbutol	1000	4.1 ± 1.0	4.9 ± 0.9	2.9 ± 1.0
BHT-CHO	1000	3.9 ± 1.0	2.6 ± 1.1	2.5 ± 0.5

[a] mitomycin C, intraperitoneal injection; the others, oral gavage.

[b] mean (± SEM) frequencies of three mice; 1000 reticulocytes scored per animal.

[c] time after oral administration of compounds.

* significantly different from concurrent control data at $\alpha = 0.05$ on binominal distribution.

[#] *p* value determined by one-tail trend test based on pooled animal data.

the test chemicals is important factor in the *in-vitro* micronuclei test. Cells were continuously treated for 48 hr with the test compounds because some micronuclei remained in the cells for several cell divisions at least, and the accumulation of micronucleated cells occurred (Matsuoka *et al.* 1993). Increase of micronuclei frequency for terbutol and its metabolites is weak compared to other carbamate insecticides such as propoxur, methomyl, and aldicarb, which cause induction of micronuclei in Chinese hamster ovary (CHO) cells and micronucleated reticulocytes in BALB/c mice (Wei *et al.* 1997). The mechanism of terbutol-induced polynuclear cells is not clear. The order of inhibition of CHL/IU cell proliferation is 4-formyl-terbutol, *N*-demethyl-terbutol > terbutol > the others. Cytotoxic effects on rat hepatocytes indicated that terbutol and its metabolites, especially *N*-

demethyl-terbutol, resulted in cell death accompanied by loss of intracellular ATP, protein thiols, and glutathione (Suzuki et al. 1997). Among mitotic disrupter herbicides, terbutol has a unique mechanism of action, where star anaphase figures were observed in onion root tips after terbutol treatment (Lelmen *et al.* 1992). Although terbutol, 4-hydroxymethl-terbutol did not increase the number of micronucleated reticulocytes in male CD-1 mice at the dose of 1000 mg/kg, 4-formyl-terbutol significantly induced micronucleated reticulocytes. 4-Formyl-BHT, which is a hydrolysis product of 4-formyl-terbutol, did not increase micronucleated reticulocytes at the dose of 1000 mg/kg. A previous study (Suzuki *et al.* 1995b) has shown that oxidation of 4-methyl group of terbutol is a major metabolite pathway in rat hepatocytes resulting in the formation of the major metabolite 4-hydroxymethyl-terbutol. Because the oxidation of hydroxymethyl group at 4-position of benzene ring is a rate-limiting step in liver, it might be reason able to assume that both terbutol and 4-hydroxymethyl-terbutol are non-genotoxic in male CD-1 mice. Negative genotoxicity for tertutol was obtained using Ames test with *Salmonella typhimurium,* chromosomal aberration with CHO-10B4 cells, and DNA repair test with *Bacillus subtilis* (Dainippon Ink & Chemicals Inc. 1992). Teratogenicity of tertutol was also negative using rats and rabbits (Dainippon Ink & Chemicals Inc. 1992). Although this *in-vivo* experiments with male CD-1 mice were preliminary, the results of 4-formyl-terbutol were dose-dependent and reproducible. The concentration of 4-formyl-terbutol was more than ppt levels in golf course drainage and ground water after terbutol application (Suzuki *et al.* 1995a). Further examination for administration route and dose, and treatment times will be necessary to estimate genotoxicity of terbutol and its degradation products.

Acknowledgments. We thank Dr. A. Matusoka and M. Hayashi (NIHS, Tokyo) for technical advice.

REFERENCES

Dainippon Ink & Chemicals Inc (1992) Review for toxicity tests of MBPMC. Nouyaku Jihou, separate volume 30-33

Hayashi M, Morita T, Kodama Y, Sofuni T, Ishidate M Jr (1990) The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. Mut Res 245 : 245-249

Hayashi M (1991) The micronucleus test. Scientific Inc., Tokyo

Hoffman JC, Vaugh KC (1994) Mitotic disrupter herbicides act by a single mechanism but vary in efficacy Protoplasma 179 : 16-25

Lehnen LP, Vaughan LC, Vaughan KC (1992) Terbutol affects spindle microtuble organizing centers. J Exp Bot 41 : 537-546

Lindholm C, Norppa, H, Hayashi M, Sorsa M (1991) Induction of micronuclei and anaphase aberrations by cytochalasin B in human lymphocyte cultures, Mut Res 260, 369-375.

Matsuoka A, Yamazaki N, Suzuki T, Hayashi M, Sofuni T (1993) Evaluation of the micronucleus test using Chinese hamster cell line as an alternative to the conventional in vitro chromosomal aberration test. Mut Res 272 : 223-236

Suzuki T, Yaguchi K, Ohnishi K, Suga T (1995a) Identification of degradation products of terbutol in environmental water from golf courses. J Agric Food Chem 43 : 1712-1717

Suzuki T, Yaguchi K, Nakagawa Y, Suga T (1995b) Metabolism of 3,5-di-*t*-butyl-4-methylphenyl *N*-methylcarbamate, terbucarb, on isolated rat hepatocytes. Bull Environ Contam Toxicol 54 : 737-744

Suzuki T, Yaguchi K, Nishima T, Suga T (1996) Degradation of terbutol in soils from turfgrass and drainage basins on golf courses. J Agric Food Chem 44 : 1599-1602

Suzuki T, Yaguchi K, Suga T, Nakagawa Y (1997) Cytotoxic effects of 2,6-di-*tert*-butyl-4-methylphenyl *N*-methylcarbamate (terbutol) herbicide on hepatocytes and mitochondria isolated from male rat. Environ Toxicol Pharmacol 3 : 167-173

Wei LY, Chao JS, Hong CC (1997) Assessment of the ability of propoxur, methomyl, and aldicarb, three carbamate insecticides, to induce micronuclei in vitro cultured Chinese hamster ovary cells and in vivo in BALB/c mice. Environ Mol Mut 29 : 386-393