Comparison of *in vitro* hormone activities of selected phthalates using reporter gene assays

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**A B S T R A C T**

Phthalates are widely used in the plastic industry and food packaging, imparting softness and flexibility to normally rigid plastic medical devices and children's toys. Even though phthalates display low general toxicity, there is increasing concern on the effects of endocrine system induced by some of phthalate compounds. The hormone activity of dibutyl phthalate (DBP), mono-n-butyl phthalate (MBP) and di-2-ethylhexyl phthalate (DEHP) were assessed using the luciferase reporter gene assays. The results showed that DBP, MBP and DEHP, not only exhibited potent antiandrogenic activity, with IC$_{50}$ value of 1.05 × 10$^{-6}$, 1.22 × 10$^{-7}$ M and exceeding 1 × 10$^{-4}$ M respectively, but also showed the androgenic activity with EC$_{50}$ value of 6.17 × 10$^{-6}$, 1.13 × 10$^{-5}$ M and exceeding 1 × 10$^{-4}$ M respectively. We also found that all the three related chemicals possessed thyroid receptor (TR) antagonist activity with IC$_{50}$ of 1.31 × 10$^{-5}$, 2.77 × 10$^{-6}$ M and exceeding 1 × 10$^{-4}$ M respectively, and none showed TR agonist activity. These results indicate that TR might be the targets of industrial chemicals. In the ER mediate reporter gene assay, three chemicals showed no agonistic activity except for DBP, which appeared weakly estrogenic at the concentration of 1.0 × 10$^{-4}$ M. Together, the findings demonstrate that the three phthalates could simultaneously disrupt the function of two or more hormonal receptors. Therefore, these phthalates should be considered in risk assessments for human health.

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1. Introduction

Exogenous compounds known as endocrine-disrupting chemicals are of special interest because they mimic, block or in some way alter the activities of endogenous hormones. It has been well documented that several chemicals from agricultural, industrial, and household sources possess endocrine-disrupting properties that are a potential threats to human and wildlife reproduction (Colborn, 1995; Colborn et al., 1993; Jensen et al., 1995). A major mechanism of endocrine disruption is the action of chemicals as receptor agonists or antagonists through direct interaction with hormonal receptors, thus altering endocrine function. In particular, chemicals mimicking endogenous estrogen via estrogen receptor (ER) have been the focus of research for the last 20 years. Meanwhile, recent studies have shown that several chemicals may exert antiandrogenic and anti-thyroid hormone effect by interfering with androgen (AR) and thyroid hormone (TR) receptors, respectively (Sohoni and Sumpter, 1998; Vinggaard et al., 1999; Zoeller, 2005).

Phthalates are a family of chemicals used in many consumer products, including building materials, cosmetics, clothing, pharma-ceuticals, medical devices, children's toys, food packaging, cleaning materials and insecticides (Schettler, 2006). Annually, more than three million metric tonnes of phthalates are produced globally (Bizzari et al., 2000). Because of their widespread use, all populations of people, domestic animals, and wildlife are regularly exposed to phthalates. People may be exposed in the work environments (Pan et al., 2006; Wormuth et al., 2006). In addition, several biomonitoring studies have revealed a widespread exposure of the nonoccupational human population (Silva et al., 2005; Hauser and Calafat, 2005) via food from plastic containers and via inhalation of dust in domestic environments. Dibutyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) are high production volume plasticizers and are regularly detected in aquatic ecosystems, due to their continuous release into the environment. These compounds are listed as priority substances in the European Union and therefore subject to an environmental risk assessment. Endocrine-disrupting effects are suspected for phthalate diesters, though to occur by *in vivo* metabolism to phthalic acid monoesters (Eigenberg et al., 1986). Recently, we made the observation that the reproductive system was damaged, severely by DBP resulting in the developmental condition of hypospadiac male offspring (Jiang et al., 2007). Several *in vitro* studies have shown that phthalates are capable of binding to estrogen receptor-α (ERα), inducing ERα-mediated gene expression, and enhancing the proliferation...
of MCF-7 human breast cancer cells expressing abundant ERα (Andersen et al., 1999; Zacharewski et al., 1998). Meeker et al. showed that DEHP metabolites may alter thyroid hormone levels in men (Meeker et al., 2007). Although, the effects of these compounds on the transcriptional activity of hormonal receptors have been extensively studied, it still remains to be determined whether various phthalates simultaneously disrupt the function of two or more hormonal receptors.

Promoter–reporter gene assay has been widely used as an in vitro method for clarifying the ligand–receptor interaction by receptor agonists and antagonists. Based on the above mechanism, AR mediated, ER mediated and TR mediated reporter gene assays had been used to screen anti/estrogenic, anti/androgenic and anti/thyroid hormone chemicals. The method is rapid, sensitive, and reproducible. Recently, we screened several chemicals for activities of hormonal receptors, and demonstrated that various pesticides are endocrine disruptive (Sun et al., 2006, 2007, 2008a,b). Using this assay, we investigated the effects of DBP, mono-n-butyl phthalate (MBP) and DEHP on androgenic, estrogenic and thyroid hormone activities. Here we provide evidence that DBP, MBP and DEHP are endocrine-disrupting chemicals with mixed androgenic/antiandrogenic, anti-thyroid hormone activities and no estrogenic activity except for DBP. Furthermore, our results indicated that the three tested phthalates simultaneously act as agonists and/or antagonists via two or more hormonal receptors.

2. Materials and methods

2.1. Chemicals

Tested chemicals were the highest grade available for sale or environmental analysis. The source, purity, CAS and abbreviation of chemicals are listed in Table 1. 17β-Estradiol (E2, ≥99%), 5α-dihydropredosterone (5α-DHT, purity >99%) and L-3,5,3′-triiodothyronine (T3; ≥98%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The structures of the chemicals tested in this study are indicated in Fig. 1. All chemicals were dissolved in dimethyl sulfoxide (DMSO), stored at −20°C, and diluted to desired concentration before use. The final concentration of DMSO in the culture medium did not exceed 0.2% (v/v) that did not affect cell yields.

2.2. MTT assay

MTT assay was performed to detect the cytotoxicity of the tested chemicals. The CV-1 cells and MDA-kb2 cells attached on culture dishes were collected and diluted to desired concentration before use. The final concentration of DMSO in the culture medium did not exceed 0.2% (v/v) that did not affect cell yields.

2.3. Reporter gene assay

2.3.1. AR reporter gene assay

The MDA-kb2 cell line (ATCC, USA), stably transformed with murine mammary tumor virus (MMTV)-luciferase was cultured in Leibovitz’s L-15 medium with 10% FBS, 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), and 0.25 µg/ml amphotericin B (Sigma) at 37°C without CO2. For experiments, cells were plated at 1 × 10^5 cells per well in 100 µl of medium in 96-well luminescence plates. When cells were attached (4-6 h), medium was removed and replaced with dosing medium. The MDA-kb2 cells were exposed to 5α-DHT (Sigma, 1.0 × 10⁻¹²-1.0 × 10⁻⁷ M in 10-fold dilution steps), solvent-control and compounds for 24 h. To distinguish agonist activity was androgen receptor or glucocorticoid receptor mediated, cells were treated with compounds alone and with flutamide, the potent antagonist of AR (24h). The model compounds were tested in a concentration range that had been shown before not to be cytotoxic. After rinsed three times with phosphate-buffered saline (PBS, pH 7.4), the cells were lysed with 5× cell culture lysis reagent (Promega, Madison, WI, USA). The cell lysates were analyzed immediately using a 96-well plate luminescence meter (Berthold Detection System, Ploerzheim, Germany). The amount of luciferase was measured with the luciferase reporter assay system kit (Promega, Madison, WI, USA) following the manufacturer’s instructions. The relative transcriptional activity was converted to fold induction above the corresponding vehicle control value (n-fold).

2.3.2. ER reporter gene assay

Green monkey kidney fibroblast (CV-1) cell line (Chinese Cell Center, Beijing) which did not contain the endogenous receptors (AR, ER and TR), was maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen Corporation, Carlshbad, CA, USA), 100 U/ml penicillin (Sigma) and 100 µg/ml streptomycin (Sigma) at 37°C in an atmosphere containing 5% CO2. The host cells were plated in 48-well microplate at a density of 5.0 × 10⁴ cells per well in the phenol red free DMEM medium containing 10% charcoal-dextran-stripped FBS (CDS-FBS). Twelve hours later the CV-1 cells were transfected with 0.5 µg of pERE-TATA-Luc+, 0.2 µg of hERα/pCI using 2.5 µg SofasTan (Sumna Company, Nainen, China) transfection reagent per well. The luciferase reporter plasmid pERE-TATA-Luc+ and rat ERα expression vector hERα/pCI were co-delivered by Dr. M. Takeyoshi (Chemicals Assessment Center, Chemicals Evaluation and Research Institute, Oita, Japan). After an incubation period of 12h, cells were treated with the tested chemicals. Exposure was during 24 h to E2 (1.0 × 10⁻¹⁻1.0 × 10⁻⁸ M in 10-fold dilution steps), solvent-controls and compounds.

2.3.3. TR reporter gene assay

The CV-1 cells were cultured and plated as described above. 12 h later CV-1 cells were transfected with 0.25 µg Gal4 responsive luciferase reporter pUAS-tk-luc, 0.1 µg pGal4-L-TR which was an expression vector coding for the ligand binding domain (LBD) of TRβ fused to the DNA binding domain of Gal4, using 2.5 µg SofasTan (Sumna Company, Nainen, China) transfection reagent per well. The luciferase reporter plasmid pERE-TATA-Luc+ and rat ERα expression vector hERα/pCI were co-delivered by Dr. M. Takeyoshi (Chemicals Assessment Center, Chemicals Evaluation and Research Institute, Oita, Japan). After an incubation period of 12h, the CV-1 cells were exposed to T3 (1.0 × 10⁻¹⁻1.0 × 10⁻⁸ M in 10-fold dilution steps), solvent-controls and compounds for 24 h.

2.4. Statistical analysis

Coefficients of variation were calculated across replicates in the experimental design. The values shown were mean ± S.D. from three independent experiments with triplicate wells for each treatment. Data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan’s multiple comparisons test when appropriate. The level of significance was set at P < 0.05. For antagonists, treatments were compared to the 5 × 10⁻⁸ M T3 or 1 × 10⁻⁸ M DHT positive control groups.
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Fig. 2. Androgenic activity of DHT and antiandrogenic activity of DEHP, DBP and MBP in MDA-kb2 cells which was stably transformed with the MMTV-luciferase. (A) Cells were treated with increasing concentrations of DHT. Data are presented as mean fold induction compared to vehicle control. Values were mean ± S.D. of three independent experiments. (B) Tested chemicals were added along with $1 \times 10^{-9}$ M of DHT. Values were mean ± S.D. of three independent experiments and were presented as percent induction, with 100% activity defined as the activity achieved with $1 \times 10^{-9}$ M of DHT. *$P<0.05$, compared with the value of 1 nM of DHT (100%).

The median inhibitory concentration (IC$_{50}$) or median effective concentration (EC$_{50}$) was calculated with GraphPad Prism 5.01 for windows (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Cytotoxicity of tested chemicals

DBP, MBP and DEHP did not show cytotoxicity neither to CV-1 cell, alone or with E$_2$ and T$_3$, nor to MDA-kb2 cell, alone or with DHT in MTT assay (data not shown). There was no statistical difference between vehicle control group and the tested chemicals treated groups across the experimental design, further indicating that there was no cytotoxicity caused by the tested chemicals at the concentrations employed.

3.2. Response to a known AR agonist

The MDA-kb2 cell line showed appropriate response to DHT, a known AR agonist. DHT ranging from $10^{-12}$ to $10^{-7}$ M induced luciferase activity in a concentration–dependent manner (Fig. 2A). From the dose–response curve, the EC$_{50}$ of DHT was $2.94 \times 10^{-10}$ M. For DHT, luciferase activity was significantly increased at $10^{-10}$ M, and the maximal induction of 9.23-fold of vehicle control was achieved at concentration of $10^{-7}$ M. Analysis of responses of six replicates showed that $10^{-7}$ M DHT induced luciferase activity with an intra-assay (within plate) coefficient of variability (CV) of 9.4% and the inter-assay (across the experiments) CV of 19.5%.

3.3. AR agonist and antagonist activity of DBP, MBP and DEHP

DBP, MBP and DEHP induced the expression of luciferase with EC$_{50}$ of $6.17 \times 10^{-6}$, $1.13 \times 10^{-5}$ M and exceeding $1 \times 10^{-4}$, respectively (Fig. 3A). The maximal induction of DBP, MBP and DEHP was 3.97-, 11.29- and 7.71-fold, respectively. The MDA-kb2 cell line contains both endogenous AR and glucocorticoid receptor (GR), both of the receptors can bind to and activate the MMTV-luc response element. Chemicals were tested against the known AR antagonist, flutamide, to distinguish between AR and GR. Cotreatment of chemicals with $1.0 \times 10^{-5}$ M flutamide significantly reduced expression of DBP, MBP and DEHP. Data are presented as mean fold induction compared to vehicle control. Values are mean ± S.D. of three independent experiments. *$P<0.05$ compared with the value of respective control (B–D).
of the luciferase gene (Fig. 3B–D). These data showed that the chemicals displayed their agonist activity through AR, and the metabolite MBP possessed stronger agonist activity than DBP.

When the tested chemicals were co-administered with 1.0 × 10^{-9} DHT, each suppressed the luciferase expression significantly (Fig. 2B). The IC_{50} value of DBP, MBP and DEHP was 1.05 × 10^{-6}, 1.22 × 10^{-7} M and exceeding 1 × 10^{-4} M, respectively. The strength of antiandrogenicity of the tested phthalates was DEHP < DBP < MBP.

3.4. Response to E_2

E_2 induced luciferase activity in a concentration-dependent manner ranging from 10^{-12} to 10^{-8} M (Fig. 4A), which indicated the system had appropriate response to the natural ligand, E_2. For E_2, the maximal induction of 15-fold of vehicle control was achieved at concentration of 10^{-8} M. From the dose–response curve, the EC_{50} value of E_2 was 1.76 × 10^{-10} M. Analysis of responses of six replicates shows that 10^{-8} M E_2 induced luciferase activity with an intra-assay (within plate) coefficient of variability (CV) of 8.5% and the inter-assay (across the experiments) CV of 15.5%.

3.5. ER agonist and antagonist activity of DBP, MBP and DEHP

For DEHP and MBP, the induction of luciferase was not significantly higher than vehicle control, which indicated neither had estrogenic activity, while DBP displayed weak estrogenic activity when it reached the maximal induction of 2.6-fold at concentration of 10^{-4} M (Fig. 4B).

3.6. Response to T_3

CV-1 cell reporter system showed appropriate response to the natural TR ligand T_3. T_3 induced luciferase activity in a concentration-dependent manner ranging from 10^{-10} to 10^{-6} M (Fig. 5A). From the dose–response curve, the EC_{50} of T_3 was 1.50 × 10^{-8} M. For T_3, it was noticeable that the maximal induction of vehicle control achieved 346-fold at concentration of 10^{-6} M. Analysis of responses of six replicates shows that 10^{-8} M T_3 induced luciferase activity with an intra-assay (within plate) CV of 7.4% and the inter-assay (across the experiments) CV of 18.5%.

3.7. TR agonist and antagonist activity of DBP, MBP and DEHP

We tested the chemicals for their inhibitory effect to the activity of 5 × 10^{-9} M T_3. As showed in Fig. 5B, DBP, MBP and DEHP possessed antagonist activity with IC_{50} of 1.31 × 10^{-5}, 2.77 × 10^{-6} M and exceeding 1.0 × 10^{-4} M, respectively. DBP and MBP exhibited the stronger antagonist activity than DEHP, especially the metabolite MBP. And for the agonist activity, none of them could induce the expression of the luciferase (data not shown), which indicated that they could not activate TR.

4. Discussion

The value of reporter gene assays as a mechanistic tool to characterize receptor mediated endocrine activity, and an important screening assay for endocrine disruptors have been well recognized (Freyberger and Schmuck, 2005). In the present study, we utilized three different reporter gene assays to investigate the potent hormone activity of chemicals. The results showed that the tested phthalates possessed mixed androgenic/antiandrogenic activity,
antagonist activity of thyroid hormone, and weak estrogenic activity except for MBP and DEHP.

The AR disrupting activity of the three chemicals was studied using MDA-kb2 human breast cancer cells, stably expressing an androgen-responsive luciferase reporter gene, MMTV-luc. Antagonist activity was detected in competition against $1 \times 10^{-9}$ M DHT. All the chemicals elicited AR antagonistic effects, and the metabolite MBP possessed the stronger antiandrogenic activity than its precursor DBP. This result indicated that metabolizing to MBP enhanced the antiandrogenic activity of DBP. Our result are consistent with previous studies, where DBP and DEHP showed antiandrogenic activity in AR-EcoScreen cells (Araki et al., 2005), and DBP possessed antiandrogenic activity in transiently transfected CHO-K1 cells using a pIND-ARE reporter vector and the AR expression plasmid pZeoSV2AR (Takeuchi et al., 2005). In contrast, no effect of DBP and DEHP on the AR activity was observed in transiently transfected CHO-K1 cells using MMTV-LUC reporter vector and the AR expression plasmid pSVAR0 (Krüger et al., 2008) nor in stable transfected CHO-K1 cells (Roy et al., 2004). The discrepancy could be a result of different cell line and consequent variant sensitivity of the various assays.

It was noteworthy that the three tested phthalates also appeared the androgenic activity with the maximal fold induction of 4, 11.3 and 7.7 for DBP, MBP, and DEHP, respectively. Because of the presence of AR and GR in MDA-kb2 cells, cells were exposed to the tested chemicals alone and with Butamidine, which is the potent AR antagonist, to distinguish agonist activity mediated by AR or GR. And we found butamidine reduced AR-mediated luciferase activity of DBP, MBP and DEHP. The three tested chemicals in our study not only displayed androgenic activity, but also the antiandrogenic actions. With respect to mixed agonist/antagonist activity of compounds, it was reported that some androgen antagonists can act as agonists depending on ligand binding affinity, concentration of ligand, and the presence of competing natural ligands (Wong et al., 1995). It is therefore important to pay close attention to the mixed activity of the tested phthalates, because the potential adverse effects of these chemicals on humans or animals will depend not only on their antiandrogenic activity, but also on their potential to act as agonist in the presence of environmental and/or endogenous androgen. DEHP and DBP are considered antiandrogenic endocrine disruptors because of their possible effect on animal gonads and reproduction (Harris et al., 1997; Ema and Miyawaki, 2001). Using the Hershberger assay, Lee and Koo (2007) found that DBP and DEHP possessed antiandrogenic activity in vivo. In rodents, perinatal exposure to DEHP and other phthalates results in major alterations in Sertoli and Leydig cells and consequent disruption of testicular function and androgen-dependent development of male offspring (Fisher et al., 2003; Mylchreest et al., 2002; Gray et al., 2000; Parks et al., 2000). Co-administration of DBP and DEHP in utero significantly reduced testosterone levels in rat foetal testis (Martino-Andrade et al., 2008). When MBP was measured in urine with high-performance liquid chromatography and tandem mass spectrometry, the results suggested that there were dose–response relationships between MBP and sperm motility and sperm concentration (Duty et al., 2003).

We utilized the CV-1 cell line which was transiently transfected with rat ERα, expression vector rERα, and ERE-response reporter gene to study the interaction of rERα and chemicals. As a result, only DBP displayed relatively weak estrogenic activity with the maximal induction of 2.58-fold of vehicle control at the concentration of $1 \times 10^{-8}$ M. We previously reported that comparing to the hERα, rERα showed little effect on the estrogenic activity of alkylphenols (Sun et al., 2008b). Our results were consistent with Harris et al. (1997) who reported that DEHP showed no estrogenic activity whereas DBP was markedly estrogenic in the recombinant yeast screen and proliferation of MCF-7 and ZR-75 cells assays. Our data support the view that the shorter chain phthalates, such as DBP, possessed the stronger estrogenic activity. In this paper, only one mechanism of action was investigated, but this may be just one of many pathways that might lead to harmful reproductive effects on human and/or animals exposed to these chemicals. Thus, the absence of estrogenic activity of DEHP and MBP observed in the assay does not eliminate the adverse effects of these compounds on reproductive system. Moreover, exposing to DEHP and MBP may alter the natural hormone balance, because estrogen responsive sites such as the reproductive tract or neuroendocrine centers are highly sensitive to these chemicals. DEHP and DBP have been shown to interfere with the estrogen-signaling pathway by competitive binding to estrogen receptors (Buteau-Lozano et al., 2008; Jobling et al., 1995). Comparing structural differences among these three tested chemicals, we found a common aromatic ring. Our results of the tested estrogenic activity of DBP and antiandrogenic activity of DBP, MBP and DEHP supported the conclusion that the most of the estrogenic and antiandrogenic chemicals contained at least one aromatic ring (Kuiper et al., 1998).

To test thyroid hormone-disrupting potencies, we used a recombining CV–1 cell line containing a T3-dependent reporter gene, UAS-tk-Luc, which was cotransfected with pG4-L-TR into CV–1 cell line, to detect the thyroid system-disrupting activity of the three chemicals. Anti-thyroid hormone activity of DEHP, DBP and MBP was observed in our study. In addition, compared to DBP, the metabolite MBP showed greater antagonistic activity. DEHP is known to decrease the plasma thyroxine level in rats (Brucker-Davis, 1998), an effect has been presumed to be due to alterations in thyroidal activity (Hinton et al., 1986) or the control of the hypotalamus–pituitary–thyroid axis (Jekat et al., 1994). Elevated urinary MBP exposure was associated with low serum thyroxine and free thyroxine in pregnant women during the second trimester (Huang et al., 2007). Using a Xenopus laevis cell line which was transfused with a self-inactivating lentivirus vector containing a luciferase gene, Sugiyama et al. (2005) evaluated possible T3 antagonist activity of several phthalates, including DBP. Our data from CV–1 cell line cotransfected with pUAS-tk-luc, and pG4-L-TR were in agreement with their in vitro study. There results indicated that the luciferase assay was a useful method for the primary screening of thyroid system–disrupting chemicals. Ishihara et al. (2003) obtained the capability of inhibiting by 40–50% of DEHP for bullfrog TR LBD. They stated DEHP may possibly act, by binding to TR, as a thyroid-disrupting chemical.

In conclusion, we demonstrated mixed androgenic/antiandrogenic, anti-thyroid hormone activity of DEHP, DBP and MBP, and weakly estrogenic activity of DBP, using three in vitro reporter gene assays. Based on the results, it could be suggested that the tested chemicals disrupted the endocrine system through interfering with several hormonal signaling pathways simultaneously. Our laboratory has been working on the relationship between urinary metabolites of phthalates and male infertility. However, further in vivo assays of hormone activities of phthalates are still necessary to extrapolate in vitro results to human population.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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