Prediction of the metabolic clearance of benzophenone-2, and its interaction with isoeugenol and coumarin using cryopreserved human hepatocytes in primary culture

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Abbreviations

BP2, benzophenone-2; CHHP, cryopreserved human hepatocytes in primary culture; UDPGA, uridine 5'-diphospho-glucuronic acid; UGT, UDP-glucuronosyltransferase; HPLC, high pressure liquid chromatography; XME, xenobiotic metabolizing enzymes.

Abstract

Benzophenone-2 (BP2) is widely used as a UV screen in both industrial products and cosmetic formulations, where it is frequently found associated with fragrance compounds, such as isoeugenol and coumarin. BP2 is now recognized as an endocrine disruptor, but to date, no information has been reported on its fate in humans. The intrinsic clearance (Cl_{int}) and metabolic interactions of BP2 were explored using cryopreserved human hepatocytes in primary cultures. In vitro kinetic experiments were performed to estimate the Michaelis-Menten parameters. The substrate depletion method demonstrated that isoeugenol was cleared more rapidly than BP2 or coumarin (Cl_{int} = 259, 94.7 and 0.40µL/min/10^6cells respectively). This vitro model was also used to study the metabolic interactions between BP2 and isoeugenol and coumarin. Coumarin exerted no effects on either isoeugenol or BP2 metabolism, because of its independent metabolic pathway (CYP2A6). Isoeugenol appeared to be a potent competitive substrate inhibitor of BP2 metabolism, equivalent to the specific UGT1A1 substrate: estradiol. Despite the fact that inhibition of UGT by xenobiotics is not usually considered to be a major concern, the involvement of UGT1A1 in BP2 metabolism may have pharmacokinetic and pharmacological consequences, due to the its polymorphisms in humans and its pure estrogenic effect.

1. Introduction

Benzophenone-2 (BP2) is one of the 12 main benzophenone derivatives (BP1 to BP12), which are widely used as UV screens to protect from light induced damage. These compounds are also used as photo stabilizers in cosmetics and personal care products, as well as sunscreens to protect skin from UV irradiation. Skin creams, lotions and perfumes are complex formulations which include numerous fragrance compounds, nanomaterials and various other elements. As these products are intended for direct application to the skin, human exposure to these complex mixtures can occur through dermal absorption. In many EU countries and America, BP2 is no longer permitted for use in sun lotions due to the high concentrations (±10%) traditionally used in these formulations. However, BP2 is still used in plastics, printing ink and cosmetics, to prevent UV induced damage. Often found in wastewater BP-2 is considered an emerging contaminants of concern by the US EPA. It has therefore been suggested that human skin may have daily contact with BP2 (Asimakopoulos et al. 2014) but no information on other possible routes of exposure to BP-2 is available (Buck Louis et al. 2014; Gao et al. 2015). This concept warrants further investigation, not least because the incidence of skin cancer and the photo damaging effects of UV radiation have increased the use of UV protection products. Furthermore, BP2 and other benzophenone derivatives have previously been classed as endocrine disruptors (EDC) (Muncke 2011). Thirdly presence of BP2 was detected in human tissues samples (Asimakopoulos et al. 2014; Buck Louis et al. 2014; Gao et al. 2015). Several studies have also highlighted the estrogenic effects of BP2 both *in vivo* in rats, *in vitro* in fish models and in coral (Weisbrod et al. 2007; Kim et al. 2011; Downs et al. 2014). Although the potency of this compound as a hormone receptor agonist or antagonist is low, when compared to natural ligands, BP2 has been described as one of the most potent of the BP family, and can elicit harmful biological effects by interacting directly with androgen and estrogen receptors (Molina-Molina et al. 2008), and with the thyroid hormone axis (Schmutzler et al. 2007; Hofmann et al. 2009). A recent study found a clear association of a higher male BP-2 concentration with diminished couple fecundity (Buck Louis et al. 2014). Elimination of these xenobiotics from the body chiefly occurs through Phase I, II and

III detoxification processes, however, metabolic interactions between personal care components and BP2 may interfere with its elimination.

Among the multitude of compounds frequently found in cosmetic formulations, coumarin and isoeugenol are commonly associated with BP2. Though coumarin metabolism has been investigated in human and animal models (Felter et al. 2006), BP2 and isoeugenol metabolic pathways are less well documented, and only described in animals.

For all BP congeners, the possible average daily intake (PADI) value was estimated to be 0.33mg/day/kg but this was possibly an over-estimation (Jeon et al. 2008). For isoeugenol and coumarin, the PADI values were estimated to be 0.2mg/day/ kg and 0.1mg/day/kg respectively. These were deemed to be far below the values expected to elicit adverse effects in susceptible species. This is particularly true for coumarin, the toxicity of which depends on metabolism via a detoxification pathway in humans (7-hydroxylation) followed by glucurono- and sulfo-conjugation, or an activation process in rat and mouse models (3, 4 epoxidation), which can be inactivated through glutathione conjugation (Lake 1999).

The pharmacokinetics and metabolism of BP2 and isoeugenol were studied in rats, and it was demonstrated that, after gavage, BP2 was rapidly metabolized to BP2-glucuronide and BP2-sulfate. However, despite the rapidity of the metabolism, estrogenic effects were still observed in the rat uterus (Molina-Molina et al. 2008). Metabolic patterns of BP-2 in numerous cell lines was also investigated showing in HepaRg cells a predominance of a direct glucuronoconjugation. As BP2 is a component of many cosmetics, daily human exposure has been confirmed by biomonitoring, which showed the presence of BP2 in human urine (Asimakopoulos et al. 2014; Buck Louis et al. 2014) Although the metabolic pathways associated with BP2 and isoeugenol have been investigated, little is known regarding their biotransformation in humans or their interactions with other cosmetic components.

The aim of this study was to investigate metabolism of BP2, isoeugenol and coumarin in human hepatocytes and to assess the metabolic interactions induced by isoeugenol and coumarin when co-administered with BP2. The metabolic information obtained in this study may be integrated into

physiologically based pharmacokinetic (PBPK) models to predict the fate of BP2 and metabolites in the human body.

2 Materials and methods

2.1 Chemicals

Culture medium and fetal calf serum (FCS) were obtained from Life Technologies Inc. (St Aubin, France). [Ring-14C(U)]-isoeugenol (8mCi/mmol specific activity), [4-14C]-coumarin (14.5 mCi/mmol specific activity) and [3H]-BP2 (20 Ci/mmol specific activity) were purchased from Bioactif (Strasbourg, France). Radiochemical purity was ±96%. 2,2′,4,4′-tetrahydroxybenzophenone (BP2), isoeugenol and coumarin were from obtained from Sigma-Aldrich Inc. (St Quentin Fallavier, France). All other chemicals were of the highest quality available from commercial sources. Centrifree ultrafiltration tubes (YMT membrane, 30,000 molecular weight cutoff) were supplied by Millipore. (Merck, France). All cell culture plastics were obtained from Falcon (Merck Eurolab, Strasbourg, France).

2.2 Human primary hepatocytes preparation

The tissue liver was obtained from the Digestive Unit, Archet 2 Hospital, University Hospital of Nice, France. With informed consent of the tissue donor, and following the ethical guidelines, piece of liver were collected from patient undergoing partial hepatectomy. Patient suffering from infectious disease (hepatitis, HIV) or cirrhotic and steatotic livers were excluded. Only one liver was taken from the program of multi -organ donors due to no transplant receiver. In a studies of Vondran et al. (2008) and Hewes et al. (2006) they indicate that there is no influence of previous chemotherapy on the isolation outcome or subsequent hepatocytes function. Period between chemotherapy and surgery may have allowed functional hepatocyte recovery.

Human hepatocytes were isolated, cryopreserved and thawed as previously described by de Sousa et al. (1997; de Sousa et al. 1996). Briefly, upon removal of piece of liver (CHU l'Archet, Nice, France), the tissue part tumor free was placed in cold sterile L-15 medium and immediately transferred to the laboratory. Hepatocytes isolation was performed under sterile conditions using a three-step collagenase

perfusion. The piece of liver was firstly perfused using an isotonic buffer containing EGTA, followed by the same buffer without EGTA. For tissue dissociation, the liver was perfused with isotonic buffer containing calcium and collagenase 0.05% (Roche Diagnostics, Meylan, France) in recirculation mode for 12-17 min. After a mechanical disruption of the tissue, the cells were washed by centrifugation (35 g, 3min). The pellet containing mainly hepatocytes is then cryopreserved as described by de Sousa et al.

Human hepatocytes (Table 1) from different donors were cultured into 48-wells Corning® Costar® plates (VWR, France), previously coated with rat tail collagen, at about $\pm 0.6 \times 10^5$ cells/well in an atmosphere of 5% CO_2 and 95% relative humidity in presence of 25 to 32 % of percoll depending the liver to allow only viable to settle down. After 30-60 min, depending the attachment rate of the cells estimated by visual examination, the medium in each well was removed and replaced by 200 µL of Williams E medium containing 10% of fetal bovine serum (FBS), 1 X of a mix of insulin, selenium and transferrin (ITS) and 0.5% of a mix of streptomycin (10,000 µg/mL) and penicillin (10,000 unit/mL) (Life Technologies, Saint–Aubin, France). After 24 hours, the cells are washed with William's E medium containing no FCS and no phenol red but supplemented with human serum albumin (HSA, 0.24µg/ml; which correspond to ±3.6 µM). Incubation with test compounds was performed in the same medium. Previously to the first experiments, BP2, isoeugenol and coumarin stability in the incubation medium was tested, as well as the nonspecific binding on culture plate. All experimental procedures were done in compliance with French laws and regulations as defined by the National Ethics Committee. Table 1 described the characteristics of donors.

2.3 Nonspecific Binding to Microsomes and Ultrafiltration Tubes.

Solutions of BP-2 were added to medium containing +/- BSA to give final concentrations of 10µM. 1 ml, in triplicate, of these solutions were added to Centrifree ultrafiltration tubes (excluding any air bubbles) and centrifuged (2000g) using a 33°C fixed angle rotor for 30 min. Binding to the Centrifree tube membrane and collection cup was determined by measuring radioactivity.

2.4 Design of experiments

The stock solutions and successive dilutions of BP2, isoeugenol and coumarin were freshly made for each experiment. They were prepared in DMSO as a 400 times concentrated solution and then diluted in William's E medium containing no FCS and no phenol red but supplemented with HSA (0.25% DMSO final concentration). The test solutions were incubated in a polypropylene tube for 10 min at 37°C. Incubations were started by the addition of 200 µl of prewarmed test solutions. Due to the high turnover monolayer cultures were gently shaken for experiment with BP2 and isoeugenol. No sign of any detachment of hepatocytes from collagen was observed during the incubation with compounds. At various intervals times (Table1), which depend on preliminary experiments, reactions were stopped by addition of 200µL of cold acetonitrile. Cells were scraped and stored at -20 °C until analysis. Prior to HPLC analyses the medium/ACN containing cells were submitted to centrifugation to remove proteins. Three to four independent preparations of cryopreserved human hepatocytes in primary culture (CHHP) were used for BP2 and isoeugenol metabolic studies, and a pool of three human cryopreserved hepatocytes batches were used for coumarin.

To quantify the number of hepatocytes, 2 to 3 blank wells were fixed with iced MeOH during 2-3 min and after coloration by the fluorescent dye HOESCHST 33342 (Sigma Aldrich) in phosphate buffer, the adherent cells were counted by using an Arrayscan XTI (Thermo Fisher Scientific, Courtaboeuf, France). Viable cells were distinguished from dead cells based on the size and the intensity produced by the fluorochrome of their nucleus.

2.5 Inhibition of BP2 metabolism by isoeugenol

To evaluate the competitive substrate inhibitory potency of isoeugenol on BP2 metabolism, cryopreserved human hepatocytes in primary culture were exposed to 10µM of BP2, alone and in the presence of 6 concentrations of isoeugenol (0, 2.5, 5, 10, 30, 60 and 120µM). Reactions were stopped after 20, 40, 60 min of exposure. In addition, a representative time-course effect of estradiol was also performed (2.5, 5, 10, 25, 50 and 100µM). Inhibition experiments were conducted on cryopreserved human in primary culture of a pool of three independent human hepatocytes batches (two experiments in duplicate).

2.6 Sample analysis

The whole hepatocyte extracts from incubations of compounds were analyzed by HPLC with radiochemical detection, after centrifugation. Quantification of BP2, isoeugenol and coumarin was performed with a Synergi 4 µm Hydro-RP 80 Å (250×4.6 mm) (Phenomenex, Le Pecq, France) by a Dionex liquid chromatography system with a P580 pump and an UVD 3405, coupled with flow scintillation analyzer 500 TR Series (Packard Biosciences Company, CT, USA). The mobile phase consisted of two solvent: A, H_2O for isoeugenol and BP2 and acetate buffer 20 mM (pH = 4.5) for coumarin; and B, 25%/75% mix of MeOH/AcN. Same column was used for the three chemicals, with only differences in solvent gradient in order to optimize separation of metabolites from parent molecules (supplemental data). Unchanged chemicals and total metabolites were quantified.

In order to analyze the overall metabolism of isoeugenol when used as an competitive substrate inhibitor in metabolic interaction studies, quantification of its unchanged form was performed from its absorbance at 270nm. A calibration curve was established and its linearity was evaluated by least-squares linear regression analysis. The calibration was performed from the T0 solution for each independent experiment.

2.7 Data analysis

Data are expressed as mean ± S.D of at least three independent hepatocyte's experimentations and from two independent preparations in duplicate for interaction studies. The data obtained were analyzed based on Michaelis-Menten kinetics, substrate-velocity curve was calculated using statistical software (Statistica V12.0, Stasoft, France) and GraphPad Prism (GraphPad Software V5.0, San Diego, CA) was used to determine K_m and V_{max} values using nonlinear regression analysis fitting : $V=(V_{max} \times S)/(1+K_m)$. Data are expressed as mean ± S.D.

To evaluate the inhibition potency of isoeugenol, a relative activity, BP2 metabolism in the absence of competitive substrate inhibitor, was set to 100%. The enzyme inhibition parameter Ki was calculated by fitting the experimental data to a 4 parameters sigmoid curve, where x represent the isoeugenol concentration. Statistical differences were determined using one-way analysis of variance with LSD Fischer post hoc tests. Data are presented as means ± standard deviations.

3 Results

3.1 *In vitro* metabolism of BP2, Isoeugenol and Coumarin

As hepatocytes represent the most appropriate model for the evaluation of integrated xenobiotic metabolism, cryopreserved human hepatocytes in primary culture (CHHP, see supplemental data N°2), from different donors, were exposed to BP2, isoeugenol and coumarin in a dose and time dependent manner. Metabolite formation was determined from human hepatocytes, using [^{3}H] BP2, [^{14}C] isoeugenol and [^{14}C] coumarin (Figure 1), with HPLC and radiochemical detection. Typical representative radiochromatograms of samples obtained by incubating BP2 with human hepatocytes are represented in Figure 2. BP2 was rapidly metabolized by human hepatocytes, mainly as a metabolite co-eluting with the solvent front (Figure 2) and a minor one. Comparison of radiochromatographic profiles from before and after treatment of the extracellular medium with B-glucuronidase from E.Coli, indicated that this metabolite was formed by direct conjugation of BP2. Isoeugenol also underwent rapid metabolism to produce four or five metabolites, the chief of which co-eluted with the solvent front. As observed for BP2, when incubated with B-glucuronidase from E.Coli, this metabolite appeared to be a direct conjugation of isoeugenol. In comparison with BP2 and isoeugenol, coumarin is much more slowly metabolized into 3 metabolites, and as before, the major metabolite is co-eluted with the solvent front. At the concentrations used, no toxic effects, from any of the compounds, were observed.

A 10µM solution of BP-2 in Medium ± BSA was chosen to investigate nonspecific binding to protein. No differences were found between radioactivity measurements made on medium with or without serum, we supposed that only a marginal degree of nonspecific binding of compounds occurred to hepatocytes or HSA. Indeed, we have previously exposed human hepatocytes to BP2 and isoeugenol for longer exposure times, which led to the total consumption of these products (data not shown). Moreover, total recovery of radioactivity in the extracellular medium was almost 95% for all conditions and both plastic binding and the radioactivity content of the hepatocytes were negligible with no detectable presence of any metabolites or BP-2. Similar results were made by Le Fol et al. (Le Fol et al. 2015)

3.2 Time course and concentration response relationships of BP2, isoeugenol and coumarin: total metabolite formation in CHHP

To further examine the depletion rate of BP2, isoeugenol and coumarin in CHHP, we examined the kinetic appearance of total metabolites at different concentrations and exposure times. All compounds were found to be stable in the media in the absence of cells. As the human exposure to these molecules is estimated to be low, concentrations used were in the range of 2.5µM to 50µM, to closely mimic *in vivo* levels. Figure 3 displays the depletion rates of BP2, isoeugenol and coumarin, represented by the rate of formation of all metabolites. As the metabolism of a chemical via the xenobiotic metabolizing enzymes (XME) can be viewed as an enzymatic reaction, it therefore can be described by Michaelis-Menten kinetics. Moreover, there is usually more than one XME present, and each have their own individual V_{max} and K_m values, which contribute to the apparent K_m and V_{max} (Figure 4) calculated from the dose and time responses in Figure 3.

The apparent Michaelis-Menten parameters calculated for the depletion of BP2 were estimated as being 34.2 ± 9.8 µM for the affinity constant (K_m) and 3.24 ± 0.49 nmol/min/10^6 hepatocytes for the V_{max}, giving an intrinsic clearance of 94.7 µl/min/10^6 hepatocytes. For isoeugenol, the estimated $K_m$was 16.0 ± 6.1 µM and the V_{max} were 4.14 ± 0.63 nmol/min/10^6 hepatocytes. These values lead to an estimated *in vitro* intrinsic clearance of 259 µl/min/10^6 hepatocytes. Coumarin was the compound with the lowest turn-over V_{max} (0.065 ± 0.0015 nmol/min/10^6 hepatocytes) and K_m (1.60 ± 0.14 µM), leading to an estimated intrinsic clearance value of 0.40 µl/min/10^6 hepatocytes. The Michaelis-Menten parameters, obtained from the observed rates and kinetics are summarized in Table 2. As the total hepatocyte volume was low, when compared to published data (4µl/10^6 cells), we can estimate that the cell volume was 0.29µL per 200µL extracellular medium in our experiments, which was considered to be negligible and therefore no correction for Cl_{int} was made (Jones and Houston 2004).

3.3 The effect of isoeugenol on BP metabolism

Due to the large differences in *Vm*, K_m and Clint values between isoeugenol and BP2, and coumarin, we only investigated the effect of isoeugenol on BP2 metabolism. Preliminary experiments showed that

coumarin had no effect on BP2 metabolism and did not potentiate the inhibition provoked by isoeugenol. A unique concentration of BP2 (10µM), lower than the K_m, was incubated in the presence of six concentrations of isoeugenol (2.5, 5, 10, 30, 60 and 120µM) for 20, 40 or 60 min of exposure with human hepatocytes. We estimated that the Ki values were 11.17 µM, 18.0 µM and 18.46µM after 20, 40 and 60 min of incubation respectively. Preliminary studies revealed that only estradiol was capable of totally inhibiting benzophenone metabolism at a concentration of 100µM after 20 min of exposure to CHHP, whereas morphine exerted only a very slight effect. We have therefore compared the estradiol competitive substrate inhibitor potency to that of isoeugenol, in one single experiment. At concentrations of up to 10µM, isoeugenol and estradiol exhibited the same effects on BP2 metabolism (Figure 5). Thus, considering that estradiol is a specific substrate of UGT1A1 and to a lesser extent, UGT2B7, we can postulate that UGT1A1 is the main Phase II enzyme implicated in the direct detoxification of BP2 (see supplemental data for UGT1A1 immunolocalisation in human hepatocytes). The analysis of isoeugenol depletion products from the same sample used for BP2 metabolism showed that isoeugenol, as predicted by its $Cl_{int,}$ was rapidly metabolized by CHHP. Indeed after 60 minutes of exposure to CHHP, most of the isoeugenol was metabolized up to a dose of 30µM (Figure 6).

4 Discussion

Benzophenone-2 is an additive which acts as a UV filter in personal-care products and as a light stabilizer for plastics, coatings, ink and adhesives. Despite its classification as nonpersistent chemical its presence in in human asks the question where this compound is really coming from (Buck Louis et al. 2014; Schlumpf et al. 2010). BP2 has been classed as an endocrine disruptor and, because of its presence in our environment, it is now considered to be a "contaminant of emerging concern" (Downs et al. 2014). BP2 has been shown to have pure estrogenic effects (Seidlova-Wuttke et al. 2004), to interfere with the thyroid hormone axis in rats (Schmutzler et al. 2007) with anti-androgenic potencies (Molina-Molina et al. 2008) and to impaired human couples fecundity (Buck Louis et al. 2014).

The work presented here was undertaken initially to evaluate the metabolic stability of BP2 with the aim of predicting its human metabolic clearance rate, using the substrate depletion approach. We further sought to study the potential metabolic interactions of BP2 with isoeugenol and coumarin, as cosmetic formulations frequently contain BP2 in combination with these fragrance compounds (Schlumpf et al. 2010). Indeed, it is now well recognized that information on the metabolism of a chemical is of crucial importance for evaluating its toxicity and potential impact on endocrine function (Jacobs et al. 2008). As the metabolism of xenobiotics occurs mainly in the liver, human hepatocytes in primary culture were used in our study. Indeed these cells are considered to be the gold standard model for evaluating hepatic metabolism, because they represent intact cellular systems, containing a functional membrane that encompasses all components of phase I, II and III metabolizing enzymes and transporters (Lam and Benet 2004). This model could help enhance the understanding of the BP2 metabolic pathway, as well as elucidate potential interactions with other molecules (Fasinu et al. 2012; Brown et al. 2007). To our knowledge, BP2 and isoeugenol metabolism were until now never investigated in human hepatocytes. Previous rat and in vitro studies showed that these two compounds are directly metabolized mainly by phase II enzymes to glucuronide - and sulfate-conjugates (Le Fol et al. 2015; Schlecht et al. 2008; Badger et al. 2002). In this study, we have not been able to characterize all metabolites formed after exposure to

human hepatocytes. Nevertheless, we have shown that the main BP2 metabolite has been formed, by a direct conjugation, through the action of UGT1A1, to UDPGA. This was confirmed by the competitive substrate inhibitor potency of estradiol, which in human hepatocytes is mainly metabolized into estradiol-3-glucuronide through UGT1A1 (Watanabe et al. 2003; Williams et al. 2002; Donato et al. 2010) and to a much lesser extent, morphine inhibition via UGT2B7 (Liu et al. 2011). BP2 elimination (after glucuronidation) is rapid in rats. As our results indicate that BP2-glucuronide represents: ± 30%, ± 46.2% and ± 56.32% after 20 min, 40 min and 60 min of exposure respectively, and total metabolites correspond to ± 35%, ± 55% and 74% of the total radioactivity, when human hepatocytes are exposed to 10µM BP2, the same conclusion can be drawn for humans.

Due to the critical role of glucuronidation in the detoxification of BP2 and isoeugenol, we have used human hepatocytes in primary culture after a culture period of 24 hours, which allows the recovery of the cofactors required for phase II reactions (Li 2007). In contrast to experiments using cryopreserved human hepatocytes in suspension, this system does not require any UDPGA supplementation (Kuester and Sipes 2007). Furthermore, the substrate depletion method is now a popular approach for determining the *in vitro* metabolic clearances of drugs or xenobiotics. There is no nonspecific binding of BP-2 to HSA. When used at low ratios, as in our study (± 0.35 10^6 cells/ml), predictions obtained from hepatocytes were shown to be more accurate than those from microsomes and didn't necessitate nonspecific binding studies (Jones and Houston 2004; Ito and Houston 2004). Moreover, it was found that clearances predicted from human liver microsome incubations underestimated the *in vivo* hepatic clearance rates for compounds that undergo phase II metabolism (Boase and Miners 2002; Engtrakul et al. 2005; Soars et al. 2002).

In contrast, coumarin exhibits a very slow turnover, therefore requiring hepatocyte exposure of up to 24 hours (CL_{int} = 0.40 µL/min/10^6cells). For this reason, this compound was not investigated further in our study. Only BP2 and isoeugenol metabolic interactions were examined over a shorter period of time, during which the expression of xenobiotic metabolizing enzymes were well maintained. In the rat, BP2 biotransformation is rapid, with a dose dependent ratio of BP2-glucuronide to unchanged BP2 of 10:60.

For isoeugenol, following oral or *iv* administration, more than 90% of the dose was eliminated from the body, as only metabolites, and no parent isoeugenol, were detected in the blood (Badger et al. 2002).

To our knowledge, this is the first time that CL_{int} has been estimated for BP2 and isoeugenol using human hepatocytes in primary culture. The results reported here support data obtained from rat studies, which showed a more rapid clearance for isoeugenol than for BP2 (CL_{int} for isoeugenol 259 µL/min/10^6cells and CL_{int} for BP2 94.7 µL/min/10^6cells). Our results also support the conclusion that these two derivatives undergo extensive metabolism and that the predominant metabolic route is glucuronidation, despite the fact that other unidentified metabolites were found in the incubation medium. These two molecules compete, at least in part, for the same UGTs. At a concentration of 10 µM or less, the effects of isoeugenol are similar to those exerted by estradiol, which inhibits the glucuronidation of BP2 and its other unidentified metabolites.

The important role of UDP-glucuronosyltransferases (UGTs) in the metabolic elimination of xenobiotics and endogenous substances has been drawing more and more attention in recent years. As BP2 glucuronide is dependent upon UGT1A1, we hypothesized that in type I Crigler-Najjar syndrome patients, who are deficient in hepatic UGT1A1, BP2 clearance can be slower (Strassburg 2008), which may further exacerbate the EDC properties observed in normal phenotypes, and interfere with the elimination of isoeugenol or bisphenol A (Trdan Lusin et al. 2012) and endogenous compounds such as bilirubin and estradiol.

Numerous studies have shown that within the benzophenone (BP) family, all 12 of the main derivatives have been used as UV filters, and some have estrogenic potencies in different species of mammals and fish. They are able to interact with estrogen, androgens and the thyroid receptor signaling pathwayss. Biologically, BP2, which is now considered to be an EDC, has been shown to be one of the most potent compounds of the benzophenone series. As with metabolism, the transcutaneous absorption rate of BP2 in humans is rapid, but rat studies have proved that despite rapid clearance from the organism, BP2 can still exert its EDC effects, even at low levels of unchanged BP2 (Schlecht et al. 2008). Aside from this referenced work, most of the studies that have examined the EDC properties of BP2 have used

subcellular hormone receptor ligand binding methods, proliferation induction in hormone responsive cell lines, transactivation systems in mammalian cell lines (Molina-Molina et al. 2008) and docking analysis (Kerdivel et al. 2013) methods. In these studies, no biotransformation characterization or intrinsic metabolic capacity investigations of these cell lines were undertaken. Indeed, Phase I, II and III steps which detoxify and eliminate xenobiotics are also implicated in the metabolism of endobiotics and more particularly endogenous steroids. Inadequate information on the fate of a possible EDC could therefore give rise to false positive (lack of metabolism) or false negative (lack of activation) data.

Furthermore, all living organisms are exposed to a large variety of xenobiotics with EDC properties. Human EDC exposure is commonly as a result of contact with compound mixtures, which, due to the concentration addition law (Silva et al. 2011; de Sousa et al. 2014), may produce significant effects, even if each chemical present is at a concentration low enough to avoid individual provocation of undesirable reactions. This concept is discussed further in the Kortenkamp study (Kortenkamp et al. 2014) which implicates BP2 in the decline of male reproductive health.

In conclusion, in contrast to coumarin, BP2 and isoeugenol undergo extensive hepatic metabolism. Their predominant route of elimination is via a direct conjugation to UDPGA, by the action of the UGT1A1. At the anticipated levels of human exposure to BP2 and isoeugenol, saturation of the detoxification process is unlikely to occur. However, in this case, given the lack of knowledge about the human exposome, which would traditionally characterize the total effects of external human exposure to a compound, we cannot entirely anticipate the fate of BP2 in the human body or deny the possibility that even at low doses, in combination with other EDCs, it may act as an endocrine disruptor.

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Figure legends

Figure 1. Chemical structures of substrates and competitive substrate inhibitor used in the study. 1) benzophenone-2, 2) isoeugenol, 3) coumarin, 4) estradiol.

Figure 2. Representative HPLC radiochromatograms of BP2 metabolites, incubated at 10µM ([^{14}C] BP2) for 20 min with cryopreserved human hepatocytes in primary culture. Effect of competitive substrate inhibitor: isoeugenol and estradiol at the specified concentrations.

Figure 3. Dose and time dependent metabolites formation of BP2 (A), isoeugenol (B) and coumarin (C) in CHHP. The line represents the best fit of the data to a polynomial equation in order to determine the initial velocity. Data are represented as the mean of three to four independent CHHP preparations and the bars represent the ± SD.

Figure 4. Michaelis-Menten study of BP2, isoeugenol and coumarin in human cryopreserved human hepatocytes in primary culture. Insert Lineweaver-Burke plots for each profile.

Figure 5. Dose and time effects of isoeugenol ( ) and estradiol ( ) on BP2 metabolism. BP2 was incubated at 10 µM on CHHP in the presence of estradiol and isoeugenol at the specified concentration for 20, 40 and 60 min. Line is drawn point to point. Data are represented as the mean of two independent experiments in duplicate (except for estradiol). One way analysis of variance was followed by a LSD Fischer post hoc multiple comparisons test to control. *P < 0.05; ***P < 0.001 compared to CHHP exposed to BP2 alone.

Figure 6. Total metabolites (%) of isoeugenol, at identical concentration as in Figure 5, formed in the presence of 10µM of BP2, at 20, 40 and 60 min time of exposure.

Tables

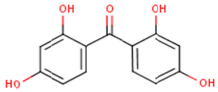
Table 1. Characteristics of donors. *: hepatocytes used for interaction studies.

Lot number	Age	Sex	Disease
HuF31*	31	F	Colorectal Metastasis
HuF82	82	F	Colorectal Metastasis
HuM66*	66	M	Colorectal Metastasis
Hu1195	M	49	Not available
Crystal*	No information, the liver was anonymized due to multi-organ removal (French Law).		

Table 2. Estimated values (average ± standard deviation) of the kinetic constants V_{max} and K_m and the ratio V_{max}/K_m of the depletion of BP2, isoeugenol and coumarin in cryopreserved human hepatocytes in primary culture.

	V_{max} (nmol/min/10^6cell)	K_m (µM)	V_{max}/K_m (µL/min/10^6cells)	V_{max}/K_m (mL/min/kg)
Benzophenone-2	3.24 ± 0.49	34.2 ± 9.8	94.7	243.7
Isoeugenol	4.14 ± 0.63	16.0 ± 6.1	259	666.7
Coumarin	0.065 ± 0.0015	1.60 ± 0.14	0.40	1.3

Figure 1



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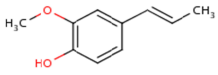

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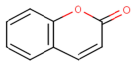

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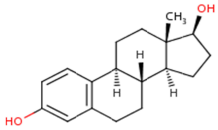

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Figure 2

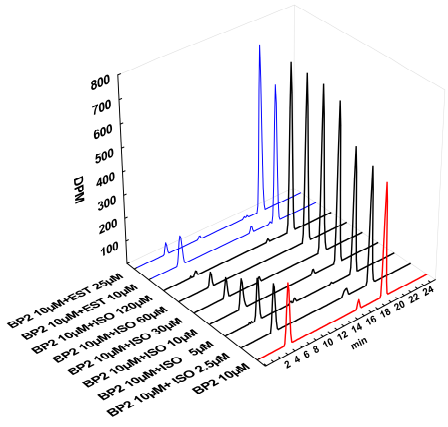


Figure 3

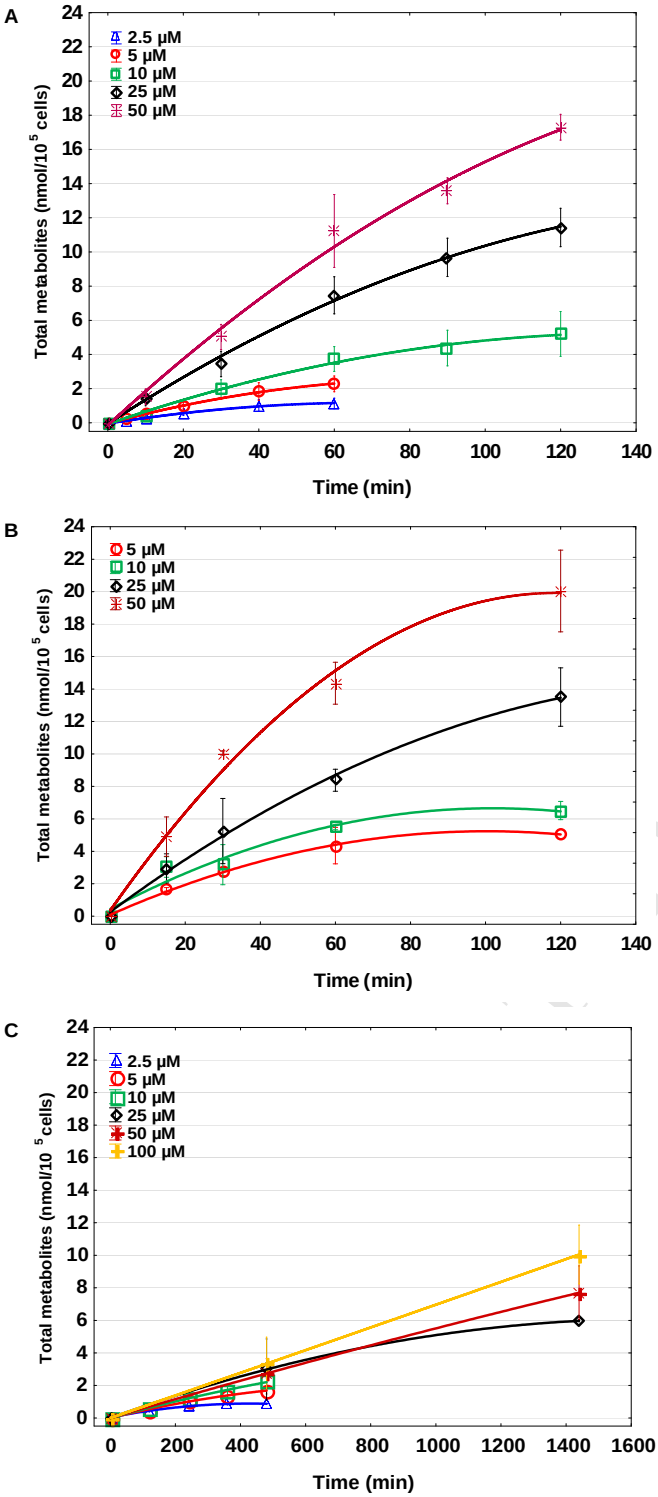
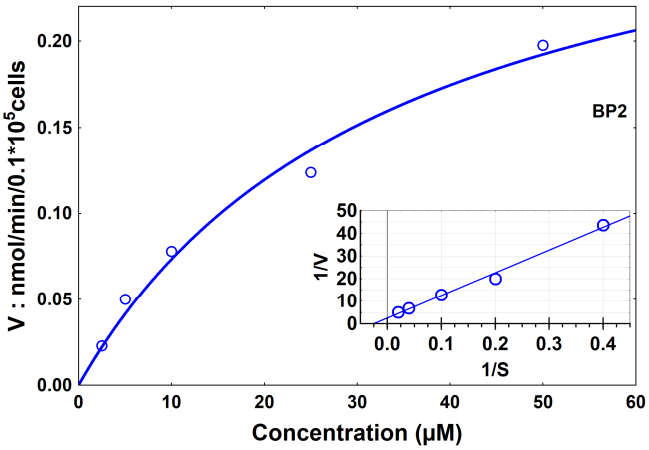
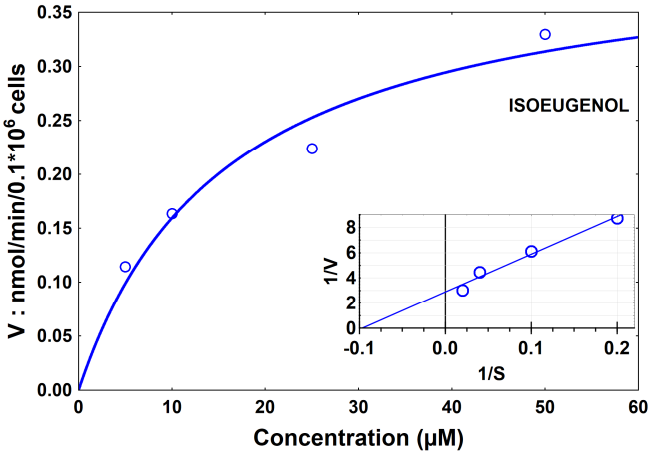

A
2.5 µM
5 µM
10 µM
25 µM
50 µM
Total metabolites (nmol/10 5 cells)
Time (min)
B
5 µM
10 µM
25 µM
50 µM
Total metabolites (nmol/10 5 cells)
Time (min)
C
2.5 µM
5 µM
10 µM
25 µM
50 µM
100 µM
Total metabolites (nmol/10 5 cells)
Time (min)

Figure 4


BP2
V : nmol/min/0.1*10^5cells
Concentration (μM)
1/V
1/S


ISOEUGENOL
V : nmol/min/0.1*10^6 cells
Concentration (μM)
1/V
1/S

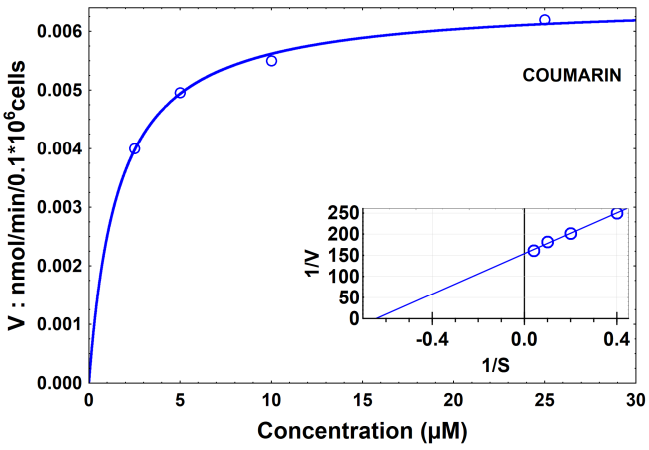
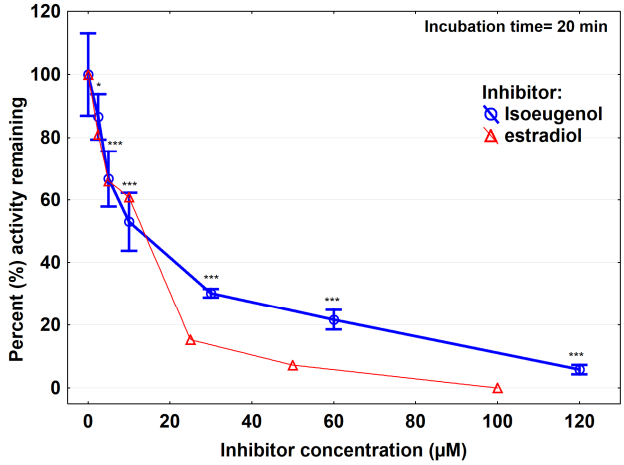
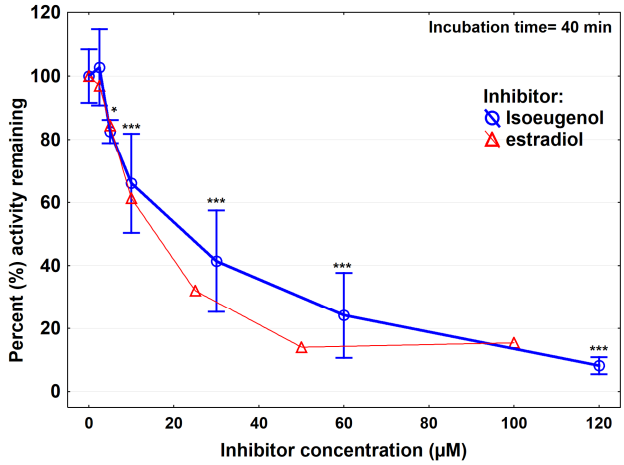

COUMARIN
V : nmol/min/0.1*10^6cells
Concentration (μM)
1/V
1/S

Figure 5


Incubation time= 20 min
Inhibitor:
Isoeugenol
estradiol
Percent (%) activity remaining
Inhibitor concentration (μM)
0
20
40
60
80
100
120
*
***
***
***
***
***


Incubation time= 40 min
Inhibitor:
Isoeugenol
estradiol
Percent (%) activity remaining
Inhibitor concentration (μM)
0
20
40
60
80
100
120
*
***
***
***
***

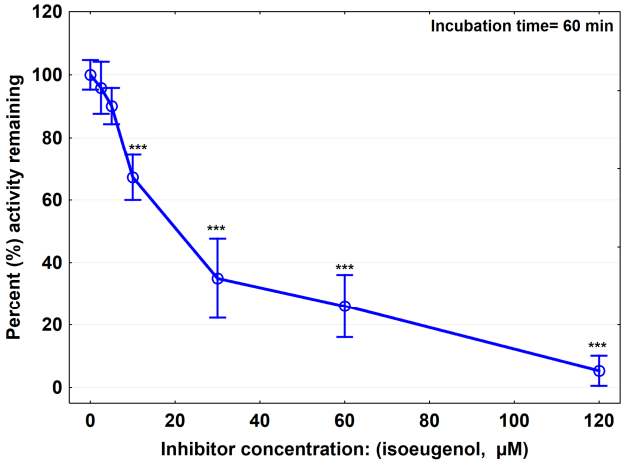
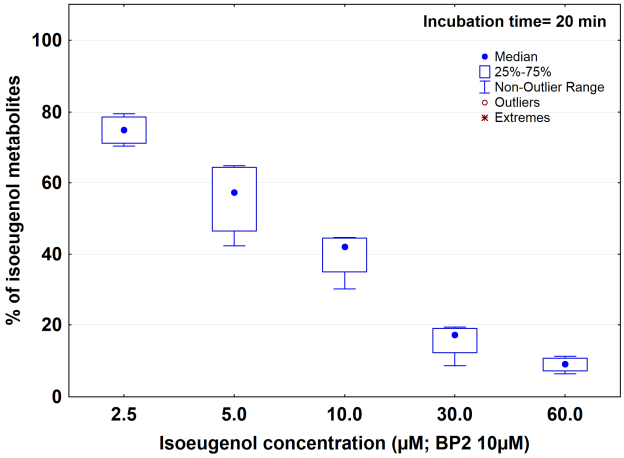
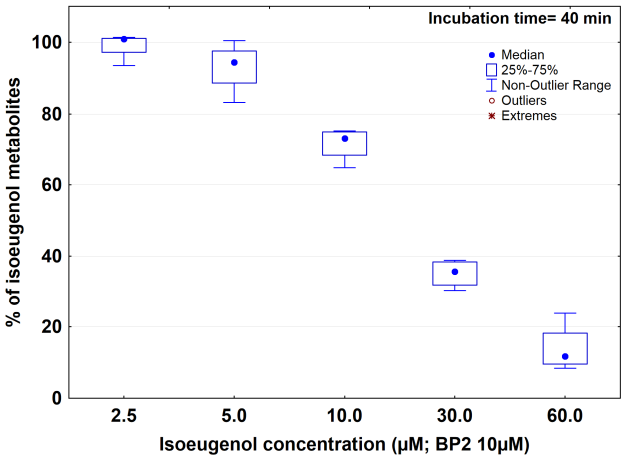
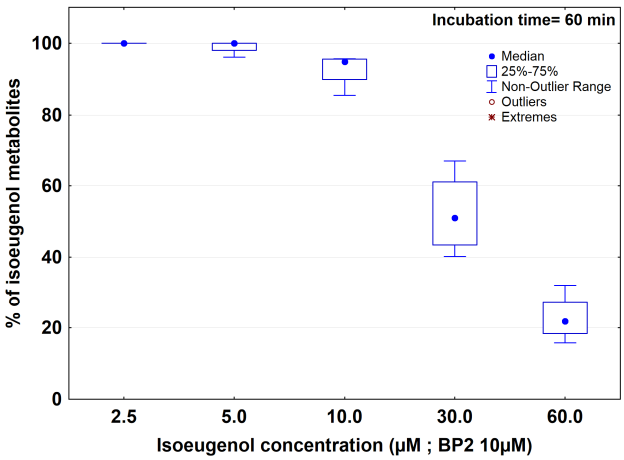

Incubation time= 60 min
Percent (%) activity remaining
Inhibitor concentration: (isoeugenol, μM)
0
20
40
60
80
100
120
***
***
***
***

Figure 6


Incubation time= 20 min
Median
25%-75%
Non-Outlier Range
Outliers
Extremes
% of isoeugenol metabolites
0
20
40
60
80
100
2.5
5.0
10.0
30.0
60.0
Isoeugenol concentration (µM; BP2 10µM)


Incubation time= 40 min
Median
25%-75%
Non-Outlier Range
Outliers
Extremes
% of isoeugenol metabolites
0
20
40
60
80
100
2.5
5.0
10.0
30.0
60.0
Isoeugenol concentration (µM; BP2 10µM)


Incubation time= 60 min
Median
25%-75%
Non-Outlier Range
Outliers
Extremes
% of isoeugenol metabolites
0
20
40
60
80
100
2.5
5.0
10.0
30.0
60.0
Isoeugenol concentration (µM ; BP2 10µM)

Highlights

•Benzophenone-2 have a pure estrogenic effect and it is used in numerous formulations with fragrances agents

•In contrast to coumarin, benzophenone-2 and isoeugenol are rapidly metabolized in human hepatocytes.

•Benzophenone-2 is metabolized by UGT1A1

•Isoeugenol strongly inhibits the metabolisation of benzophenone-2, which could result in slowing down its elimination

.