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The estrogenic effects of benzylparaben at low doses based on uterotrophic assay in immature SD rats

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ABSTRACT

Benzylparaben (BzP), a type of parabens being used as a preservative agent in cosmetics, food, and pharmaceutical products, may be ingested by humans. In this study, we performed an immature uterotrophic assay using Sprague Dawley (SD) rats by intragastric administration to determine the estrogenic effects of BzP and found significant increases in uterine weight with doses of 0.16 mg/kg body weight and higher ($P < 0.05$). The *in vivo* estrogenicity of BzP was supported by *in vitro* results from the human estrogen receptor α (hERα)-coactivator recruiting assay and *in silico* molecular docking analysis performed in this study. The *in vitro* estrogenic activity of BzP can be observed at concentrations of 1.0×10^{-8} M and higher. Molecular docking analysis showed that BzP fits well into the agonist pocket of hERα. The lowest observed effect dose (LOED) (0.16 mg/kg/day) of BzP is much lower than the documented LOEDs of other parabens. Actual risk may exist for people who consume a diet high in BzP or use BzP-laden cosmetics. In addition, we tested the sensitivity of Wistar rats to 17β-estradiol by immature uterotrophic assay, and no obvious uterotrophic response was observed in the rats given doses up to 100 μg/kg body weight.

1. Introduction

Endocrine-disrupting chemicals (EDCs) have become a public concern in recent decades (Marx-Stoelting et al., 2011). EDCs can disrupt the body's normal functions by mimicking or blocking hormones at low levels (Schug et al., 2011). Exposure to EDCs is known to cause many disorders, such as obesity, male and female subfertility, decline in semen quality, urogenital birth defects, feminization of male infants, delayed or early puberty, endometrial hyperplasia, endometriosis, recurrent miscarriage, polycystic ovary syndrome, and menstrual irregularity (Vom Saal et al., 2012; Diamanti-Kandarakis et al., 2009; Zama and Uzumcu, 2010; Schell and Gallo, 2010; Brouwers et al., 2011; Swan et al., 2005; Grun and Blumberg, 2009). In addition, prostate, testicular, thyroid, and breast cancers are suspected to occur more frequently worldwide because of exposure to EDCs (Hu et al., 2011; Huyghe et al., 2003; Enewold et al., 2009; Cao et al., 2010).

Many of the chemicals used in industrial solvents and lubricants, plastics, plasticizers, pesticides, fungicides, and pharmaceutical agents have been identified as EDCs in recent years. Dichlorodiphenyltrichloroethane, bisphenol A, phthalates, dioxin, polychlorinated biphenyls, 4-nonylphenol, and diethylstilbestrol etc., have been focally studied, and restricted or banned in industrial and agricultural products due to their toxicities (Colborn et al., 1993; Meeker, 2010; Elobeid and Allison, 2008; Soto and Sonnenschein, 2010; Laws et al., 2000). Parabens are reported to be estrogenic; however, they are still used extensively worldwide because there is insufficient knowledge about their toxicity at low doses.

Parabens are a family of alkyl esters of *p*-hydroxybenzoic acid mainly comprising seven chemicals (methyl, ethyl, propyl, isopropyl, butyl, isobutyl, and benzyl parabens). In 1998, Routledge et al. reported that subcutaneous injection of butyl paraben at 200 mg/kg body weight (bw) per day significantly increased the uterine weights of immature female Alpk/AP rats, although it did not significantly increase the uterine weights when administered by gavage at 1200 mg/kg bw per day by gavage (Routledge et al. 1998). Lemini et al. (2003) found that methyl, ethyl, propyl, and

Abbreviations: BzP, benzylparaben; EDCs, endocrine-disrupting chemicals; E_2, 17β-estradiol; hERα, human estrogen receptor α; bw, body weight; BAP, bacterial alkaline phosphatase; GST, glutathione S-transferase; DMSO, dimethyl sulfoxide; FDA, U.S. Food and Drug Administration; PND, postnatal day; JECFA, Joint FAO/WHO Expert Committee on Food Additives; SD, Sprague Dawley; SE, standard error; SD, standard deviation; RMSE, root mean square error; PMF, potential of mean force; LOED, lowest observed effect dose.

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butyl parabens significantly increased the uterine weights of immature CD1 mice when injected subcutaneously at doses of 16.5, 6, 20, and 7 mg/kg bw per day, respectively ($P < 0.05$). In another uterotrophic assay study, however, neither methyl, ethyl, nor propyl parabens produced any estrogenic response when administered by gavage at dose levels up to 100 mg/kg bw per day to immature B6D2F1 mice (Hossaini et al., 2000). The various animals and treatments used for uterotrophic assay play important roles in assessing the estrogenic activity of chemicals. Of concern is BzP, which is not only used as a preservative agent in cosmetics, food, and pharmaceutical products, but also widely used as an intermediate in the pharmaceutical, printing and dyeing, and pesticide industries. Thus, there are two possible routes of exposure to BzP: oral intake and skin absorption. Although Darbre et al. (2003) found that BzP significantly increased the relative uterine weight of mice following topical application of daily dose of 33 mg to dorsal skin for three days, there is no report of the *in vivo* estrogenic effects of BzP administered by gavage.

2. Materials and methods

2.1. Chemicals

The compounds 17β-estradiol (>98.0%, E_2) and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). BzP (>98%) was purchased from Tokyo Kasei Kogyo Co. Ltd., (Tokyo, Japan).

2.2. Methods

2.2.1. Binding prediction of BzP into human estrogen receptor by automated molecular docking

Scigress (Ultra Version 2.2.0, Fujitsu, USA) is a very useful pre-screening tool for developing novel estrogen receptor ligands, and has been used to dock flexible ligands into the active site of a rigid protein (Zhang et al., 2012). The three-dimensional structure of the hERα-LBD (ligand-binding domain of human estrogen receptor α) (PDB ID 1ERE) was derived from the Protein Data Bank website (http://www.rcsb.org/pdb). The protein structure was cleaned to a monomer of chain A for docking calculations. Docking calculations were evaluated with a 15 × 15 × 15 Å grid box with 0.375 Å grid spacing. The procedure was set to run 60,000 generations with an initial population size of 50, elitism of 5, crossover rate of 0.8, and mutation rate of 0.2. The potential of mean force (PMF), a knowledge-based approach that extracts pairwise atomic potentials from the structure information of known protein–ligand complexes contained in the Protein Data Bank, was used to score the binding affinity of a chemical in the active site.

2.2.2. Immature rat uterotrophic assays

Immature female Sprague Dawley (SD) rats and Wistar rats at an age of postnatal day (PND) 20 were purchased from Experimental Animal Tech Co. of Weitonglihua (Beijing, China). The weight variations of animals used were less than ±20% of the mean weight. The rats were housed two or three per stainless steel wire-mesh cage. The housing environment was controlled at a temperature of 22 ± 2 °C, a relative humidity of 40–60%, and a 12-h light/dark cycle. The rats were fed *ad libitum* with a basic diet and sufficient drinking water from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). Before the experiment, the rats were randomly assigned into the treatment and control groups. The number of animals per group are listed in Tables 1 and 2. Body weights were measured and recorded daily throughout the experiments. Intragastric administration (5 mL/kg bw, as determined daily) of control and test compounds was performed daily for 3 days beginning on PND 21. On PND 24, the rats were weighed and sacrificed under chloroform anesthesia 24 h after the final treatment. The uterus of each rat was dissected. Each uterus was blotted, and the blotted weight was recorded. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Peking University. The test chemicals were dissolved in peanut oil for intragastric administration. The vehicle group was treated with peanut oil only. E_2 was dissolved in peanut oil to final doses of 1, 5, 25, 100, 400 μg/kg bw/day for an experiment comparing the uterotrophic effects of E_2 treatment in SD and Wistar rats. BzP was dissolved in peanut oil to final doses of 0.16, 0.8, 4, 20, and 100 mg/kg bw/day for experiment 1 and to doses of 0.0064 and 0.032 mg/kg bw/day for experiment 2, both performed in SD rats to study the uterotrophic effect of BzP. To compare the uterotrophic effect of the chemical with that of the control, the relative uterine weight was calculated for each animal. The relative uterine weight was taken as the ratio of uterine weight to final body weight.

2.2.3. Human estrogen receptor α-coactivator recruiting assay

The estrogenicity of BzP was measured by a ligand-dependent coactivator recruiting assay with glutathione S-transferase (GST)-tagged hERα-LBD. In the assay, the GST-tagged hERα-LBD and 6 × his (histidine)-tagged nuclear receptor interaction domain of steroid receptor coactivator 2-bacterial alkaline phosphatase (6 × his-hSRC2 NID-BAP) fusion proteins were prepared as described by Kanayama et al. (2003). E_2 and BzP were serially diluted 10-fold with DMSO to final concentration ranges of 10^{-4}–10^{-11} and 10^{-4}–10^{-10} M. The absorbance at 405 nm (BAP activity) was measured to demonstrate the chemical's binding affinity for hERα. In this assay, the wells in which only DMSO was added were used to determine background values. Graphpad Prism 4 software (GraphPad Software, Inc., San Diego, CA) was used to calculate chemical's sigmoidal concentration-effect curve. In this assay, the EC50 was calculated according to the sigmoidal concentration-effect curve. The EC50 is the concentration of the test chemical corresponding to 50% of the maximal activity.

2.3. Data analysis

The statistical program SPSS (Ver 13.0; Chicago, IL, USA) was used to analyze the data. Data are presented as mean and standard deviation (SD) unless otherwise indicated. Group differences were assessed by one-way analysis of variance and Fisher's least significant difference (LSD) method. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Automated docking of BzP in agonist pocket of hERα-LBDs

The binding potential and pose of BzP in the agonist pocket of hERα-LBDs were predicted by Scigress 3.0. The original ligand E_2 in the complex was docked into the binding site, and the PMF for E_2 was −55.745 kcal/mol, similar to our previously calculated PMF (−55.655 kcal/mol) by Scigress 2.2.0 (Zhang et al., 2012). The root mean square error (RMSE) between the original sites of E_2 and the newly calculated binding sites of E_2 was 0.2659 Å (Fig. 1A), similar to that reported for the E_2 docked into the PDB

Table 1
Number of rats used, arrival body weight, final body weight, blotted uterus weight, and blotted uterus weight/body weight in the uterotrophic assay of E_2 in Sprague Dawley (SD) and Wistar rats. Data are presented as the mean ± SD (standard deviation).

Rat strain	E_2 dose	N	Arrival weight[a] (g)	Final weight (g)	Blotted uterus weight (g)	Blotted uterus weight/bw (%)
Sprague	0 μg/kg/day (Ctrl)	9	48.1 ± 7.7	64.8 ± 8.5	0.034 ± 0.007	0.053 ± 0.009
Dawley	1 μg/kg/day	8	46.4 ± 4.9	63.8 ± 6.1	0.042 ± 0.010	0.065 ± 0.015
Wistar	5 μg/kg/day	8	49.9 ± 4.9	67.4 ± 6.2	0.056 ± 0.014	0.083 ± 0.020
	25 μg/kg/day	8	48.1 ± 3.9	66.5 ± 5.4	0.107 ± 0.025	0.161 ± 0.036
	100 μg/kg/day	8	44.2 ± 3.4	60.0 ± 4.3	0.121 ± 0.016	0.203 ± 0.033
	400 μg/kg/day	7	50.3 ± 5.7	67.7 ± 7.0	0.154 ± 0.019	0.234 ± 0.041
	0 μg/kg/day (Ctrl)	8	47.1 ± 2.7	67.1 ± 2.8	0.045 ± 0.007	0.064 ± 0.006
	1 μg/kg/day	8	48.2 ± 5.2	70.4 ± 8.5	0.054 ± 0.013	0.071 ± 0.013
	5 μg/kg/day	8	47.5 ± 5.6	68.2 ± 9.1	0.051 ± 0.012	0.069 ± 0.011
	25 μg/kg/day	8	49.9 ± 3.4	73.4 ± 6.0	0.054 ± 0.010	0.068 ± 0.013
	100 μg/kg/day	8	47. 1 ± 3.5	69.3 ± 3.9	0.055 ± 0.009	0.075 ± 0.010
	400 μg/kg/day	8	48.8 ± 3.8	72.4 ± 6.1	0.089 ± 0.020	0.118 ± 0.034

[a] The rats' body weight on the day of arrival (PND20).