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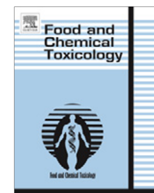

p-Hydroxybenzoate esters metabolism in MCF7 breast cancer cells

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ABSTRACT

Parabens are among the most frequently used preservatives to inhibit microbial growth and extend the shelf life of a range of consumer products. The objective of the present study was to gain insight into the metabolism of parabens in breast cancer cells (MCF7) since they have demonstrated estrogenic activity towards these cells and have been detected in breast cancer tissues. The toxicity of parabens to MCF7 cells was determined using MTT assays. Hydrolysis of methyl-, butyl and benzyl-paraben to *p*-hydroxybenzoic acid was analyzed in cultured MCF7 cells and in cellular homogenates. Glucuronidation and sulfoconjugation were studied in MCF7 homogenates, and parabens were analyzed by HPLC. Methyl-paraben was shown to be far less toxic than butyl and benzyl-paraben. Parabens were completely stable in MCF7 homogenates whereas *p*-nitrophenyl acetate, a substrate type, underwent hydrolysis. MCF7 cell homogenates did not express glucuronidation and sulfoconjugation activities toward parabens. The higher stability of parabens may explain their accumulation in breast cancer tissue as previously reported in the literature.

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1. Introduction

Parabens are alkyl esters of *p*-hydroxybenzoic acid that are extensively used as preservatives in the food, pharmaceutical and cosmetic industries due to their broad antimicrobial spectra and low toxicity (Soni et al., 2005). Parabens are considered to be relatively safe compounds with a low bioaccumulation potential (Soni et al., 2005) and are still used in a wide range of personal care products (Andersen, 2008; Yazar et al., 2011). However, it has been unequivocally demonstrated *in vivo* and *in vitro* in various screening tests that parabens have endocrine-disrupting activity that may represent a potential risk to human health (Darbre and Harvey, 2008). Parabens have been detected in human fluids i.e., blood (Sandanger et al., 2011), urine (Ye et al., 2006a), milk (Schlumpf et al., 2010), and human breast tumors (Darbre et al., 2004; Barr et al., 2012) in different populations.

Parabens can reach the circulation through oral administration and dermal application of cosmetic creams. The levels of paraben in the blood depend on the extent of exposure (Sandanger et al., 2011). BuPB peaked in the serum 3 h after topical application of a cream containing parabens, when C_{max} reached 135 ng/ml (Janjua et al., 2007), and in urine with a peak value after 8–12 h (Janjua et al., 2008). No other detailed studies have been performed on the pharmacokinetics of other parabens in human after oral or dermal exposure. Most research has focused on the determination of paraben levels in urine, mostly present as metabolites or in breast cancer tissue and little is known about intact parabens in the blood. Based on Darbre and Harvey's 2008 review, BuPB may exhibit estrogenic effects at the blood concentration determined by Janjua et al. (2007). Their calculation took into consideration the fact that BuPB is 10,000 times less potent than 17-β estradiol.

Parabens are mainly metabolized to *p*-hydroxybenzoic acid (Jewell et al., 2007a,b). The estrogenic activity of the latter is low compared to parabens (Routledge et al., 1998; Darbre et al., 2004; Byford et al., 2002). Parabens undergo phase II reactions (Ye et al., 2006b; Janjua et al., 2008), and both glucuronide and sulfate conjugates have been detected in human urine (Ye et al., 2006b).

Metabolism studies of parabens have not been performed in breast cancer tissues, in which parabens were detected in intact form, or in breast cancer cells, which may represent a valuable model to study their metabolism in cancer tissues. After analysis of 20 samples (Darbre et al., 2004) and 160 samples (Barr et al., 2012) of human breast tumor tissue, the authors found variable results regarding the most accumulated parabens which they explained by several factors including route of exposure, lifestyle,

Abbreviations: MePB, methylparaben; BzPB, benzylparaben; BuPB, butylparaben; UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase.

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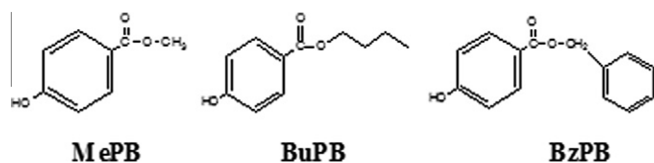

Diagram 1. Chemical structures of MePB, BuPB and BzPB.

and the level of each paraben consumed. Another factor that may play a major role is the rate of metabolism of each paraben, especially since it is well documented that human esterases (in the skin and liver), hydrolyze parabens to *p*-hydroxybenzoic acid depending on the length of the chain (Imai et al., 2006; Bando et al., 1997; Jewell et al., 2007b). The possible absence of metabolism in breast tissue may also lead to accumulation and enhancement of the effect of parabens.

The MCF-7 cell line has been used to screen estrogen disruptors in human breast cancer (Soto and Sonnenschein, 1985; Soto et al., 1995). MCF-7 is an immortalized human breast adenocarcinoma cell line that endogenously expresses predominantly ERα but also some ERβ (Brooks and Thompson, 2005; Chen et al., 2004). Cultured MCF7 cells have been widely used to demonstrate the proliferative effect of parabens (Routledge et al., 1998; Okubo et al., 2001; Byford et al., 2002; Vanparys et al., 2006). In the present study, they were as a model of breast cancer cells to evaluate the route and rate of biotransformation of parabens. The toxicity of parabens toward MCF7 cells was evaluated and the results were used to perform metabolism studies in cultured MCF7 cells at non-toxic concentrations. The stability of MePB, BuPB, with a short and long alcohol chain, respectively, and of BzPB, with an aromatic alcohol moiety (Diagram 1), was further studied in cultured MCF7 cells and by 18,000*g* supernatant fraction of MCF7 cell homogenates. In addition, glucuronidation and sulfoconjugation of these parabens were studied in MCF7 homogenates.

2. Materials and methods

2.1. Chemicals

RPMI, fetal bovine serum (FBS), D-glutamine, penicillin/streptomycin solution, sterile phosphate buffered solution (PBS), methylparaben, ethylparaben, butylparaben, benzylparaben, *p*-hydroxybenzoic acid, *p*-dihydroxybenzoic acid, *p*-nitrophenol, *p*-nitrophenyl acetate, UDP-glucuronic acid (UDPGA), dithiotreithol, 3′-phosphoadenosine-5′-phosphosulfate (PAPS), magnesium chloride, dimethylsulfoxide (DMSO) and bicinchoninic acid were obtained from Sigma–Aldrich, France.

2.2. Cell line and culture

The cell line we used is a human breast adenocarcinoma cell deposited in the American Type Culture Collection under the designation MCF7 (ATCC; ATCC number: HTB-22). The MCF7 line retains several characteristics of differentiated mammary epithelium including the ability to process estradiol via cytoplasmic estrogen receptors. MCF7 cells were cultured in RPMI medium with 1% (v/v) L-glutamin, 10% (v/v) fetal bovine serum, 1% (v/v) penicillin (10,000 IU/mL), and 1% (v/v) streptomycin (10,000 UG/mL) in a 5% CO_2 atmosphere at 37 °C.

2.3. Cytotoxicity assays

The cytotoxicity of PB on MCF7 cells was evaluated with the MTT assay in 96-well plates. Exponentially growing cells were seeded at a density of 10,000 cells per well and allowed to grow overnight. After removing the cell culture medium and washing the cells in phosphate-buffered saline (PBS, pH 7.4), MCF-7 cells were incubated for 1 h in a new medium containing serial dilutions of MePB, BuPB or BzPB. Stock solutions of parabens (PB) were prepared in DMSO. The working solutions, ranging from 2.5 to 1000 μM, were freshly prepared in RPMI and sterilized by filtration through a 0.22-μm Millipore® filter. The maximum DMSO concentration in the medium was less than 0.5% v/v including the control groups. The MTT assay involves reduction of tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) by viable cells to purple formazan. Briefly, after PB treatment, the cells were washed twice with PBS and then incubated for 3 h at 37 °C in the culture medium containing MTT at a final concentration of 0.5 mg/ml. The cells were finally lysed with DMSO to solubilize the formazan. Absorbance was measured at 492 nm.

2.4. Stability of parabens in cultured MCF7 cells

MCF-7 cells were seeded in 35-mm dishes (density 330,000 cells/ml) and cultured for 24 h at 37 °C. Cells were then exposed to concentrations of MePB (250 μM), BuPB or BzPB (10 μM) for 1 h as assessed by the MTT assay. The concentration of DMSO never exceeded 0.5%. One hundred microliters of cell culture supernatants of the four replicas were collected before exposure, at the time of exposure and after 1 h of exposure to parabens. The supernatant was then conserved at −80 °C. Aliquots of the supernatant were then used to assess HPLC analysis.

2.5. Stability of parabens in MCF7 cellular homogenates

2.5.1. Preparation of cell homogenates

MCF-7 cells were cultured to confluence in 75 cm^2 flasks. The cell suspension was centrifuged at 10,000*g* at 4 °C for 15 min. The supernatant was eliminated and the pellet was washed three times with phosphate saline buffer (pH 7.4). The cell suspension was sonicated for 30 min on ice and then centrifuged at 18,000*g* at 4 °C for 20 min. The supernatant was then conserved at −80 °C. Aliquots were used to assess hydrolysis, glucuronidation and sulfoconjugation activities toward parabens.

2.6. Esterase activity of MCF7 cell homogenates

The esterase activity of MCF7 homogenates towards *p*-nitrophenyl acetate (100 μM), a prototype substrate, was monitored between 0 and 30 min using 50 mM phosphate buffer, pH 7.4. The reaction was initiated by the addition of MCF-7 homogenates (438 μg) in a final volume of 1 ml. Phosphate buffer was used instead of *p*-nitrophenyl acetate in control assays. Media were incubated at 37 °C and the reaction was terminated by placing the tubes on ice and adding 1 ml of acetonitrile. After centrifugation at 4 °C for 15 min, the formation of the reaction product, *p*-nitrophenol, was determined by a spectrophotometer at a wavelength of 402 nm. Non-specific activity was corrected for by subtracting the control absorption from the assay absorption. The activity of esterase was expressed on micromoles per min per mg of protein.

To determine the molar extinction coefficient of *p*-nitrophenol, a stock standard solution of *p*-nitrophenol 60 mM was prepared in 0.05 mM phosphate buffer at pH 7.4; aliquots were taken to prepare standard solutions ranging from 2.5 to 600 μM in a final volume of 1 ml. Acetonitrile (1 ml) was then added and absorbance was recorded at 402 nm.

Using increasing concentrations of *p*-nitrophenyl acetate (2.5, 5.0, 7.5, 10, 25, 50, 100, 125 and 250 μM), the kinetic parameters of the esterase activity of MCF7 homogenates (438 μg) were determined. Experiments were performed in triplicate.

To determine if parabens can affect the esterase activity of MCF7 homogenates, BuPB (0, 62.25, 125, 250, 500 and 1000 μM) was incubated with MCF-7 homogenates (90.6 μg) and the esterase activity toward *p*-nitrophenyl acetate 50 μM was evaluated after 30 min incubation at 37 °C. Stock solution of BuPB was prepared in DMSO (5 mg/ml; 25742 μM). BuPB did not absorb at 402 nm. Blanks contained similar volumes of DMSO. The activity of esterase was expressed in micromoles per min per mg of protein.

MCF-7 cellular homogenate (438 μg protein) was incubated with MePB, BuPB or BzPB (250 μM) at 37 °C in phosphate buffer (67 mM, pH 7.4) in a total volume of 1.5 ml. Aliquots were removed at 0, 1, and 18 h and analyzed by HPLC as described below. Blank incubations were conducted in the absence of protein to determine spontaneous hydrolysis.

2.7. UDP-glucuronosyltransferase (UGT) activity

UGT activity toward parabens was measured in cell homogenates prepared from MCF7 cells as described above. Cell homogenates (438 μg) were incubated in 50 mM Tris–HCl buffer pH 7.4, 10 mM $MgCl_2$ with 1 mM paraben and 1 mM UDP-GA (final volume 0.35 ml) at 37 °C for 1 h. Reactions were stopped by adding 250 μl of acetonitrile and 100 μl of the internal standard and samples were centrifuged for 10 min at 10,000*g*. Supernatant samples were analyzed by HPLC.

2.8. Sulfotransferase (ST) activity

ST activity was measured in MCF-7 homogenates. MCF-7 homogenates (438 μg) were incubated in 50 mM Tris–HCl buffer (pH 7.4), 10 mM $MgCl_2$ with 1 mM paraben, 1 mM dithiotreithol and 1 mM PAPS (final volume 0.35 ml) at 37 °C for 1 h. Reactions were stopped by adding 250 μl of acetonitrile and 100 μl of the internal standard and samples were centrifuged for 10 min at 10,000*g*. Supernatant samples were analyzed by HPLC.

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