Structure-activity relationships of phthalates in inhibition of human placental 3β-hydroxysteroid dehydrogenase 1 and aromatase

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ABSTRACT

Phthalates are associated with preterm delivery. However, the mechanism is unclear. Progesterone formed by 3β-hydroxysteroid dehydrogenase 1 (HSD3B1) and estradiol by aromatase (CYP19A1) in placenta are critical for maintaining pregnancy. In this study, we compared structure-activity relationships (SAR) of 14 phthalates varied in carbon atoms in alcohol moiety to inhibit human HSD3B1 in COS1 and CYP19A1 in JEG-3 cells. There were responses in that only diphthalates with 4–7 carbon atoms were competitive HSD3B1 inhibitors and diphthalates with 6 carbon atoms were CYP19A1 inhibitors. IC50s of dipentyl (DPP), bis(2-butoxyethyl) (BBOP), dicyclohexyl (DCHP), dibutyl (DBP), and dihexyl phthalate (DHP) were 50.12, 32.41, 31.42, 9.69, and 4.87 μM for HSD3B1, respectively. DCHP and BBOP inhibited CYP19A1, with IC50s of 64.70 and 56.47 μM. DPP, BBOP, DCHP, DBP, and DHP inhibited progesterone production in JEG-3 cells. In conclusion, our results indicate that there is clear SAR for phthalates in inhibition of HSD3B1 and CYP19A1.

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1. Introduction

Phthalates are synthetic esters and are used widely as plasticizers. Phthalates include a series of structurally different chemicals with a phthalic acid moiety and a moiety of alcohol that ranges from methanol (C1) to tridecyl alcohol (C13), either as a straight chain or a branching chain. Phthalates are possible environmental pollutants [1]. Human epidemiological studies showed that maternal exposure to phthalates is associated with low birth weight [2] and the delayed timing of parturition [3]. Human studies have demonstrated that phthalates can cross the placenta and readily reach the fetus [2,4]. Study also showed that phthalate metabolites were associated with the trophoblast differentiation of the placenta [5].

The human placenta, besides providing transportation of nutrients from the maternal circulation into the fetus, serves as an endocrine gland by synthesizing peptides, neuropeptides, and steroid hormones [6]. Two of the most important steroid hormones synthesized by the human placenta are progesterone and estradiol, which are essential for the maintenance of pregnancy [7]. Progesterone maintains the correct uterine environment (e.g. the lining of the uterus), keeps the quiescence of the myometrium (e.g. the suppression of the movement of the myometrium), and antagonizes the labor-inducing effects of estrogens, prostaglandins, and oxytocin [8]. Placental estradiol stimulates placental growth and enhances placental blood flow to provide the optimal exchange of gases and nutrients required for the rapidly developing fetus [9]. Progesterone levels continuously increase throughout the course of pregnancy [10]. The progesterone biosynthesis is catalyzed by the placental mitochondrial 3β-hydroxysteroid dehydrogenase 1 (HSD3B1), which uses pregnenolone as a substrate and NAD+ as a cofactor (Fig. 1) [11]. During the early pregnancy, placental trophoblast cells do not express P450 aromatase (CYP19A1) until the formation of the syncytiotrophoblast, an outer syncytial layer of the trophoblast that actively invades the uterine wall forming the outermost fetal component of the placenta, by the fusion of cytotrophoblast cells. However, the syncytiotrophoblast requires an external source of androgens, provided by the adrenal glands of the fetus. The fetal adrenal gland produces dehydroepiandro-
terone abundantly [12], CYP19A1 is a cytochrome P450 and is the rate-limiting enzyme in estrogen biosynthesis. Through interaction with NADPH-cytochrome P450 reductase, CYP19A1 catalyzes three steps of hydroxylation to convert an androgen to an estrogen. Interfering with progesterone and estrogen levels has been suggested to be an approach to disrupt the pregnancy.

Previously, we have analyzed the structure-activity relationships (SAR) of phthalates varied in the carbon atom numbers in the alcohol moieties and found that there were structure-dependent responses in inhibition of some steroidogenic enzymes and only some phthalates inhibited several steroidogenic enzymes, including 3β-hydroxysteroid dehydrogenase isoform 2 (HSD3B2) and 17β-hydroxysteroid dehydrogenase isoform 3 in human testis, and 11β-hydroxysteroid dehydrogenase isoform 2 in human kidney [13,14]. HSD3B1 and HSD3B2 share high similarity in their protein sequences with 93.6% similarity [15]. In the present study, we performed SAR analysis to compare the potencies of 14 structurally different phthalates varied in the carbon atom number of the carbon-chain in the alcohol moieties as inhibitors of HSD3B1 in a transfected COS1 cell line and progesterone production in placental cell line JEG-3 as well as CYP19A1 in the human JEG-3 cell microsome in vitro. COS1 cell line is a fibroblast-like cell line derived from monkey kidney tissue by immortalizing CV-1 cells [16]. This cell line has no background activity of human HSD3B1. We selected the JEG-3 cell line as our placental trophoblastic model system. This cell line was derived from a human choriocarcinoma and had many of the biological and biochemical characteristics associated with syncytiotrophoblasts such as the production of progesterone and several other steroids as well as steroidogenic enzymes [17,18]. The phthalates tested in the present study include diphthalates: dimethyl (DMP), diethyl (DPE), dipropyl (DPPrP), di-n-butyl (DBP), di-n-pentyl (DPP), dicyclohexyl (DCHP), bis(2-butoxyethyl) (BBOP), diheptyl (DHP), di(2-ethylhexyl) (DEHP), di-n-octyl (DNOP), and diisononyl phthalate (DINP) as well as monophthalates: monomethyl (MMP), monobutyl (MBP), and mono(2-ethylhexyl) phthalate (MEHP).

2. Materials and methods

2.1. Materials

[^3]H)Pregnenolone ([3H-P5], [3H] progesterone, and [3H] estradiol-17β ([3H-E2) were purchased from Dupont-New England Nuclear (Boston, MA). Unlabeled pregnenolone, progesterone, androstenedione, testosterone, and estradiol were purchased from Steraloids (Newport, RI). The following phthalates were purchased from Sigma–Aldrich Company (St. Louis, MO, USA): DMP (purity, ≥99%), DEP (purity, 99.5%), DPPrP (purity, analytical standard), DBP (purity, >99%), DPP (purity, ≥99%), DCHP (purity, >99%), BBOP (purity, analytical standard), DHP (purity, >97%), DNOP (purity, ≥98%), DEHP (purity, ≥99%), DINP (purity, ≥99%), MMP (purity, ≥97%), and MBP (purity, ≥99%). MEHP (purity, ≥98%) was purchased from TCI America (Portland, OR, USA). The full names of these phthalates are shown in Table 1. COS1 and JEG-3 cell lines were obtained from ATCC (HTB-36, Manassas, VA, USA). Phthalates were dissolved in dimethyl sulfoxide.

2.2. Construction and expression of human HSD3B1 plasmid in COS1

An expression plasmid was constructed to express human HSD3B1 (HSD3B1) cDNA in pcDNA3.1 expression vector. The Escherichia coli transformants carrying an insert were selected by colony hybridization, and a clone with the insert in the correct orientation relative to the vector T7 promoter was identified by restriction mapping. All transfections were carried out on 80% confluent cultures in 12-well plates. Aliquots of 1 mg HSD3B1 pcDNA3.1 were transfected into mammalian COS1 cells with the FuGENE Transfection Reagent (Roche) according to manufacturer’s protocol. Cells were allowed to grow for 24 h in media containing 10% fetal bovine serum. Then media were removed and cells were harvested for HSD3B1 activity assay.

2.3. Preparation of mitochondria of COS1

Mitochondrial preparations of COS1 transfected with human HSD3B1 were performed as previously described [19]. In brief, COS1 cells were homogenized in cold 0.01 mM phosphate buffered saline (PBS, pH 7.2) containing 0.25 mM sucrose and centrifuged at 700g for 30 min at 4 °C to remove the cellular fragments. The COS1 cell supernatants were transferred to new tubes and were centrifuged at 10,000g for 30 min at 4°C to get mitochondrial pellets. The protein concentrations were measured by Bio-Rad Protein Assay Kit (cat# 500-0006, Bio–Rad, Hercules, CA) according to manufacturer’s pro-
2.4. Preparation of microsome

Microsomal preparation from JEG-3 cells were performed as previously described [19]. In brief, JEG-3 cells were homogenized in cold 0.01 M PBS containing 0.25 N sucrose and centrifuged at 700g for 30 min at 4 °C to remove the cellular fragments. The JEG-3 cell supernatants were transferred to new tubes and were centrifuged at 10,000g for 30 min at 4 °C to remove mitochondrial pellets. The resulting supernatants were further transferred to new tubes and were centrifuged at 105,000g for 60 min twice at 4 °C to obtain the microsomal pellets. The protein concentrations were measured as above. The protein concentrations were adjusted to 2 mg/ml. JEG-3 cell microsomes were used for measurement of CYP19A1 activity.

2.5. HSD3B1 assay

HSD3B1 activity in the mitochondrion was measured. In brief, HSD3B1 activity assay tubes contained 0.2 μM pregnenolone and 0.2 mM NAD+ in 250 μl 0.5% Tween-20 PBS. Phthalates were dissolved in dimethyl sulfoxide, and the final dimethyl sulfoxide concentration was 0.5%, at which dimethyl sulfoxide had no effects on HSD3B1 activity. We determined the linear reaction using different concentrations of the mitochondria for HSD3B1 activity. At first, the 30-min reactions were initiated by addition of 20 μg mitochondrial proteins in presence of 100 μM phthalates to determine the maximal inhibition. The selection of the concentration of 100 μM for a phthalate was based on the most appropriate and conservative point of reference for assessing a margin of exposure as 20 mg/kg/day DEHP (51.8 μM) [20]. In a pilot experiment, we had determined the velocity of HSD3B1 within the linear range in the above conditions. The reactions were stopped by adding 2 ml ice-cold ether. The steroids were extracted and the organic layer was dried under nitrogen. The steroids were separated chromatographically on thin-layer plates in chloroform and methanol (97:3, v/v), and the radioactivity was measured using a scanning radiometer (System AR2000, Bioscan Inc., Washington, DC). The percentage conversion of pregnenolone into progesterone was calculated by dividing the radioactive counts identified as progesterone by the total counts associated with pregnenolone plus progesterone.

2.6. CYP19A1 assay

CYP19A1 activity in the microsome was measured. In brief, CYP19A1 activity assay tubes contained 0.1 μM testosterone and 0.2 mM NADPH in 250 μl 0.5% Tween-20 PBS. Phthalates were dissolved in dimethyl sulfoxide, and the final dimethyl sulfoxide concentration was 0.5%, at which dimethyl sulfoxide had no effects on CYP19A1 activity. We determined the linear reaction in different concentrations of microsome for CYP19A1 activity. At first, the 30-min reactions were initiated by addition of 10 μg microsomal proteins in presence of 100 μM phthalates to determine the maximal inhibition. A pilot experiment had determined the velocity of CYP19A1 within the linear range in the above conditions. The reactions were stopped by adding 10 μl 1 N HCl. Reaction medium were collected for measurement of estradiol using radioimmunoassay as below. The percentage conversion of testosterone to estradiol was calculated.

2.7. Cell culture and phthalate treatment

JEG-3 cells were cultivated at 10 5 cells per well in 24-well plates in MEM medium with phenol red containing 10% fetal calf serum (basal) or with 2 mM 8bromo-cAMP (8Br-cAMP) at 37 °C/5% CO2 in the presence of 100 μM phthalates or without for 12 h 8Br-cAMP was used because it can penetrate the cell membrane to act like endogenous cAMP for stimulation. These media were used for progesterone assay. The JEG-3 cells were also cultivated at 10 5 cells per well in 24-well plates in MEM medium with phenol red containing 10% fetal calf serum with 10 μM androstenedione at 37 °C/5% CO2 in the presence of 100 μM phthalates or without for 12 h. The media were used for estradiol assay. Androstenedione was used as the source of androgen. For cytotoxicity analysis, cells were treated with 100 μM phthalates and the cells were harvested for trypsin blue exclusion assay according to the manufacturer’s instruction. Cells were also treated with different concentrations of phthalates for 12 h. Media were collected for the measurement of progesterone and estradiol levels.

2.8. Progesterone and estradiol measurement by radioimmunoassay

Progesterone and estradiol concentrations were measured with a tritium-based radioimmunoassay using either anti-progesterone antibody (Fitzgerald, MA) or anti-estradiol antibody (Fitzgerald, MA) as previously described [21]. Standards ranging between 10
and 2000 pg/ml progesterone or estradiol were prepared in triplicate. Standards and samples were incubated with respective tracer and antibody at 4°C overnight and charcoal-dextran suspension was used to separate the bound and free steroid. The bound steroids were mixed with a scintillation buffer and counted in a β scintillation counter (PE, USA). The minimum detectable concentrations of the assay for progesterone and estradiol were ≤ 50 pg/ml and 7.5 pg/ml, respectively. Quality control samples contain 100 pg/ml progesterone or 100 pg/ml estradiol. The intra-assay coefficients of variation were 7.82% and 7.12% for progesterone and estradiol, respectively. The inter-assay coefficients of variation were 7.54% and 7.96% for progesterone and estradiol, respectively. The cross reactivity of the anti-progesterone antibody with pregnenolone was 0.9%.

2.9. Determination of half maximal inhibitory concentration (IC50) value and mode of action

The IC50 value was determined as the concentration of an inhibitor causing 50% inhibition of an enzyme in vitro. To determine the IC50 value, different concentrations of a phthalate compound were used. The non-linear regression fit for log [inhibitor] vs. response was performed using GraphPad (Version 6.0, GraphPad Software Inc., San Diego, CA). To determine the inhibitory mode of a phthalate against pregnenolone, different concentrations of pregnenolone (0.002–10 μM) plus 0.2 mM NAD+ were added into reaction mixture (0.5% Tween-20 PBS buffer) containing 20 μg mitochondria and a phthalate (different concentrations). To determine inhibitory mode of a phthalate against NAD+, different concentrations of NAD+ concentrations (0.002–100 μM) plus 0.2 μM pregnenolone were added into 0.5% Tween-20 PBS reaction mixture and a phthalate (various concentrations). To determine the inhibitory mode of a phthalate against testosterone (CYP19A1), different concentrations of testosterone (0.002–2 μM) plus 0.2 mM NADPH was added into the reaction mixture (0.5% Tween-20 PBS buffer) containing 10 μg human JEG-3 microsome and a phthalate (different concentrations). Lineweaver-Burk plot was used to determine the mode of inhibition using GraphPad.

2.10. Statistics

Assays were repeated four to six times. Data were subjected to analysis by one-way ANOVA followed by DUNCAN multiple comparison testing to identify significant differences between two groups when three and more groups were used. All data are expressed as means ± SEM, n = 4–6. Differences were regarded as significant at P < 0.05.

3. Results

3.1. Effects of phthalates on HSD3B1 activity in the mitochondria

HSD3B1, which catalyzes the conversion of pregnenolone into progesterone, was measured in the mitochondria prepared from the transfected COS1 in the presence of 100 μM phthalates (Fig. 2). Under this condition, diphthalates (DBP, DPP, BBOP, DCHP, and DHP) with 4–7 carbon atoms in the alcohol moieties had potent inhibition of HSD3B1 enzyme activity by over 50% (Fig. 2A). Diphthalates (DMP, DEP, and DPP) with 1–3 carbon atoms in the alcohol moieties had less potent but significant inhibition of the enzyme, while those (DEHP, DNOP, and DNP) with 8–9 carbon atoms showed almost no inhibition of HSD3B1 activity (Fig. 2A). Of all monophthalates tested, MBP slightly but significantly inhibited HSD3B1 activity, while MMP and MEHP had almost no inhibition of the enzyme (Fig. 2B). The IC50 values were determined for DBP, DPP, BBOP, DCHP, and DHP, because these diphthalates inhibited HSD3B1 by over 50% at 100 μM. As shown in Fig. 3 and Table 1, IC50 values of DPP, BBOP, DCHP, DBP, and DHP in inhibition of human HSD3B1 were 50.12, 32.41, 31.40, 9.69, and 4.87 μM (Fig. 3, Table 1). This indicates that there is a clear structure-dependent inhibition for phthalates.

3.2. The modes of inhibition of phthalates on HSD3B1

The modes of phthalate-mediated inhibition of human HSD3B1 activity were examined by enzyme kinetic analysis (Lineweaver-Burk plot). The Lineweaver-Burk equation is a linear equation, where 1/V is a linear function of 1/[S]. The Lineweaver-Burk plot is readily represented graphically to determine the mode of inhibition. When different concentrations of pregnenolone were used, Lineweaver-Burk plot analysis showed that DPP, BBOP, DCHP, DBP, and DHP had the same y-intercept and different slopes x-intercepts, indicating that DPP, BBOP, DCHP, DBP, and DHP were competitive inhibitors for human HSD3B1 activity (Fig. 4), indicating that these phthalates might bind to the pregnenolone binding site of the enzyme. When different concentrations of cofactor NAD+ were used, Lineweaver-Burk plot analysis showed that DPP, BBOP, DCHP, DBP, and DHP inhibited cofactor-mediated action in a mixed manner (Fig. 5).
3.3. Effects of phthalates on CYP19A1 activity in the microsome

CYP19A1, which catalyzes the conversion of androgen into estrogen, was measured in the microsome prepared from JEG-3 cells in the presence of 100 μM phthalates (Fig. 6). Under this condition, diphthalates (DCHP and BBOP) with 6 carbon atoms in the alcohol moiety had potent inhibition on this enzyme activity by over 50% (Fig. 6A). Diphthalates with 1–5 and 7–9 carbon atoms in the alcohol moiety showed almost no inhibition of CYP19A1 activity (Fig. 6A). Monophthalates, MMP, MBP and MEHP, had also almost no inhibition of this enzyme (Fig. 6B). The IC₅₀ values were determined for DCHP and BBOP. As shown in Fig. 7, IC₅₀ values of DCHP and BBOP in inhibition of human CYP19A1 were 56.47 and 64.70 μM, respectively (Fig. 7, Table 1). This indicates that there is a clear structure-dependent inhibition of CYP19A1 for phthalates.

3.4. The modes of inhibition of phthalates on CYP19A1

The modes of phthalate-mediated inhibition of human CYP19A1 activity were examined by enzyme kinetic analysis. When different concentrations of testosterone were used, Lineweaver-Burk plot analysis showed that DCHP and BBOP were competitive inhibitors for human CYP19A1 activity (Fig. 8), indicating that these two phthalates might bind to the testosterone binding site of the enzyme.
3.5. Effects of phthalates on progesterone and estradiol production in JEG-3 cell line

Progesterone is the main steroid that is produced in the human placenta in the last two trimesters. Using the human placental cell line JEG-3, all phthalates (at 100 µM) were tested for cell viability using trypan blue exclusion assay and it was found that they had no effects on cell viability (data not shown). Of all phthalates, only DBP, DPP, BBOP, DCHP, and DHP showed a significant decrease in basal and 8Br-cAMP-stimulated progesterone secretion by ≥50% at 100 µM (Fig. 9). Monophthalates (MMP, MBP and MEHP) had no inhibition on basal and 8Br-cAMP-stimulated progesterone (Fig. 9). The data confirm the direct inhibition by these phthalates of HSD3B1 in the mitochondria. CYP19A1 requires androgen from adrenal source. We treated cells with phthalates (100 µM) in the presence of 10 µM androstenedione. As shown in Fig. 10, of both diphthalates and monophthalates tested, only DCHP and BBOP significantly inhibited estradiol production by JEG-3 cells.

4. Discussion

The present study showed structure-dependent inhibition of human HSD3B1 and CYP19A1 activities by phthalates. Diphthalates with 4–7 carbon atom numbers in the alcohol moiety showed moderate and potent inhibition of human HSD3B1. Diphthalates with 1–3 carbon atom numbers in the alcohol moiety showed less potent inhibition of human HSD3B1 and those with 8–9 carbon atom numbers had almost no inhibition when 100 µM of each compound was tested. Only two diphthalates, BBOP and DCHP, which have 6 carbon atom numbers in the alcohol moiety, inhibited human CYP19A1. Because HSD3B1 and CYP19A1 are critical enzymes for progesterone and estrogen biosynthesis in human placentas, it is
important to note that some of these phthalates possibly disrupt placental function.

In the present study, we performed SAR analysis of human HSD3B1 activity by phthalates. Diphthalates with 4–7 carbon atom numbers in the alcohol moiety were among most potent inhibitors (Fig. 2). The potencies of inhibition of the human HSD3B1 activity of these diphthalates were: DHP > DBP > DCHP > BBOP > DPP (Fig. 3 and Table 1). Diphthalates with 1–3 carbon atom numbers in the alcohol moiety were less potent inhibitors (Fig. 2). Diphthalates with 8–9 carbon atom numbers in the alcohol moiety had almost no inhibition on HSD3B1 (Fig. 2). Interestingly, SAR analysis also showed that there was structure-dependent difference in inhibition of other hydroxysteroid dehydrogenases. In the previous study, we already demonstrated the structure-dependent difference in inhibition of another HSD3B isoform, HSD3B2, in human testis potently [13]. For human HSD3B2, diphthalates with 3–6 carbon atom numbers in the alcohol moiety were more potent [13].

DPrP also inhibited human HSD3B2 more potently [13]. However, in this study, DPrP inhibited human HSD3B1 far less potent while DHP inhibited human HSD3B1 more potently than HSD3B2 (Fig. 3), indicating that there is slightly structural difference for inhibitors. Indeed, although these two human HSD3B isoforms share high similarity (93.6%) in their protein sequences [22], they had different cell locations and biochemical properties. For example, human HSD3B1 is mainly present in the placenta and peripheral tissues and has higher affinity for steroid substances, while HSD3B2 is predominantly present in gonads and adrenals and has lower affinity for steroid substrates [15]. For mono-phthalates, MMP, MBP and MEHP were all ineffective to inhibit both HSD3B isoforms.

Although in vivo animal studies for pregnancy outcomes and low birth weight have not been performed systematically for SAR analysis, the data of SAR of in vivo gestational exposure to different phthalates in the rats to cause reduction of fetal testicular testosterone and induce the malformation of male reproductive tract.
have been continuously accumulated. Generally speaking, phthalates with 1-3 and 9-12 carbon atom numbers in the alcohol moiety almost did not cause any adverse effects on male reproductive system. For example, when five diphthalates DMP, DEP, benzyl butyl phthalate (BBP), DEHP, and DINP were administered orally to pregnant female rats from gestational day 14 to postnatal day 3, it was found that BBP (6/4 carbon atoms in the alcohol moiety) and DEHP (8/8 carbon atoms) reduced pup birth weight (15%) and that BBP, DEHP, and DINP (9/9 carbon atoms) caused 84%, 82%, and 7.7% of male malformations in the reproductive system while DMP (1/1 carbon atoms) and DEP (2/2 carbon atoms) had no effects [23].

In another study, the effects on testosterone production in male rat fetus, gene expression levels and postnatal development of the male and female offspring after in utero exposure to DEP, DBP (4/4 carbon atoms), diisobutyl phthalate (DiBP, 4/4 carbon atoms), di-n-pentyl (DPP, 5/5 carbon atoms), BBP, and DEHP from gestational day 8-18 was investigated, it was found that DBP, DiBP, BBP, and DEHP were equally potent (ED50 of 440 ± 16 mg/kg/d) and DPP was approximately 3-fold more potent (ED50 of 130 mg/kg/d) while DEP had no effect on fetal testosterone production [24]. Hannas et al. also compared SAR of fetal testosterone production in rat fetal testes after in utero exposure to DiBP, diisohexyl phthalate (DiHP, 7/7 carbon atoms), DEHP, and DINP and found that DiBP and DiHP reduced fetal testosterone with equal potency to DEHP and DINP was 2.3 fold less potent [25].

The effects on steroidogenesis and pregnancy outcomes by phthalates were mostly performed in rats and only a few in primates. In a previous study of investigating the effects of MBP on newborn marmosets, the New World monkey species, administration of a single dose of 500 mg/kg MBP significantly suppressed blood testosterone levels and chronic treatment for 14-days increased Leydig cell volume per testis, consistent with MBP-mediated suppression of steroidogenesis followed by compensatory Leydig cell hypertrophy [26]. In human case-control studies, urinary metabolites of DEHP/DBP were significantly and inversely associated with boys’ AGD, a biomarker for fetal testosterone [27,28]. The associations of urinary phthalate metabolite levels with semen characteristics, sperm DNA damage, and serum reproductive hormones in men showed that there were dose–response relationships of MBP and indicative relationships of the highest MBzP (the mono-phthalate metabolite of di-benzyl phthalate) quartile and low sperm concentration while there were no relationships of MMP, MEP, or the DEHP metabolites with these semen parameters [29–32]. Because there are well documented anti-androgenic effects of certain phthalates in rodent models [33] and the associations of reduced testosterone in men with obesity

Fig. 6. Inhibition of phthalates on human aromatase (CYP19A1). 10 μg of microsomes were incubated with different concentrations of phthalates and 100 nM testosterone in the presence of 0.2 mM NADPH for 30 min. Values from six samples in a duplicate assay are represented. Panel A, diphthalates; Panel B, monophthalates. Mean ± SEM. *, **, and *** indicate significant difference compared to control at P < 0.05, 0.01, and 0.001, respectively.

Fig. 7. Concentration-dependent inhibition of some diphthalates on human aromatase (CYP19A1). 10 μg of microsomes were incubated with different concentrations of phthalates and 100 nM testosterone in the presence of 0.2 mM NADPH for 30 min. Values from four samples in a duplicate assay are represented. Mean ± SEM. BBP (Panel A), DCHP (Panel B).
and type 2 diabetes [34], the possible link between phthalates and diabetes 2 was also investigated in humans. An association of urinary phthalate MEP, MBzP, and the DEHP metabolites with the increased insulin resistance was found [35].

There are few studies on relationship of phthalates with rates of pregnancy and miscarriage. In a recent study, the associations between urinary concentrations of phthalate metabolites (14 phthalate metabolites) and outcomes of assisted reproductive technologies were investigated and it was found that the monophthalate metabolites of DnIP was associated with reduced fertilization rate and DEHP metabolites were negatively associated with clinical pregnancy and live birth [36]. Another study with 430 couples in a Danish prospective cohort reported an association between urinary DEHP metabolite levels with pregnancy loss [37].

Human investigations also have shown that maternal exposure to phthalates (DEHPDBP) was associated with low birth weight [2] and with the delayed timing of parturition [3,38]. The placental cells in vivo might be exposed to parent phthalate compounds since the previous study showed that the parent phthalate compounds such as DEHP and DBP could be detected in cord blood and meconium of new-born babies after parent exposure [2]. This may be partially contributed by the direct inhibition of placental steroidogenesis. Indeed, the production of progesterone and estrogen in human placentas is very important for maintenance of pregnancy. Apparently, some phthalates could impair placental function via direct inhibiting HSD3B1 and CYP19A1 activity.

Interestingly, many diphthalates, such as DBP and DEHP, are thought to convert more potent monophthalates, MBP and MEHP, to exert their biological actions [39]. These monophthalates had almost no inhibition on HSD3B1 activity (Fig. 2) and progesterone production in placental cell line JEG-3 (Fig. 9). Actually, the diphthalate DBP was more potent than its metabolite MBP to inhibit HSD3B1 activity and progesterone production in cell line (Figs. 2 and 9).

In the present study, we also demonstrated structure-dependent inhibition of human CYP19A1 activity by phthalates. Only two di-phthalates, BBOP and DCHP, which have 6 carbon numbers in the alcohol moiety, were moderate inhibitors (Figs. 6 and 7). Di-phthalates with 1–5 and 7–9 carbon numbers in the ethanol moiety had no effects even at 100 μM (Fig. 6). Three monophthalates tested had no inhibition on CYP19A1 activity (Fig. 6).

Structure-dependent activity of phthalates was also documented for other steroidogenic enzymes. For the inhibition on 17β-hydroxysteroid dehydrogenase 3 [13] and 11β-hydroxysteroid dehydrogenase 2 [14], di-phthalates with 3–6 carbon numbers in the alcohol moiety were also effective.

The modes of DBP, BBOP, DCHP, and DHCP in the inhibition of human HSD3B1 activity were competitive, and these were similar to the mode of DHCP to inhibit human HSD3B2 in the tesis [13]. To examine whether human DBP, BBOP, DCHP, and DHCP competed with cofactor NAD+ for HSD3B1, we assayed different concentrations of NAD+ in the presence of various concentrations of DBP, BBOP, DCHP, and DHCP. Our study showed that DBP, BBOP, DCHP, and DHCP were a mixed inhibitor against NAD+ (Fig. 6). This mode of inhibition is different from that of DHCP for human HSD3B2, in which DHCP is a noncompetitive inhibitor against NAD+ [13].
Fig. 10. Inhibition of phthalates on androstenedione-mediated estradiol production in JEG-3 cells. Panel A, diphthalates; Panel B, monophthalates. Values from four samples in four assays are represented. *, **, and *** indicate significant difference compared to control at P < 0.05, 0.01, and 0.001, respectively.

The modes of BBOP and DCHP in the inhibition of human CYP19A1 activity were competitive, and these were similar to the mode of DCHP to inhibit human HSD3B1. This indicates that BBOP and DCHP bind to the active site of steroid substrate of CYP19A1.

In conclusion, in the present study, we performed the structure-activity relationship analysis of 14 phthalates in inhibition of human placental HSD3B1 and aromatase and found that DBP, BBOP, DCHP, and DHP are potent HSD3B1 inhibitors and BBOP and DCHP are moderate CYP19A1 inhibitors.

Transparency document

The Transparency document associated with this article can be found in the online version.

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