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Estrogenic potency of benzophenone and its metabolites in juvenile female rats

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Abstract The estrogenic activity of benzophenone and its metabolites, benzhydrol and \( p \)-hydroxybenzophenone, were investigated in vitro by estrogen receptor (ER) competitive ligand binding assay and in vivo by uterotrophic assay in juvenile female Sprague-Dawley (SD) rats. \( p \)-Hydroxybenzophenone as well as diethylstilbestrol and bisphenol A, known xeno-estrogenic compounds, competed with fluorescein-labeled \( 17\beta \)-estradiol to bind human recombinant ER\(_2\) in a concentration-dependent manner. Fifty percent inhibitory values (IC\(_{50}\)) of diethylstilbestrol, bisphenol A, and \( p \)-hydroxybenzophenone were approximately \( 10^{-8} \), \( 10^{-7} \), and \( 5 \times 10^{-5} \) M, respectively. However, neither the parent compound nor benzhydrol at concentrations from \( 10^{-9} \) to \( 5 \times 10^{-4} \) M impaired the binding of \( 17\beta \)-estradiol to ER\(_2\). Juvenile female rats (21-days-old) were given s.c. injections of benzophenone, its metabolites, and \( 17\beta \)-estradiol for 3 days. Administration of \( p \)-hydroxybenzophenone (100–400 mg/kg) elicited an increase in absolute and relative uterine weights in a dose-dependent manner and \( 17\beta \)-estradiol (10 \( \mu \)g/kg) increased uterine weight approximately fourfold relative to control. The uterine response caused by both compounds was accompanied by an increase in luminal epithelial height and stromal cells in the uterus and an increase in thickness of vaginal epithelial cell layers with cornification. In contrast, benzophenone and benzhydrol at a dose of 400 mg/kg affected neither uterine weight nor histological changes of the uterus and vagina. These results indicate that \( p \)-hydroxybenzophenone, a metabolite of benzophenone, exhibits intrinsic xeno-estrogenic activity in the female reproductive tract.

Key words Benzophenone · Metabolites · Uterotrophic assay · Competitive binding assay · Female rats

Introduction

Benzophenone is an ultraviolet light-absorber which has been used in medicine and industry for more than 30 years. Its 12 derivatives, designated benzophenone-1 to benzophenone-12, are widely used today in cosmetic products as a photostabilizer, as a sunscreen in lotions, and hair sprays to protect the skin and hair from UV irradiation. In addition, benzophenone is used in the manufacture of a fragrance ingredient, insecticides, agricultural chemicals, and pharmaceuticals and is an additive for plastics and coatings. Benzophenone occurs naturally in plants such as muscat grape and mango, and as a series of aryl ketones is used in flavorings. Benzophenone has been investigated in vivo and in vitro to assess its toxicological properties and metabolism. It has been reported that large amounts (100 or 500 mg/kg per day for 4 weeks) of benzophenone increase liver and kidney weights in rats (Burdo et al. 1991; Freeman et al. 1994) and cause hepatocellular necrosis accompanied by disorganization of lobular architectures in guinea pigs (Dutta et al. 1993).

Recently, it was reported that the liver and kidney were identified as target organs of toxicity induced by the compound in rats and mice; treatment-related increases in liver weights were attributed to hypertrophy and/or cytoplasmic vacuolization of hepatocytes, and increased kidney weights were associated with a spectrum of renal changes with foci of tubule regeneration (National Toxicology Program 2000). On the other hand, benzophenone primarily undergoes reduction to benzhydrol, followed by conjugation and urinary excretion in rabbits (Robinson 1958; Robinson and Williams 1957). In addition, Stoklinski et al. (1980) demonstrated that a small amount, approximately 1\%, of benzophenone after oral administration is converted to \( p \)-hydroxybenzophenone in rats.
Although many natural and synthetic compounds are ubiquitous in the environment, little is known about the potential risks to humans of exposure to known xenoestrogens. Recently, considerable attention has focused on bisphenol A as well as other phenolic compounds as endocrine disrupting chemicals having weak estrogenic activity in bioassays both in vivo (Nagel et al. 1997; vom Saal et al. 1998) and in vitro (Gaido et al. 1997). The metabolic pathway and toxicity of benzophenone have been studied; however until now, the relationship between metabolism and estrogenic activity has not been investigated. Benzophenone is listed among “chemicals suspected of having endocrine disrupting effects” by the World Wildlife Fund, the National Institute of Environmental Health Sciences in the USA, and the Japanese Environment Agency.

In a previous study, we reported that benzophenone at a low toxic level was enzymatically converted to at least three metabolites: benzhydrol, \( p \)-hydroxybenzophenone, and a sulfate, in isolated rat hepatocytes (Fig. 1; Nakagawa et al. 2000). The effect of \( p \)-hydroxybenzophenone on the proliferation of MCF-7 cells was weakly estrogenic compared to that of \( 17\beta \)-estradiol, whereas the parent compound and benzhydrol were essentially inactive at the concentrations used. In the present study, we investigate potential estrogenic activities of benzophenone and its intermediates, \( p \)-hydroxybenzophenone and benzhydrol, using an in vitro competitive binding assay for estrogen receptor and an in vivo uterotrophic assay in immature female rats. The mechanisms are discussed of the activities of benzophenone and its metabolites.

### Materials and methods

#### Reagents

The chemical compounds used were obtained as follows: benzophenone, \( p \)-hydroxybenzophenone, benzhydrol and bisphenol A (BPA; purities of \( >97\% \)) from Tokyo Kasei Co. (Tokyo, Japan); \( 17\beta \)-estradiol (E\(_2\)) and diethylstilbestrol (DES) from Sigma Chemical Co. (St. Louis, Mo., USA). All other chemicals used were of the highest purity commercially available.

![Fig. 1 Proposed metabolism of benzophenone in isolated rat hepatocytes: A benzophenone, B benzhydrol, C \( p \)-hydroxybenzophenone and D sulfate of \( p \)-hydroxybenzophenone. \( \alpha \) Ketone reductase, \( \beta \) monooxygenase, \( \gamma \)-sulfotransferase](image.png)

#### Competitive binding assay

Competitive binding between \( 17\beta \)-estradiol and various compounds was assayed using a nonradioactive estrogen-R(\( x \)) competitor screening kit (Wako Pure Chemicals, Osaka); the kit consists of human recombinant estrogen receptor \( x \) (ER\(_ x \)) coated on the bottom of 96-well plates and fluorescein-labeled \( 17\beta \)-estradiol as competitor. DES and other compounds dissolved in dimethylsulfoxide (DMSO) were added to a reaction solution containing \( 17\beta \)-estradiol, and the mixture (100 \( \mu \)l) was added to each well. After 2 h of incubation at room temperature, the mixture, which contained free compounds and the \( 17\beta \)-estradiol unbound to ER\(_ x \) in wells, was aspirated and exchanged with the assay solution (100 \( \mu \)l). The concentration of \( 17\beta \)-estradiol bound to ER\(_ x \) on the plates was measured in a CytoFluor 4000 fluorescence plate reader (PerSeptive Biosystems, Framingham, Mass., USA) with filters set for 485-nm excitation and 535-nm emission. The results are expressed as a percentage of the fluorescence value for the reaction solution without sample.

#### Animals and the 3-day uterotrophic assay

Female Sprague-Dawley (SD) rats, 7 days pregnant, were obtained from Charles River Japan (Yokohama, Japan) and housed in plastic cages with heat-treated laboratory grade fir shavings as bedding. Animals were maintained under controlled temperature (23–24°C), relative humidity (45–55\%) and light (12 h light/12 h dark) conditions with food (CE-2, Clea Japan, Tokyo, Japan) and water available ad libitum. At Day postpartum all female litters were culled to 10 pups in order to assure normal growth rates in all pups. Pups were weaned at 21 days of age and weighed, weight-ranked and placed into treatment groups such that the mean body weight (46.4 g), for all groups was comparable. During the remainder of the study, all animals were housed under conditions identical to those described for the timed-pregnant rats.

Immature 21-day-old females were used to compare uterotrophic effects of \( 17\beta \)-estradiol, benzophenone, \( p \)-hydroxybenzophenone, benzhydrol, and DMSO as a vehicle. All animals were dosed once per day for 3 days by subcutaneous injection on the dorsal surface and were killed 6 h after the last dose. Dosing solutions for the immature animals were prepared in DMSO at a concentration of 100, 200, or 400 mg/ml and used at a dosing volume of 100 \( \mu \)l/100 g body weight. The dose level of \( 17\beta \)-estradiol as a positive control was 10 \( \mu \)g/kg body weight. The body of the uterus was cut just above its junction with the cervix and at the junction of the uterine horns with the ovaries, and was weighed. The uterus and vagina were placed in buffered formalin solution, and paraffin sections were prepared and stained with hematoxylin and eosin for histological examination.

#### Statistical analysis

Statistical analysis was performed by one-way analysis of variance, followed by a Dunnett’s multiple comparison test. Statistical significance was assumed at \( p < 0.05 \).

#### Results

Previously, we reported that benzophenone at a low level of toxicity was enzymatically converted to at least three metabolites: benzhydrol, \( p \)-hydroxybenzophenone and a sulfate in isolated rat hepatocytes (Fig. 1). The effect of \( p \)-hydroxybenzophenone on the cell proliferation of MCF-7 was weakly estrogenic compared with that of \( 17\beta \)-estradiol, whereas the parent compound and benzhydrol were essentially inactive at the concentrations used. To compare affinities among benzophenone,