Toxicology Letters 209 (2012) 146–153

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

The estrogenic potential of salicylate esters and their possible risks in foods and cosmetics

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ARTICLE INFO

Article history:
Received 6 September 2011
Received in revised form 9 December 2011
Accepted 10 December 2011
Available online 16 December 2011

Keywords:
Estrogenicity
Salicylate esters
Endocrine-disrupting chemicals
Benzyl salicylate
Uterotrophic effect
Health risk

ABSTRACT

Salicylate esters (SEs), a class of chemicals extensively used as flavor and fragrance additives in foods, beverages and a wide variety of consumer products, are suspected to have estrogenic activity based on chemical analysis of *in silica* molecular docking. We evaluated the estrogenic potentials of phenyl salicylate (PhS), benzyl salicylate (BzS), phenethyl salicylate (PES), ethyl salicylate (ES) and methyl salicylate (MS) using an *in vitro* human estrogen receptor α (hERα)-coactivator recruiting assay and *in vivo* immature rodent uterotrophic bioassays. We found that PhS, BzS and PES showed obvious *in vitro* hERα agonistic activities; BzS in particular exhibited a higher estrogenic activity compared to bisphenol A (BPA). The uterine weights were significantly increased in mice treated with 11.1, 33.3, 100 and 300 mg/kg/day BzS and 33.3 mg/kg/day PES and rats treated with 3.7, 11.1, 33.3 and 100 mg/kg/day BzS for 3 days ($P < 0.05$). Finally, we transformed the daily intakes and the dermal exposures of SEs in the real world into estradiol equivalent concentrations (EEQs). We found that the EEQ of BzS daily intake in consumers in the U.S. and the EEQs of dermal BzS and PES exposure among high-volume users worldwide were higher than the maximum secure daily estradiol intake recommended by the U.S. Food and Drug Administration (FDA). In particular, the EEQ for dermal BzS exposure was up to 162 ng EEQ/kg, which is 3.3 times higher than the maximal acceptable daily E_2 intake recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

1. Introduction

Endocrine disruption has become a serious public health concern over the past decades (Hotchkiss et al., 2008). A variety of endocrine-disrupting chemicals (EDCs) have the potential to mimic, block or interfere with hormones in the body and subsequently affect development and reproduction in humans (Zama and Uzumcu, 2010; Bourguignon and Parent, 2010; Fisher, 2004; Cooper and Kavlock, 1997; Colborn et al., 1993). Many disorders, such as reduction of sperm quality, impairment of fertility, increased rates of irregular menstruation, endometriosis, spontaneous abortion, increased numbers of birth defects in the male sex organs, child obesity and precocious puberty, are suspected to be caused by widespread exposure to EDCs (Bourguignon and Parent, 2010; Fisher, 2004; Colborn et al., 1993; Meeker, 2010; Trasande et al., 2009; Newbold, 2010; Elobeid and Allison, 2008; Walvoord, 2010). The occurrence of some cancers, such as breast cancer, endometrial cancer, testicular cancer and prostate cancer, have been detected more frequently in many industrialized countries and have been linked to EDC exposure (Fisher, 2004; Meeker, 2010; Walvoord, 2010; Soto and Sonnenschein, 2010; Darbre and Charles, 2010).

Many chemicals, particularly some pesticides, plasticizers and drugs, have been identified as EDCs. Among them, dichlorodiphenyltrichloroethane, bisphenol A (BPA), phthalates, dioxin, polychlorinated biphenyls, 4-nonylphenol and diethylstilbesterol have been implicated as causes of developmental and reproductive disorders or cancers at hormonally sensitive sites in the body (Colborn et al., 1993; Meeker, 2010; Elobeid and Allison,

Abbreviations: BzS, benzyl salicylate; PhS, phenyl salicylate; PES, phenethyl salicylate; ES, ethyl salicylate; MS, methyl salicylate; SE, salicylate ester; EEQ, estradiol equivalent concentration; E_2, 17β-estradiol; hERα, human estrogen receptor α; bw, body weight; BPA, bisphenol A; BAP, bacterial alkaline phosphatase; DMSO, dimethyl sulfoxide; TIF2, transcriptional intermediary factor 2; REC10, 10% relative effective concentration; FDA, the U.S. Food and Drug Administration; PND, postnatal days; JECFA, the Joint FAO/WHO Expert Committee on Food Additives.

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doi:10.1016/j.toxlet.2011.12.004

2008; Soto and Sonnenschein, 2010; Laws et al., 2000). These substances are commonly detected in various environmental media and can be easily absorbed into the body. However, most of these well-known EDCs are trace chemicals in environmental samples, and to date, there is still no firm evidence that certain environmental EDCs cause health problems at low levels of exposure. Moreover, although many of these EDCs have been banned or restricted in use, all of the endocrine-disruption-related public health disorders described above continue to increase in frequency. Therefore, it is possible that we may not recognize that some EDCs in large doses are seriously adversely affecting human health.

Salicylate esters (SEs) are a group of suspected EDCs, as described above. SEs are predicted to have estrogenic effects based on findings from an automated docking method, and some SEs have demonstrated estrogenic activities, as reported in previous studies that used *in vitro* assays. For example, benzyl salicylate (BzS) was reported to possess estrogenic activity in assays using the estrogen responsive MCF7 human breast cancer cell line (Charles and Darbre, 2009). Many SEs have been extensively used as flavoring agents or fragrances in a variety of foods, beverages and consumer products, and humans are exposed daily to large doses (Surburg and Panten, 2006). For instance, as flavoring agents in foods, the estimated daily intakes of methyl salicylate (MS), ethyl salicylate (ES) and BzS for the U.S. population were 740, 29 and 0.5 μg/kg body weight (bw) daily, respectively (Adams et al., 2005). As a fragrance additive used in cosmetics and fragrances, BzS has been calculated to be applied to the skin in concentrations up to 402.3 μg/kg bw daily (Belsito et al., 2007; Lapczynski et al., 2007). However, to date, there is little knowledge on the toxicology of SEs, and no *in vivo* studies on the estrogenicity of SEs have been reported. No standard for SEs with respect to estrogenicity nor information on the estrogenic risk of SEs in foods and personal care products is available. Therefore, the objectives of this study were to evaluate the estrogenic potencies of the commonly used SEs using *in vitro* and *in vivo* assays, to calculate the maximal acceptable daily exposure concentrations and to assess the potential risks associated with some applications of SEs with respect to their estrogenicity.

2. Materials and methods

2.1. Chemicals

The compounds 17β-estradiol (>98.0%, E_2), phenethyl salicylate (>97.0%, PES) and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). BPA (>99.0%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). BzS (>99.0%) was purchased from Acros Organics (NJ, USA). MS (>99.5%), ES (>99.0%) and phenyl salicylate (>99.0%, PhS) were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). The structures of the SEs tested in this study are shown in Table 1.

2.2. Methods

2.2.1. Automated molecular docking

Scigress (Ultra Version 2.2.0, Fujitsu, USA) is a very useful pre-screening tool for developing novel estrogen receptor ligands (Kiss and Allen, 2007) and was used to dock flexible ligands into a rigid protein active site. The three-dimensional structure of the hERα-LBD (ligand-binding domain of human estrogen receptor α) (PDB ID 1ERE) was downloaded from the Protein Data Bank web site (http://www.rcsb.org/pdb). The protein structure was cleaned and reduced to a monomer of chain A. The active site of chain A was used 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 structure information of known protein-ligand complexes contained in the Protein Data Bank, was used to score the binding affinity of a compound in the active site. The original ligand in the complex, 17β-estradiol (E_2) was docked into the binding site. PMF for E_2 was −55.655 kcal/mol, similar to the reported PMF (−55.745 kcal/mol) of another hERα-LBD template (PDB ID 1A52) by Kiss and Allen (2007). The root mean square error (RMSE) between the previously reported and newly calculated binding sites of E_2 was 0.2659 Å, which was similar to that reported for E_2 docked into the PDB ID 1A52 template (0.2532 Å) (Kiss and Allen, 2007). The low RMSE indicated the reliability of this *in silico* method. The PMF value for BPA, a well-known estrogenic compound, was calculated to be −53.694 kcal/mol. Therefore, we assumed that compounds with higher PMFs than that of BPA are "high-affinity" compounds for the hERα ligand binding site.

2.2.2. Human estrogen receptor α-coactivator recruiting assay

The estrogen receptor agonist activity of the SEs was measured using a ligand-dependent coactivator recruiting assay with glutathione S-transferase (GST)-tagged hERα-LBD (Kanayama et al., 2003). Preparation of 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 and the ligand binding assay were conducted according to Kanayama et al. (2003). Stock solutions of test chemicals were subjected to a 10-fold serial dilution with DMSO to prepare eight concentrations in the range of 10^{-3}–10^{-10} M. Finally, the binding affinities of the tested chemicals for hERα were expressed as the absorbance at 405 nm (BAP activity). The wells with only DMSO added were used as background values for this assay. A sigmoidal concentration-effect curve for each of the tested chemicals was calculated using the Graphpad Prism 4 software (GraphPad Software, Inc., San Diego, CA). The 10% relative effective concentration (REC10), the maximal acceptable daily exposure, and the estradiol equivalent concentrations (EEQ) of the SEs were calculated based on a sigmoidal concentration-effect curve of E_2 standards obtained using the same plates. The REC10 is the concentration of the test chemical corresponding to 10% of the maximum activity of E_2; the maximal acceptable daily exposure is the concentration of the test chemical showing the same agonist activity as 0.05 μg/kg bw of E_2, which was determined by the Joint FAO/WHO Expert Committee on Food Additives (JEFCA) as the maximal acceptable daily E_2 intake (JECFA, 1999). The EEQ values are the concentrations of E_2 showing the same agonist activities with the test chemicals.

2.2.3. Immature mouse uterotrophic assays

Immature female CD-1 mice at an age of 19 postnatal days (PNDs) were obtained from the Experimental Animal Tech Co. of Weitonglihua (Beijing, China). They were housed in stainless steel wire-mesh cages in a temperature-controlled room on a 12 h light: 12 h dark cycle. The animals were housed three to a cage, fed *ad libitum* with a basic diet from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China) and were provided water *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Peking University. The mice were weighed, weight-ranked and assigned randomly to each of the treatment and control groups; each group consisted of 12 mice. Body weights were recorded daily throughout the study. The dose of 300 mg/kg/day was selected as the highest dose for each of the SE chemicals based on available information from the literature suggesting that this dose would likely cause no visible toxicity in the animals. Stock solutions of test chemicals were subjected to a 3-fold serial dilution in peanut oil to prepare the doses. Intragastric administration (20 mL/kg bw) of control and test compounds to each mouse was performed daily for 3 days beginning on PND 21 according to the weight of the mouse. At PND 24, the mice were weighed and sacrificed by cervical dislocation, and their uteri were dissected. Each uterus was blotted, and the wet weight was recorded. Groups treated with peanut oil only were used as vehicle controls, and E_2 was used as the positive control at doses of 10, 50 and 400 μg/kg bw/day. The immature mouse uterotrophic assay was performed in several experiments, and in each experiment, one or two chemicals were tested.

2.2.4. Immature rat uterotrophic assays

Immature female Sprague Dawley rats at an age of 20 PNDs were obtained from the Experimental Animal Tech Co. of Weitonglihua (Beijing, China). The animals were housed two or three to a cage and then acclimatized in a controlled environment with a temperature of 22 ± 2 °C, a relative humidity between 40% and 60% and an artificial lighting at 12 h light: 12 h dark cycle. The animals were fed *ad libitum* with a basic diet from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China), and drinking water was provided *ad libitum*. Before experiments, the rats were randomly assigned into each of the treatment and control groups. Body weights were recorded daily throughout the study. Intragastric administration (5 mL/kg bw) of control and test compounds to each rat was performed daily according to the weight for 3 days beginning on PND 21. Groups treated with peanut oil only were used as vehicle control, and E_2 was tested at doses of 1, 5, 25, 100 and 400 μg/kg bw/day. Doses of 1.23, 3.70, 11.1, 33.3 and 100 mg/kg/day were selected for BzS. On PND 24, the rats were weighed and sacrificed under chloroform anesthesia 24 h after the final treatment. Their uteri were dissected. Each uterus was blotted, and the wet weight was recorded. All procedures were approved by the Institutional Animal Care and Use Committee of Peking University.

2.2.5. Data analysis

Data are expressed as the mean ± standard deviation (SD), unless otherwise stated. The statistical program SPSS (Ver 13.0; Chicago, IL, USA) and Excel (Microsoft, NY, USA) were used to analyze the data. Group differences were evaluated by one-way Analysis of Variance and Fisher's Least Significant Difference (LSD) test. For the group treated with BzS in the immature rat uterotrophic assays, a chi-square test was used to determine the difference in the number of rats with uterine weights