In Vitro Transformation of Chlorinated Parabens by the Liver S9 Fraction: Kinetics, Metabolite Identification, and Aryl Hydrocarbon Receptor Agonist Activity

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We investigated the kinetics of in vitro transformation of a dichlorinated propyl paraben (2-propyl 3,5-dichloro-4-hydroxybenzoate; Cl2PP) by the rat liver S9 fraction and assessed the aryl hydrocarbon receptor (AhR) agonist activity of the metabolite products identified in HPLC and GC/MS analysis and by metabolite syntheses. The results indicated that the chlorination of Cl2PP reduced its degradation rate by approximately 40-fold. Two hydroxylated metabolite products showed AhR agonist activity of up to 39% of that of the parent Cl2PP when assessed in a yeast (YCM3) reporter gene assay. The determination of the metabolic properties of paraben bioaccumulation presented here provides further information on the value of risk assessments of chlorinated parabens as a means to ensure human health and environmental safety.

Key words preservative; disinfection by-product; metabolic reaction; standard chemical synthesis; aryl hydrocarbon receptor

Alkyl esters of 4-hydroxybenzoic acid (parabens) are widely used as preservatives in pharmaceuticals and personal care products, because they are stable over a wide pH range, are sufficiently soluble in water, and also exhibit a broad spectrum of antimicrobial activity.1 In Japan, parabens are permitted for use in all cosmetic products at concentrations up to 1% (w/w).

Compounds, including parabens, that contain phenolic hydroxyl groups exhibit favorable chlorination kinetics; thus, parabens are easily chlorinated by chlorine in tap water and by sodium hypochlorite, a commonly used bleaching agent.2 In recent years, the increasing use of parabens has led to concerns related to the potential for adverse effects associated with their widespread occurrence in the environment. Several monitoring studies have now detected chlorinated parabens in effluents, pool water, and even river water.3,4

Organochlorine compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polychlorinated biphenyls (PCBs) are hydrophobic and persist in the environment for significant periods of time. This exposure and persistence may adversely affect humans and other organisms. The toxicity of TCDD and PCBs is associated with their interaction with the aryl hydrocarbon receptor (AhR), which is a ligand-dependent transcription factor.5 Activation of AhR by TCDD and dioxin-like compounds can induce various biological responses, including activation of the CYPs system (particularly CYP1A1) and disruption of normal hormone signaling pathways, and can lead to developmental and reproductive toxicities, immunotoxicity, and mutagenicity.6 Recently, chlorinated parabens were suggested to affect toxicity through interaction with AhR.7 Parabens are hydrolyzed mainly to 4-hydroxybenzoic acid in mammals; this metabolite can thereafter be conjugated with sulfate, glucuronide, or glycine, prior to excretion in urine.8 However, not all metabolite products and metabolic reactions that can arise from paraben exposure in mammals have been chemically determined. Moreover, the biological activity especially the endocrine disrupting properties, of these metabolites, are most often not known. The main reason for this is the lack of chemical standards to support identification of all metabolite products arising from bio-transformations of parabens.

The aim of the present study was to carry out an assessment of the metabolic kinetics of in vitro transformation of chlorinated paraben by a rat liver S9 fraction and to identify associated metabolite products and their toxicities. AhR agonists only need to meet minimal requirements in hydrophobicity, size, and planar shape to fit into the receptor-binding pocket to induced endocrine disrupting properties.8,9 Here, we focused particularly on paraben metabolites containing a phenolic hydroxyl group. AhR agonist activity was measured using a yeast reporter gene assay. For our model compound, we selected the dichlorinated derivative (Cl2PP) of propyl paraben, the most frequently used paraben in pharmaceuticals and personal care products (Fig. 1). Cl2PP has been detected at levels up to 0.18nm in pool and river water in Japan.3,7

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Cl2PP shows AhR agonist activity (EC_{25}=1300 nM) and more acute toxicity toward invertebrates (EC_{50}=33 µM) than that by the corresponding parent paraben. 7,11) Cl2PP is a common and toxic paraben in the water environment and it is therefore important to evaluate its metabolic fate.

Results and Discussion

Kinetics

To investigate the degradation rate of propyl paraben and its chlorinated derivative ln(C/C_0) values were plotted against reaction time, as shown in Fig. 2. Correlation coefficients (r^2) were 0.864 and 0.915, respectively. From these values, it was established that the metabolic reaction for the paraben and its chlorinated derivative is first order. The rate constant k and the half-life t_{1/2} for the paraben were 0.800 per min and 0.87 min, respectively; for the chlorinated derivative, the corresponding values were 0.0195 per min and 36 min, respectively. The degradation rate of chlorinated paraben was 1/40-fold that of the paraben in the rat liver S9 fraction treatment; the results suggested that chlorination of the paraben may enhance accumulation of the paraben in the biota.

Syntheses and Identification of Metabolites

The reaction products of Cl2PP after exposure to in vitro transformation by a rat liver S9 fraction were assessed by GC/MS. The total ion chromatogram (TIC) and the MS data for trimethylsilyl (TMS) derivatives of the reaction products, and the associated synthetic compounds (which fulfill structural criteria for endocrine disrupting properties) are shown in Figs. 3 and 4. In the MS spectrum of peak 2, the molecular ion peaks (M^+) at m/z 374 and m/z 376 presented the typical isotopic pattern of a mono-chlorinated compound and indicated the substitution of an –OH group with a Cl atom in the TMS derivative of Cl2PP. We therefore identified peak 2 as propyl 3-chloro-4,5-dihydroxybenzoate (2) and completed its synthesis (see Experimental).

The molecular ion peaks (M^+) at m/z 408, observed in the spectra of peaks, 3, 5, and 6 indicated the substitution of an –OH group with a H atom in the TMS derivative of Cl2PP. The MS spectrum of peak 4 was dominated by the mass fragment ions at m/z [M^+–15], resulting from the loss of a methyl moiety in the derivatives rather than by the molecular ion peaks (M^+). Each of the ion peaks at [M^+–15] and (M^+) for all products also presented the typical isotopic pattern corresponding to dichlorinated compounds. We therefore completed the synthesis of four compounds that fit these analytical data.
AhR Agonist Activity of Metabolites

Table 1 shows the AhR agonist activities of the metabolites as measured using the yeast reporter gene assay. Two hydroxylated metabolite products (4) and (6) of the dichlorinated propyl paraben showed AhR agonist activity, when assessed in the yeast (YCM3) reporter gene assay, of up to 18 to 39% of that of Cl$_2$PP. When assessed in the yeast reporter gene assay, of up to 18 to 39% of that of Cl$_2$PP. and the presence of Cl$_2$PP; as such, they retained AhR agonist activity when tested in a murine reporter cell line (0.1% of the activity of β-naphthoflavone (BNF)). Studies in vitro have demonstrated that MCDF has a proteasome-dependent degradative effect on ER protein levels. Therefore, our current data warrant further studies to investigate AhR-ER crosstalk in the presence of Cl$_2$PP. Some chlorinated parabens occur widely in aquatic environments and, as such, organisms may be continuously exposed. The determination of the metabolic properties of chlorinated parabens can provide information on their potential for bioaccumulation and whether a xenobiotic is likely to be converted to a more potent or less potent toxin. It is probable that risk assessments of chlorinated parabens to ensure human health and environmental safety will require analysis of all their metabolite products in the manner exemplified here.

**Experimental**

**Materials** Propyl paraben was purchased from Tokyo Chemical Industry, Tokyo, Japan. Cl$_2$PP was synthesized using previously reported procedures (purity (GC)>99%). The male Sprague-Dawley rat liver S9 mix induced with phenobarbital and 5,6-benzoflavone was purchased from Kykman Corporation, Noda, Japan. d-Glucose 6-phosphate disodium salt (G-6-P-Na$_2$) and β-nicotinamide adenine dinucleotide phosphate, oxidized form, monosodium salt (β-NADP$^+$-Na) were purchased from Oriental Yeast Company Limited, Osaka, Japan. Dimethyl sulfoxide (DMSO) was purchased from Dojindo Laboratories, Kumamoto, Japan and was of fluorometric grade. N,O-Bis(trimethylsilyl)-trifluoroacetamid (BSTFA) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All chemicals used were of analytical grade or HPLC grade.

Propyl 3-chloro-4,5-dihydroxybenzoate (2) was synthesized via 3 steps from chlorinated hydroxybenzoate (a-c in Chart 1). Isopropyl 3,5-dichloro-2,4-dihydroxybenzoate (3) was synthesized via the 2 steps from commercially available hydroxybenzoic acid (d-e in Chart 1). 2-Hydroxypropyl 3,5-dichloro-4-hydroxybenzoate (4) was synthesized by esterification of 3,5-dichloro-4-hydroxybenzoic acid (1) suspended in benzene with 1-bromo-2-propanol in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as the catalyst under an atmosphere of nitrogen (f in Chart 1). 2-Hydroxy-
1-methylethyl 3,5-dichloro-4-hydroxy-benzoate (5) was synthesized by chemoselective benzoylation of 1,2-propanediol using 1.5 M equivalent of triphenylphosphine (Ph$_3$P) and diethyl azodicarboxylate (DEAD) (g in Chart 1). 3-Hydroxypropyl 3,5-dichloro-4-hydroxy-benzoate (6) was synthesized by a transesterification of chlorinated 4-hydroxybenzoate (h in Chart 1). Analytical data for synthetic metabolites and their intermediate substances are as follows:

**Compound (2b)**
Yield 89%. $^{1}$H-NMR (CDCl$_3$) $\delta$: 1.04 (3H, t, $J$ = 7.5 Hz), 1.77–1.84 (2H, m), 4.30 (2H, t, $J$ = 6.8 Hz), 8.28 (1H, d, $J$ = 2.5 Hz), 8.60 (1H, d, $J$ = 2.0 Hz). MS m/z: 261 (M$^+$+2), 259 (M$^+$).

**Compound (2c)**
Yield 96%. $^{1}$H-NMR (500 MHz, CDCl$_3$) $\delta$: 1.02 (3H, t, $J$ = 7.5 Hz), 1.72–1.79 (2H, m), 4.30 (2H, t, $J$ = 6.8 Hz), 8.28 (1H, d, $J$ = 2.5 Hz), 8.60 (1H, d, $J$ = 2.0 Hz). MS m/z: 261 (M$^+$+2), 259 (M$^+$).

**Compound (2)**
Yield 57%. Purity (GC) > 99%. $^{1}$H-NMR (CDCl$_3$) $\delta$: 0.93 (3H, t, $J$ = 7.3 Hz), 1.63–1.70 (2H, m), 4.11 (2H, t, $J$ = 6.8 Hz), 7.26 (1H, d, $J$ = 1.5 Hz), 7.37 (1H, d, $J$ = 1.5 Hz). High resolution-time-of-flight (HR-TOF)-MS (M$^+$–H): 229.0348 (Calcd for C$_{10}$H$_{10}$O$_4$35Cl: 229.0268), 231.0313 (Calcd for C$_{10}$H$_{10}$O$_4$37Cl: 231.0238).

**Compound (3b)**
Yield 63%. $^{1}$H-NMR (CDCl$_3$) $\delta$: 7.80 (1H, s).

**Compound (3)**
Yield 12%. Purity (GC) > 99%. $^{1}$H-NMR (CDCl$_3$) $\delta$: 1.39 (6H, d, $J$ = 6.3 Hz), 5.24–5.32 (1H, sept, $J$ = 1.3 Hz), 7.78 (1H, s). HR-TOF-MS (M$^+$–H): 262.9872 (Calcd for C$_{10}$H$_{9}$O$_4$35Cl: 262.9870), 264.9844 (Calcd for C$_{10}$H$_{9}$O$_4$37Cl: 264.9840), 266.9818 (Calcd for C$_{10}$H$_{9}$O$_4$37Cl: 266.9820).

**Compound (4)**
Yield 67%. Purity (GC) > 99%. $^{1}$H-NMR (CDCl$_3$) $\delta$: 1.26 (3H, d, $J$ = 5.5 Hz), 4.12–4.30 (3H, m), 7.94 (2H, s). HR-TOF-MS (M$^+$–H): 262.9842 (Calcd for C$_{10}$H$_{9}$O$_4$35Cl: 262.9870), 264.9841 (Calcd for C$_{10}$H$_{9}$O$_4$37Cl: 264.9840), 266.9812 (Calcd for C$_{10}$H$_{9}$O$_4$37Cl: 266.9820).

**Compound (5)**
Yield 51%. Purity (GC) 97%. $^{1}$H-NMR (CDCl$_3$) $\delta$: 1.48 (3H, d, $J$ = 6.5 Hz), 3.77–3.85 (2H, m), 5.22–5.29 (1H, m, $J$ = 6.0 Hz), 8.00 (2H, s). HR-electron ionization (EI)-MS (M$^+$): 263.9955 (Calcd for C$_{10}$H$_{9}$O$_4$35Cl: 263.9956), 265.9928 (Calcd for...
was incubated for 18 h at 30°C in a 96-well culture plate. 7) The dose–response concentration of the maximal response (EC25). The dose–response curve of each compound was derived using logistic regression of the mean±standard deviation (S.D.) from a minimum of three assays performed in duplicate.

Yield 55%. Purity (GC)>99% 1H-NMR (CDCl3) δ: 2.00 (2H, t, J=3.3 Hz), 3.72–3.77 (2H, q, J=6.0 Hz), 4.45–4.48 (2H, t, J=6.0 Hz), 7.94 (2H, s). HR-TOF-MS (M+–H): 262.9879 (Calcd for C10H10O4Cl2: 262.9870), 264.9849 (Calcd for C10H9O4Cl2: 264.9840), 266.9810 (Calcd for C10H9O4Cl2). Yeast Reporter Gene Assay AhR agonist activity of compounds was measured using a reporter assay based on the recombinant yeast YCM3 strain. 20) After the yeast cells were pre-incubated at 30°C overnight in a modified synthetic dextrose medium lacking tryptophan and leucine, a solution (1.25μL) of the test compound in DMSO was mixed with 125μL of synthetic galactose medium (2% galactose, lacking tryptophan and 100× dextrose medium lacking tryptophan and leucine, a solution (2.5 mL of S9 mix, 35 mg of G-6-P-Na2, 70 mg of β-NADP+–Na, 50 mL of Z buffer, and 200 μL of N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) buffer, pH 7.2) at 37°C and 100 rpm. Its initial concentration (C0) C1P was 100 μM. Reaction samples (100 μL) were digested by zymolyase 20 T, and then chlorophenol red-galactopyranoside (CPRG) was added. 9) β-Galactosidase activity was assessed by measuring the absorbance at 600 nm (A600) using a microplate reader. The absorbance at 578 nm (A578) and calculated using the equation: β-galactosidase activity=[(1000×A578)/[A600×0.01 (cell volume, mL)×reaction time (min)]].

BNF (β-naphthoflavone) was used as a positive control. AhR agonist activity was recorded as the 25% effective concentration (EC25). Statistical Analysis Data are expressed as the mean±standard deviation (S.D.) from a minimum of three assays performed in duplicate. Metabolic Reaction and Extraction DMSO solution (2 mL) of ClP was incubated with 48 mL of the S9 mix solution (2.5 mL of S9 mix, 35 mg of G-6-P-Na2, 70 mg of β-NADP+–Na, 50 mL of Z buffer, and 200 μL of N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) buffer, pH 7.2) at 37°C and 100 rpm. Its initial concentration (C0) C1P was 100 μM. Reaction samples (100 μL) were filtered through a 0.45 μm membrane filter (Minisart RC, Sartorius Japan K.K., Tokyo) and were subjected to HPLC for ClP quantification. Reaction samples (2 mL) also were adjusted to pH 2 with 2 M hydrochloric acid and extracted in ethyl acetate (3×1.2 mL). The ethyl acetate extracts were combined and dried over anhydrous sodium sulfate. After removing the solvent under reduced pressure, 10 μL of BSTFA and 100 μL of ethyl acetate were added to the test tubes containing the extracts, and the solution was allowed to stand for 1 h at room temperature to complete trimethylsilylation. After the reaction was complete, samples were examined using GC/MS with a mass-selective detector (HP 5973N Series; Agilent Technologies, Palo Alto, CA, U.S.A.) on an HP-5 fused-silica capillary column (J&W Scientific, Folsom, CA, U.S.A.), according to our previously described method. 10) Acknowledgment This work was supported by the Grant-in-Aid for Scientific Research (C) No. 26410195 from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. Conflict of Interest The authors declare no conflict of interest.

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