Bull, Natl. Inst. Health Sci., **129**, 68-75（2011） **Original**

A chemical category approach of genotoxicity studies for branched alkylphenols

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A chemical category is a group of chemicals whose toxicological properties are expected to be similar or follow a regular pattern as a result of structural similarity. The category approach is beneficial for decreasing in the resource of risk assessment for huge amount of unevaluated existing chemicals, and also in the use of all kinds of animal tests including even *in vivo* genotoxicity tests from a point of view of the animal welfare. The present paper reports the results of *in vivo* micronucleus tests of *o-sec*-butylphenol（CAS：89-72-5）and 2-isopropyl-5-methylphenol（CAS：89-83-8）and discusses genotoxic potential of seven alkylphenols, *o-sec*-butylphenol, 2-isopropyl-5-methylphenol, *p-sec*-butylphenol（CAS：99-71-8）, 2-*tert*-butylphenol（CAS：88-18-6）, 2, 4-di-*tert*-butylphenol（CAS：96-76-4）, 4-*tert*-butylphenol（CAS：98-54-4）and 6-*tert*-butyl-*m*-cresole（CAS：88-60-8）by the category approach. Based on the negative results of *in vivo* micronucleus tests, it can be concluded that these category chemicals are not likely clastogenic *in vivo*. Further *in vivo* micronucleus assays on untested substances may not be required by using the category approach, but further supporting information such as physicochemical profiles and（Q）SAR predictions may be necessary to strengthen the rationale for the category approach.

Keywords：category approach, alkylphenol, genotoxicity

Introduction

A chemical category is a group of chemicals whose toxicological properties are expected to be similar or follow a regular pattern as a result of structural similarity. A category approach is used in many chemical programmes such as the OECD High Production Volume（HPV）programme[1], the US HPV Challenge programme[2] and the EU Existing Substances programme[3]. The overall data set can allow the estimation of the hazard for the untested endpoints. Data gap filing can be done from one or more tested chemicals to an untested chemical. The category approach is effective for hazard identification and hazard estimation, and it is beneficial for decreasing in the resource of risk assessment for huge amount of unevaluated existing chemicals, and also in the use of all kinds of animal tests including even *in vivo* genotoxicity tests from a point of view of the animal welfare.

Structurally similar alkylphenols shown in Table1 are listed in the most recent OECD HPV List of chemicals to be investigated for environment and human health effects[4] and were selected as target substances for the Safety Examination of Existing Chemicals in Japan in order to obtain reliable information in compliance with the OECD Test Guidelines and in accordance with the principles for GLP[5]. Of these chemicals, 4-*tert*-butylphenol（CAS：98-54-4）and 6-*tert*-butyl-*m*-cresole（CAS：88-60-8）were already assessed under the OECD HPV programme[6]. In the OECD HPV programme, screening information data sets（SIDS）for at least two different genotoxic endpoints have been required for the initial assessment[7], and the Ames assays and *in vitro* chromosome aberration assays for these chemicals were performed.

Table 1 shows summary results of genotoxicity studies of the branched alkylphenols. All the chemicals showed negative results in the Ames assays with and

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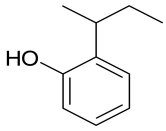
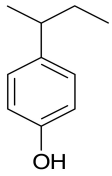
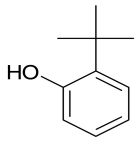
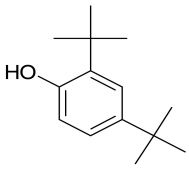
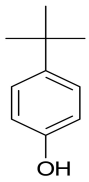
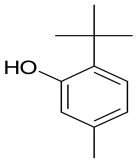
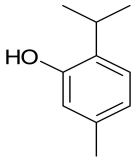
Table 1 Summary results of genotoxicity studies of branched alkylphenols conducted under the Safety Examination of Existing Chemicals in Japan

Substance name (CAS No.)	Structure	S9	Ames	In vitro Chromosome Aberration		In vivo micronucleus
				[C] [a]	[P] [b]	
o-sec-Butylphenol (89-72-5)		w/o	−	+	−	[current study]
		with	−	+	−	
p-sec-Butylphenol (99-71-8)		w/o	−	±	−	
		with	−	±	−	
2-*tert*-Butylphenol (88-18-6)		w/o	−	−	−	− [e]
		with	−	+	+	
2,4-di-*tert*-Butylphenol (96-76-4)		w/o	−	−	−	
		with	−	+	−	
4-*tert*-Butylphenol [c] (98-54-4)		w/o	−	−	+	−
		with	−	+	+	
6-*tert*-Butyl-*m*-cresole [c] (88-60-8)		w/o	−	−	−	− [d]
		with	−	+	−	
2-Isopropyl-5-methylphenol (89-83-8)		w/o	−	−	−	[current study]
		with	−	+	−	

[a] [C] : Clastogenicity ; [b] [P] : Polyploidy ; [c] The initial assessment of the chemical was already assessed under the OECD HPV programme ; [d] The in vivo micronucleus test was carried out by the Chemicals Evaluation and Research Institute, Japan. ; [e] The result form the German Chemical Society-Advisory Committee on Existing Chemicals of Environmental Relevance (Beratergremium fur Umweltrevante Alstoffe : BUA) [21]

without metabolic activation. On the other hand, an equivocal result on *p-sec*-butylphenol (CAS : 99-71-8) and positive results on the other six chemicals were observed for clastogenicity in the *in vitro* chromosome aberration assays with and/or without metabolic activation. Polyploidy was also observed for 2-*tert*-butylphenol (CAS : 88-18-6) and 4-*tert*-butylphenol with and/or without metabolic activation. However, *in vivo* micronucleus tests on 4-*tert*-butylphenol (not publically available) and 6-*tert*-butyl-*m*-cresole[6] showed negative results, and these findings seem to suggest that these branched alkylphenols can be non-genotoxic *in vivo* although *in*

vitro genotoxicity is equivocal.

These branched alkylphenols are widely used as antioxidants in rubbers, plastics, foods and oils to inhibit or slow oxidative process, and they are also used as intermediates for synthesis of resins, plasticizers, surface-active agents, perfumes and other products[8]. Consumer exposure to these branched alkylphenols can occur through the use of products containing these chemicals. The similarities in structure, use and *in vitro* genotoxicity seem to support grouping these chemicals into one category. To ascertain robustness of the chemical category of these branched alkylphenols on genotoxicity, additional *in vivo* micronucleus tests were assessed on 2-isopropyl-5-methylphenol（CAS：89-83-8）as a target of isopropyl substitutions and *o-sec*-butylphenol（CAS：89-72-5）as a target of *sec*-butyl substitutions. The present paper reports the results of *in vivo* micronucleus tests of 2-isopropyl-5-methylphenol and *o-sec*-butylphenol and discusses genotoxic potential of these chemicals by the category approach.

Materials and Methods

In vivo micronucleus test in mouse bone marrow

The test was performed according to the Guideline for Screening Mutagenicity Testing of Chemicals, Japan and OECD TG 474[9] and in compliance with GLP requirements[5]. 2-Isopropyl-5-methylphenol（Purity> 98%；Lot No.CAN1119）was obtained from Wako Pure Chemical Industries, Ltd., Japan and cyclophosphamide（CAS No.50-18-0；Lot No.73H0846）obtained from Sigma Chemical Co. was used as a positive control. Crj：BDF1 mice, 8-weeks of age, from Charles River Laboratories, Japan were used after more than 1 week acclimatization. Mice were housed in a temperature-and humidity-controlled room（23±1℃；55±5%）with a light-dark（12 h-12 h）cycle. In a dose finding study, 5 male and 5 female mice were singly given 2-isopropyl-5-methylphenol by gavage at 500, 750, 1000, 1250, 1750 or 2000 mg/kg bw, in which deaths were observed at 1500 and 1750 mg/kg bw in females while no death was observed in males for four days（data not shown）. Subsequently, a single dose of 2-isopropyl-5-methylphenol at 1500-2000 mg/kg bw caused deaths in males in a preliminary study（data not shown）；therefore, 1250 mg/kg bw was set as the highest dose in the main test. Sampling time was set at 24 h after administration according to the preliminary study, in which no differences were observed in a sampling time of 24, 48, or 72 h.

Mice（5/sex/dose）were received single oral gavage administration of 2-isopropyl-5-methylphenol at 0（control：olive oil）, 156.3, 312.5, 625, or 1250 mg/kg bw. Positive control mice（5/sex）received single oral doses of cyclophosphamide at 50 mg/kg bw. All groups of mice were killed 24 h after treatment. Bone marrow samples were prepared according to the method of Schmid[10,11] for the control, 312.5, 625, or 1250 mg/kg bw groups. Samples were stained with 0.04 mg/mL acridine orange. According to the method of Hayashi et al.[12], the incidence of micronuclei was determined. Two thousand polychromatic erythrocytes（PCE）for each animal were observed for the incidence of micronucleated erythrocytes, and the proportion of PCE among the total erythrocyte population was also determined from a sample of 500 total erythrocytes for each animal.

Data were analyzed using the Fisher's exact test with Bonferroni correction for group mean comparisons[13]. Dose-dependent increases of the number of micronucleated polychromatic erythrocyte per total number of PCE（MNPCE）was detected using the Cochran-Armitage test[14]. Proportion of PCE among the total erythrocyte population was analyzed by the t-test with Bonferroni correction.

In vivo micronucleus test in rat bone marrow

The test was performed according to OECD TG 474[9] and Guideline for Genotoxicity Tests on Drugs[15], and in compliance with GLP requirements[5]. *o-sec*-Butylphenol（Purity 99.15%）was obtained from Honshu Chemical Industry, Japan and cyclophosphamide monohydrate（CAS No.6055-199-2；Lot No.036K1225）obtained from Sigma-Aldrich Co. was used as a positive control. Crl：CD（SD）rats, 7-weeks of age, from Charles River Laboratories, Japan were used after one week of acclimatization. Rats were housed in a temperature-and humidity-controlled room（21.8-22.9℃；46.6-62.2%）with a light-dark（12 h-12 h）cycle. The animals were given commercial food and water *ad libitum*. In a dose finding study, 3 male and 3 female rats were given *o-sec*-butylphenol by gavage at 150, 300, 600, 1200 mg/kg bw once a day for two days（24 h interval）, in which deaths were observed at 1200 mg/kg bw/day and clinical changes were observed at 600 mg/kg bw/day in both sex（data not shown）. Therefore, 600 mg/kg bw was set as the highest dose.

Table 2 Results of the micronucleus test in mice after gavage dose of 2-isopropyl-5-methylphenol (CAS: 89-83-8)

Dose	Number of mice	MNPCE (%)[a]	PCE/ (PCE+NCE) (%)[b]
Male			
0 mg/kg (Solvent control: olive oil)	5	0.12 ± 0.08[c]	48.6 ± 8.6
312.5 mg/kg	5	0.20 ± 0.10	55.7 ± 5.4
625 mg/kg	5	0.19 ± 0.16	48.2 ± 12.3
1250 mg/kg	5	0.15 ± 0.12	53.6 ± 10.5
50 mg/kg (Positive control: CP)	5	1.57 ± 0.70*	45.6 ± 13.1
Female			
0 mg/kg (Solvent control: olive oil)	5	0.17 ± 0.14	63.8 ± 4.8
312.5 mg/kg	5	0.14 ± 0.07	60.6 ± 8.0
625 mg/kg	5	0.15 ± 0.09	62.8 ± 4.8
1250 mg/kg	5	0.11 ± 0.04	64.2 ± 8.2
50 mg/kg (Positive control: CP)	5	1.43 ± 0.35*	54.9 ± 6.2

PCE: Polychromatic erythrocyte, MNPCE: Micronucleated PCE, NCE: Normochromatic erythrocyte, CP: Cyclophosphamide
*: Significantly different from the solvent control ($P<0.01$)
[a]: Number of micronucleated polychromatic erythrocytes/ total number of polychromatic erythrocytes observed.
[b]: Number of polychromatic erythrocytes/ total number of erythrocytes observed.
[c]: Values are given as mean ± S. D.

All rats were weighed prior to dosing and preparation of bone marrow samples. Clinical signs of toxicity were observed at 1 and 3 h after treatment, and prior to dosing and preparation of bone marrow samples. Rats (5/sex/dose) were received oral gavage administration of *o-sec*-butylphenol twice with 24 h intervals at 0 (control: corn oil), 75, 150, 300 or 600 mg/kg bw. Positive control rats (5/sex) received two oral doses of cyclophosphamide (24 h intervals) at 20 mg/kg bw/ day.

All groups of rats were killed 24 h after last treatment. One femur was removed from each rat, and bone marrow cells were flushed out with 10% neutral buffer formalin. Excess serum was removed by centrifugation. Bone marrow samples were stained with 0.05 w/v% acridine orange. According to the method of Hayashi et al.[12], the incidence of micronuclei was determined. Two thousand PCE for each animal were observed for the incidence of micronucleated erythrocytes, and the proportion of PCE among the total erythrocyte population was also determined from a sample of 1000 total erythrocytes for each animal.

Data were analyzed using the Kastenbaum and Bowman's method[16] for group mean comparisons. Dose-dependent increases of the MNPCE were detected using the Cochran-Armitage test[17]. Body weight and proportion of PCE among the total erythrocyte population were analyzed by the MiTOX® (Mitsui Engineering & Shipbuilding Co., Ltd).

Results

In vivo micronucleus test in mouse bone marrow after gavage dose

Table 2 shows a result of the micronucleus test in mice after gavage doses of 2-isopropyl-5-methylphenol. There were no deaths at any doses of 2-isopropyl-5-methylphenol although signs of toxicity were observed at 1250 mg/kg bw. A frequency of MNPCE was not significantly increased in males and females up to the dose of 1250 mg/kg bw while a frequency of MNPCE was significantly increased in the positive controls in both sexes. Proportion of PCE among the total erythrocyte populations was not changed in any dosing groups.

In vivo micronucleus test in rat bone marrow after gavage dose

Table 3 shows a result of the micronucleus test in rats after gavage doses of *o-sec*-butylphenol. One male showed diarrhea, and two males showed ataxic gait and a decrease in locomotor activity at 600 mg/kg bw/day. Four females showed ataxic gait and three of them also showed a decrease in locomotor activity at 600 mg/kg bw/day. One female in the 300 mg/kg bw/day group died before the sampling due to the incorrect administration. Body weights were not statistically changed in both sexes at any doses. A frequency of MNPCE was not changed in females at any doses. On the other hand, gavage dose of *o-sec*-butylphenol significantly increased a frequency of MNPCE compared to the solvent control

Table 3 Results of the micronucleus test in rats after gavage dose of *o-sec*-butylphenol（CAS：89-72-5）

Dose	Number of mice	MNPCE（%）[a]	PCE/（PCE+NCE）（%）[b]
Male			
0 mg/kg（Solvent control：corn oil	5	0.06 ± 0.08[c]	51.0 ± 5.2
150 mg/kg	5	0.10 ± 0.05	54.7 ± 3.4
300 mg/kg	5	0.14 ± 0.09	52.9 ± 5.1
600 mg/kg	5	0.20 ± 0.05*[d]	56.6 ± 1.4
20 mg/kg（Positive control：CP）	5	5.45 ± 1.25*	44.7 ± 5.1
Female			
0 mg/kg（Solvent control：corn oil）	5	0.11 ± 0.05	55.0 ± 5.7
150 mg/kg	5	0.13 ± 0.08	57.0 ± 3.5
300 mg/kg	4[e]	0.10 ± 0.07	53.8 ± 5.8
600 mg/kg	5	0.11 ± 0.04	52.7 ± 3.0
20 mg/kg（Positive control：CP）	5	3.19 ± 1.30*	25.8 ± 4.0*

PCE：Polychromatic erythrocytes, MNPCE：Micronucleated PCE, NCE：Normochromatic erythrocyte, CP：Cyclophosphamide
*：Significantly different from the solvent control（$P<0.05$）
[a]：Number of micronucleated polychromatic erythrocytes/ total number of polychromatic erythrocytes observed.
[b]：Number of polychromatic erythrocytes/ total number of erythrocytes observed.
[C]：Values are given as mean ± S. D.
[d]：The frequency of MNPCE（0.20 ± 0.05%）was within background control data from 2001 to 2007 of the laboratory（Mean ± 3SD=0.13 ± 0.24%；n=449）.
[e]：One female in the 300 mg/kg bw/day group died before the sampling due to the incorrect administration.

at 600 mg/kg bw/day in males. Proportion of PCE among the total erythrocyte populations was not changed.

Discussion

Equivocal results on *in vitro* genotoxicity of branched alkylphenols were obtained in the previous studies. Müller and Sofuni[18] indicated that some chemicals produce chromosome aberration *in vitro* but do not produce positive results in Ames assays. The clastogenic response of such chemicals is often associated with high cytotoxicity[19], high osmolality and pH extremes[20]. There are also chemicals that show positive results in the *in vitro* chromosome aberration tests but negative in the rodent micronucleus tests. The numerical proportions of positive results in the Ames assays, *in vitro* chromosome aberration assays and *in vivo* micronucleus assays were reported to be 7.7%（23/298）, 28.9%（77/266）and 6.7%（19/283）, respectively in pharmaceutical chemicals[18]. To ascertain if genotoxic potential of branched alkylphenols can be expressed in animals, additional *in vivo* micronucleus tests were performed on 2-isopropyl-5-methylphenol and *o-sec*-butylphenol.

After gavage doses of 2-isopropyl-5-methylphenol, a frequency of MNPCE was not significantly increased in males and females up to 1250 mg/kg bw while a frequency of MNPCE was significantly increased in the positive controls in both sexes. Proportion of PCE among the total erythrocyte populations was not changed；indicating inhibition of bone marrow cell proliferation was not induced under the test conditions. These results indicate that 2-isopropyl-5-methylphenol does not induce genotoxic effects *in vivo*.

After gavage doses of *o-sec*-butylphenol, a frequency of MNPCE was not changed in females at any doses. In contrast, dose of *o-sec*-butylphenol significantly increased a frequency of MNPCE compared to the solvent control at 600 mg/kg bw/day in males. However, the frequency of MNPCE（0.20 ± 0.05%）was within background control data from 2001 to 2007 of the laboratory（Mean ± 3SD=0.13 ± 0.24%；n=449）. Therefore, the increase in MNPCE was considered to be due to low MNPCE in the control group. Proportion of PCE among the total erythrocyte populations was not changed；indicating inhibition of bone marrow cell proliferation was not induced under the test conditions. These results indicate that *o-sec*-butylphenol does not induce genotoxic effects *in vivo*.

The previous assessments under the HPV programme also showed that gavage doses of 6-*tert*-butyl-m-cresole up to 125 mg/kg bw, the maximum tolerated dose, did not induce micronucleus in bone marrow cells nor suppress their proliferation in ICR mice[6], and 4-*tert*-butylphenol did not induce micronucleus in bone

Table 4 The category approach on genotoxicity of alkylphenols

Substance name	Molecular weight	Log Kow[a)]	Ames	In vitro Chromosome Aberration	In vivo micronucleus
o-sec-Butylphenol	150.22	3.27	negative	positive	negative (current study)
p-sec-Butylphenol	150.22	3.08	negative	equivocal	negative (read across)
2-*tert*-Butylphenol	150.22	3.31	negative	positive	negative[b)]
2, 4-di-*tert*-Butylphenol	206.32	5.19	negative	positive	negative (read across)
4-*tert*-Butylphenol	150.22	2.4-3.4	negative	positive	negative
6-*tert*-Butyl-*m*-cresole	164.24	4.11	negative	positive	negative[c)]
2-Isopropyl-5-methylphenol	150.22	3.3	negative	positive	negative (current study)

[a)]Data from NITE (2010)[31)] and OECD (2010)[6)].
[b)]Data from BUA (2003)[21)].
[c)]Data from OECD (2010)[6)].

marrow cells nor suppress their proliferation at up to the maximum tolerated dose of 50 mg/kg bw in ICR mice (not publically available). In addition, the German Chemical Society-Advisory Committee on Existing Chemicals of Environmental Relevance (Beratergremium fur Umweltrevante Alstoffe: BUA) also stated that 2-*tert*-butylphenol does not induce any micronuclei in the bone marrow of mice at toxic dosages *in vivo*, while it is non-mutagenic in bacteria but is clastogenic *in vitro* in mammalian cells[21)].

There are numerous reasons why activity shown *in vitro* may not be observed *in vivo*; for example, lack of absorption, inability of the active metabolite to reach DNA, rapid detoxication and elimination[22)]. There are only a few data available on toxicokinetics for the whole body of these branched alkylphenols, but no direct information in the target cells of bone marrow. 4-*tert*-Butylphenol was rapidly excreted as glucouronide and sulfate conjugates in urine and feces in rats[23, 24)]. In workers handling 4-*tert*-butylphenol, most of the chemical was excreted within 24 hours, and metabolites in the urine was correlated with exposure levels of the chemical[25)]. 2-Isopropyl-5-methylphenol is readily absorbed from the intestine and excreted rapidly as glucouronide and sulfate conjugates in humans, dogs, rabbits and rats[26-28)]. After a single dose of 2-isopropyl-5-methylphenol, peak plasma concentrations were reached after 2 hours and eliminations half-life was 10.2 hours in humans. Sulphate and glucronide conjugates of 2-isopropyl-5-methylphenol, but not free 2-isopropyl-5-methylphenol, were corrected in urine.

The physicochemical properties and chemical structure can be used to make some predictions regarding the ADME of substances. A range of Log Kow of these category chemicals is 2.4-5.19 (Table 4), which suggests that the substances could readily absorbed and distributed in physiological fluids[29)]. The alkylphenols are expected to have slightly higher acid dissociation constants (pKa) than phenol (pKa 10.0 at 25℃); therefore, will not be ionized significantly at physiological pH's[30)]. Alkylphenols which contains phenol moieties are likely to undergo Phase II conjugation and systemic exposure to unchanged substance may be limited[29)]. Based on available data, the rapid conjugation and excretion of these chemicals may explain why genotoxicity was not observed *in vivo* although *in vitro* clastogenicity was increased with S9 mix. However, there is a possibility that active metabolites did not reach the target cells of born marrow at high concentration and could react to chromosomes in hepatic cells. An *in vivo* genotoxic assay for hepatic cells may be useful for further evaluation.

In the present paper, we showed that 2-isopropyl-5-methylphenol and *o-sec*-butylphenol were not clastogenic *in vivo* under the test conditions, and existing data also showed that 6-*tert*-butyl-*m*-cresole, 4-*tert*-butylphenol and 2-*tert*-butylphenol were not clastogenic *in vivo*[6, 21)]. Based on the weight of evidence, it can be concluded that these branched alkylphenols are not genotoxic *in vivo* (Table 4). The use of the category approach is useful to identify common or trend properties of members of the category and to use measured data to similar untested chemicals without further testing to fill data gap. In conclusion, further *in vivo* micronucleus assays on *p-sec*-butylphenol and 2,4-di-*tert*-butylphenol may not be required by using the category approach, but further supporting information such as physicochemical profiles and (Q)

SAR predictions may be necessary to strengthen the rationale for the category approach.

Acknowledgements

The studies were supported by the Ministry of Health, Labour and Welfare, Japan. The *in vivo* micronucleus tests were performed at the Hatano Research Institute, Food and Drug Safety Center, Japan for 2-isopropyl-5-methylphenol and Mitsubishi Chemical Safety Institute, Japan for *o-sec*-butylphenol.

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