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Endocrine Disrupting Activity of Seven Phthalate Analogues *in vitro*

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ABSTRACT : **Phthalate analogues are a plasticizer and solvent used in industry. Phthalates were reported to be a potential carcinogen classified in the category of suspected endocrine disruptors. Most common human exposure to these compounds may occur with contaminated food. They may migrate into food from plastic wrap or may enter food from general environmental contamination. Since these substances are not limited to the original products, and enter the environment, they have become widespread environmental pollutants, thus leading to a variety of phthalates that possibly threaten the public health. Concern about their use has been mounting. To screen and elucidate the endocrine disrupting activity and their mechanism of phthalate analogues, first of all, E-screen assay was performed in MCF7 human breast cancer cells with seven phthalate analogues. In this cell proliferation assay, only dibutyl phthalate (DBP) showed weak estrogenic activity. Also the yeast-based transcription assay to assess the interactions of DBP with the estrogen, androgen, and progesterone receptors was conducted. DBP in the concentration ranges from 10^{-16} to 10^{-11} M was active in the estrogen transcriptional assay, but it did not show the effect on β-galactosidase activity in the progesterone and androgen transcriptional assays. These data indicate that DBP shows estrogenic potential and can be classified as weak and/or suspected endocrine disrupting chemicals.**

Keywords : **phthalate, endocrine disruptor, E-screen assay, receptor-binding assay, yeast-based transcription assay**

Introduction

Phthalate analogues (esters of 1,2-benzyenedicarboxylic acid) are widely used as plasticizers to increase the flexibility and workability of high-molecular-weight polymers. Their low melting point and high boiling point make them very useful as heat transfer fluids and carriers. The world wide production of phthalates approximates 2.7 million metric tons a year (Bauer and Herrmann, 1997). Phthalates are used in plastic goods (e.g., in children's toys, paints, lacquers, cosmetics, as well as food wrappings). Furthermore, many medical items, such as blood bags, tubes, and filtering membranes, may contain a considerable amount of phthalates. Since these substances are not limited to the original products, and they may enter the environment and have become widespread environmental pollutants, thus leading to a variety of phthalates that possibly threaten the public health. Concern about their use has been mounting.

It has been suggested that substances present in the environment may contribute to the development of hormone-dependent cancers and comprise reproductive capacity in humans and wildlife (Colborn *et al.*, 1993; Davis *et al.*, 1993; Eubanks, 1997). Phthalates are often mentioned as suspected endocrine disruptors, i.e., some phthalates are blamed for causing damage to the testes and decreasing sperm production (Gray *et al.*, 1982; Hardell *et al.*, 1997) and are reported to be a potential carcinogen. Among many phthalate compounds, butyl-benzyl-phthalate and di-n-butyl-phthalate (DBP) may act as xenoestrogens or antiandrogens (Bradbury, 1996; Mylchreest *et al.*, 1998). Estrogenic activity of phthalate esters was investigated *in vitro* assay (Harris *et al.*, 1997). Di-(2-ethylhexyl)-phthalate was demonstrated to increase the frequency of proliferating cells in a human breast-cancer cell line (Blom *et al.*, 1998). Also, DBP was reported to be genotoxic on human mucosa of the upper aerodigestive tract (Kleinsasser *et al.*, 2000).

To screen and elucidate the endocrine disrupting activity, first of all, E-screen assay was performed with MCF7 human breast cancer cells with seven phthalate analogues. This *in vitro* assay compares the cell yield between cultures of breast tumor-derived MCF7 cells treated with 17β-estradiol (Soto and Sonnenschein,

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1985) and cultures treated with different concentrations of xenobiotics suspected of being estrogenic (Soto *et al.*, 1995). We also subjected yeast-based assay to assess the interactions of DBP with the estrogen, androgen, and progesterone receptors (Routledge and Sumpter, 1996, 1997; Gaido *et al.*, 1997).

In this study, we investigated the endocrine-disrupting activity of seven phthalate analogues by using *in vitro* E-screen and yeast transcription assay.

Materials and Methods

1) E-Screen assay

a) Chemicals

Diallyl phthalate (CAS No. 131-17-9), benzyl *n*-butyl phthalate (85-68-7), di-*n*-octyl phthalate (117-84-0), detridecyl phthalate (119-06-2) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Diisodecyl phthalate (26761-40-0), di-*n*-nonyl phthalate (84-76-4) were obtained from Merck (Darmstadt, Germany). Dibutyl phthalate (CAS No. 84-74-2) and 17β-estradiol (CAS No. 50-28-2) were obtained from Sigma-Aldrich Co. (St. Louis, USA). They were dissolved in ethanol immediately before use. The final concentration of ethanol used in the medium was below 1%.

b) Cell line and cell culture conditions

Human breast cancer estrogen-sensitive MCF7 cell line was kindly donated by Dr. Soto in Tufts University School of Medicine. For routine maintenance, cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% CO_2/95% air under saturating humidity (Soto and Sommemschein, 1985).

c) Cell proliferation experiments

MCF7 cell line was tested with slight modification described by Soto *et al.* (1995). Cells were trypsinized and plated in 12-well plates at an initial concentration of 3×10^4 cells per well in 5% FBS in DMEM. The cells were allowed to attachment for 24 hr, then 5% charcoal dextran-treated fetal bovine serum (CDFBS) supplemented phenol red-free DMEM was substituted for the seeding medium. Appropriate concentrations of the test compounds were added. The assay was stopped after 168 hr by mixing with 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the plate was further incubated for 3 hr at 37°C. The medium was removed and the formed formazan crystals were dissolved with 1 ml of dimethyl-sulfoxide (DMSO). Finally, the aliquots were transferred to a 96-well plate to read optical density (OD) in a microtiter plate reader at 540 nm. Linearity of optical density was evaluated in the MTT assay.

2) Yeast transcription assay

a) Chemicals

Dibutyl phthalate (CAS No. 84-74-2) and 17β-estradiol (CAS No. 50-28-2) were obtained from Sigma-Aldrich Co. (St. Louis, USA). Progesterone and testosterone were provided by Dr. Chung in our institute and diluted by ethanol. Chlorophenol red-β-d-galactopyranoside (CPRG) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Oxylalicticase was obtained from Enzogenetics (Corvallis, USA). All other chemicals were obtained from Sigma-Aldrich Co. (St. Louis, USA).

b) Yeast strain

A recombinant yeast strain was developed in the Genetics Department at Glaxo for use in a test to identify compounds that can interact with the human estrogen receptor (hER). This strain was donated from Dr. Routledge in Department of Biology and Biochemistry of Brunel University (Routledge and Sumpter, 1996).

In this system, the hER is expressed in a form capable of binding to estrogen-responsive element sequences (ERE). These sequences were situated within a strong promoter sequence on the expression plasmid. Upon binding to an active ligand, the estrogen-occupied receptor interacts with transcriptional factors and other transcriptional components to modulate gene transcription. This causes expression of the reporter gene *lac-Z* and the enzyme produced (β-galactosidase) is secreted into the medium, where it metabolizes the yellow chromogenic substrate CPRG into a red product that can be measured by absorbance at 540 nm.

The YPH500 strain which is donated from Dr. Gaido in Chemical Industry Institute of Toxicology, and used for androgen and progesterone receptor assays (Sikorski and Hieter, 1989). This yeast transformations were carried out following the lithium acetate transformation protocol (Ito *et al.*, 1983). Yeast transformants used for measuring progesterone and androgen receptor activity contain three separate plasmids: (1) an expression plasmid which contains the CUP1 metallothionein promotor fused to either the human progesterone or androgen receptor (Vegeto *et al.*, 1992), (2) a reporter plasmid carrying two copies of a progesterone/androgen responsive element at upstream of the structural gene for â-galactosidase and (3) a plasmid encoding for RSP5 (Reversion of *Spt* Phenotype) in the progesterone receptor assay, or SPT3

which is part of the multicomponent protein named SAGA (*Spt-Ada-Gcn5* Acetyltransferase) in the androgen receptor assay (Vegeto *et al.*, 1992).

c) Growth of yeast

For the estrogen receptor assay, growth medium was prepared by adding 5 ml of 20% glucose solution, 1.25 ml of l-aspartic acid solution (4 mg/ml) and 0.5 ml of vitamin solution. Vitamin solution was prepared adding 8 mg of pyridoxine, 8 mg of pantothenic acid, 40 mg of inositol, and 20 ml of biotin solution (2 mg/100 ml) to 180 ml of double-distilled water. 0.4 ml of l-threonine solution (24 mg/ml), and 125 μl of copper (II) sulfate solution (20 mM) were added to 45 ml of single strength minimal medium in a sterile conical flask. The growth medium was then inoculated with 0.25 ml of the concentrated stock yeast and incubated at 28°C for approximately 24 hr on an orbital shaker (250 rpm with a 50 mm throw) until the absorbance at 640 nm was reached to 1.0. The assay medium was prepared by adding 0.5 ml of the chromogenic substance CPRG (10 mg/ml) to 50 of fresh growth medium. The medium was seeded with 2 ml of yeast from the yeast solution cultured for 24 hr with the absorbance at 640 nm of 1.0 prior to use.

YPH500 yeast transformants were grown overnight at 30°C with vigorous orbital shaking at 300 rpm in the selective medium containing yeast nitrogen base without amino acids (6.7 g/l) plus dextrose (20 g/l), lysine (30 mg/l), uridine (40 mg/l), adenine (40 mg/l), and histidine (20 mg/l) for yeast transformed with the androgen or progesterone receptors. After incubation for overnight, the yeast were then subcultured in fresh medium and allowed to grow until early-mid-log phase ($OD_{600\ nm}$ approximately 1.0).

3) Estrogen receptor assay

For the estrogen receptor assay, yeast was carried out within a typeII laminar air flow cabinet to minimize aerosol formation. Chemicals were serially diluted and 10 μl aliquots of each concentration were then transferred to a optically flat bottom 96-well microtiter plate. Chemicals dissolved and diluted in absolute ethanol were allowed to evaporate to dryness on the assay plate. Chemicals that were insoluble in ethanol were dissolved and serially diluted in medium. Aliquots (200 μl) of the seeded assay medium (medium containing recombinant yeast) and the chromogenic substance CPRG was then dispensed to each sample well using a multichannel pipettor. Each plate contained at least one row of blank (assay medium only), as well as a standard curve for 17β-estradiol. The yeast mixture was incubated at 32°C in a low temperature incubator. After incubation for 2 days, the color development of the medium was checked periodically at 540 nm, to obtain data with the best contrast. After incubation, control wells appear as light orange in color due to the background expression of β-galactosidase, and turbid due to growth of the yeast. Positive wells (containing no growth) indicate lysis of the cells, and the color may vary (Routledge and Sumpter, 1995).

4) Androgen receptor assay

For the androgen receptor assay, yeast from early-mid-log phase growth ($OD_{600\ nm}$ approximately 1.0) were diluted to an $OD_{600\ nm}$ of 0.03 in selective medium plus quoted into 5 ml conical screw cap tubes and doses for either steroid or test chemical in ethanol were applied. Dose-response curve for testosterone as positive control was performed in experiments for the androgen transcriptional assay. The cultures were incubated overnight at 30°C with vigorous orbital shaking (300 rpm). After incubation the yeast culture samples were diluted in the appropriate selective medium to an $OD_{600\ nm}$ of 0.25 and 100 μl of the aliquot was added to each well of a 96-well microtiter plate. Each sample was assayed in triplicate. To each well, 100 μl of assay buffer (60 mM Na_2HPO_4 and 40 mM NaH_2PO_4), 10 mM KCl, 1 mM $MgSO_4$ 2 mg/ml 2-nitrophenyl-β-d-galactosidase (ONPG), 0.1% SDS, 50 mM β-mercaptoethanol, and 200 U/μl oxalyticase were added. The change in concentrations of *o*-nitrophenol, the yellow product that results from β-galactosidase cleavage of ONPG, was measured using a kinetic microtiter plate reader. β-galactosidase activity is expressed as V_{max} ($OD_{420\ nm}$/min) divided by cell density ($OD_{590\ nm}$) (Gaido *et al.*, 1997).

5) Progesterone receptor assay

Yeast was diluted to an $OD_{600\ nm}$ of 1.0 in the selective medium and 50 aliquots were added to each well of 96 well microplate which also contained 50 μl selective medium, 100 μM $CuSO_4$, and 2×dose of progesterone or DBP in ethanol (50 μM $CuSO_4$, and 1×dose final concentration). Vehicle control (0.1% ethanol) was included in each experiment. A progesterone dose-response curve was performed in every experiment as a positive control. The cultures were incubated at 30°C for 4 hr

because progesterone inhibited the cell growth of yeast in a progesterone receptor-dependent manner. After incubation the yeast culture samples were diluted in the appropriate selective medium to an OD_{600nm} of 0.25 and 100 μl of its aliquot was added to each well of a 96-well microtiter plate. Each sample was assayed in triplicate. To each well, 100 μl of assay buffer (60 mM Na_2HPO_4 and 40 mM $NaH_2PO_{4)}$, 10 mM KCl, 1 mM $MgSO_4$, 2 mg/ml 2-nitrophenyl-β-d-galactosidase (ONPG), 0.1% SDS, 50 mM β-mercaptoethanol, and 200 U/μl of oxalyticase were added. The change in concentration of *o*-nitrophenol, the yellow product that results from β-galactosidase cleavage of ONPG, was measured using a kinetic microtiter plate reader. β-Galactosidase activity is expressed as V_{max} ($OD_{420\ nm}$/min) divided by cell density ($OD_{590\ nm}$).

Results & Discussion

Recently, phthalate esters, a group of phthalic acid-derived compounds have been shown to induce estrogen receptor (ER)-mediated responses. *In vitro* studies demonstrated that selected phthalate esters, namely, di-2-ethylhexyl phthalate (DEHP), di-n-butyl phthalate (DBP), and butylbenzyl phthalate (BBP), were capable of competing with 17β-estradiol for binding to rainbow trout hepatic ER (Jobling et al., 1995). However, this is not sufficient evidence to conclude that these phthalate esters are estrogenic. In addition, competitive ligand-binding data cannot indicate agonist or antagonist activities of a chemical. Therefore, to further investigate the ER-mediated activities of the seven phthalates listed in Table 1, were examined using recombinant receptor/reporter gene assays in mammalian cells and yeast. These assays have been previously used to investigate the ER-mediated activities of a number of chemicals, natural products and complex mixtures (Balaguer *et al.*, 1996a,b; Clemons *et al.*, 1998; Connor *et al.*, 1995, 1997; Fielden *et al.*, 1997; Moore *et al.*, 1997; Ruh *et al.*, 1995; Zacharewski, 1997; Zacharewski *et al.*, 1995).

Seven phthalate analogues were tested for estrogenic potency (Table 1). In E-screen assay, among seven phthalate analogues, 10^{-4} M DBP, 10^{-5} M BBP and 10^{-5} M DAP revealed high values of relative proliferative effect (RPE), 106, 109 and 80%, respectively, in MCF7 human breast cancer cells. These suggest that DBP, BBP and DAP have the activity of a partial agonist capable of interacting with the ligand-binding domain of the human ER although concentrations are higher compared with 17β-estradiol in E-screen assay. However, remaining four phthalate analogues used in this study revealed very low relative proliferative potency (RPP) values. These results are consistent with a previously published report demonstrating that DBP and BBP were capable of inducing a vitellogenin A2 ERE-regulated luciferase reporter in transiently transfected MCF7 cells and inducing gene expression of an estrogen response element (ERE)-regulated reporter gene and promoting ZR75 human breast cancer cell proliferation (Jobling *et al.*, 1995). Also, Nakai *et al.* (1999) reported that dialkyl phthalates with an alkyl chain of more than C_3 (n=3), for example, diallyl phthalate, dibutyl phthalate and dicyclohexyl phthalate exhibited a distinct full receptor binding on the basis of chemical structures.

So, we performed yeast-based steroid hormone receptor gene transcription assays to confirm other hormonal

Table 1. Estrogenic effect of seven phthalate analogues measured by the E-screen assay

Compound	Concentration	RPE (%)[a]	RPP (%)[b]
17β-Estradiol	0.001 μM	100	100
Benzyl butyl phthalate (BBP)	10 μM	109	0.001
Dibutyl phthalate (DBP)	100 μM	106	0.001
Diallyl phthalate (DAP)	10 μM	80	0.001
Di-n-octyl phthalate (DOP)	100 μM	35	0.0001
Dinonyl phthalate (DNP)	100 μM	35	0.0001
Diisodecyl phthalate (DiDP)	100 μM	19	0.0001
Ditridecyl phthalate (DTP)	100 μM	67	0.0001

The lowest concentration needed for maximal cell yield. [a]The relative proliferative effect (RPE) is calculated as 100× proliferative effect (PE), which is 100 times the ratio between the highest cell yield obtained with the chemical and with 17β-estradiol, of the test compound/PE of 17β-estradiol. [b]Relative proliferative potency (RPP) is the ratio between 17β-estradiol and xenoestrogenic dose needed to produce maximal cell yields × 100. All compounds designated as full or partial agonist increased cell yields significantly over the hormoneless control.

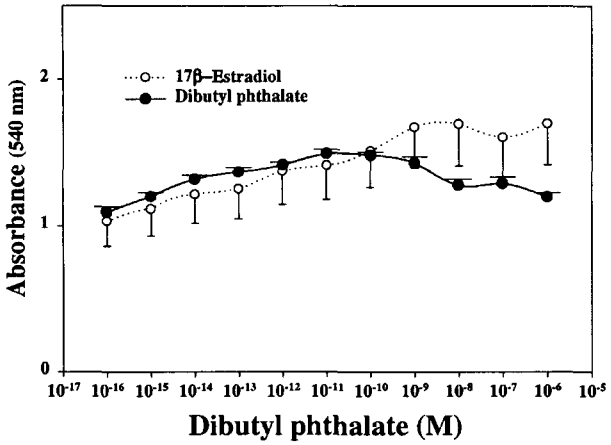

Fig. 1. Yeast assay for estrogen receptor activity. The graph shows the log concentration of 17β-estradiol (serially diluted from 10^{-6} M) plotted against the absorbance of the medium after 2 days incubation. Values represent the mean ± SE (n=3). In most cases, the SEM was too small to illustrate.

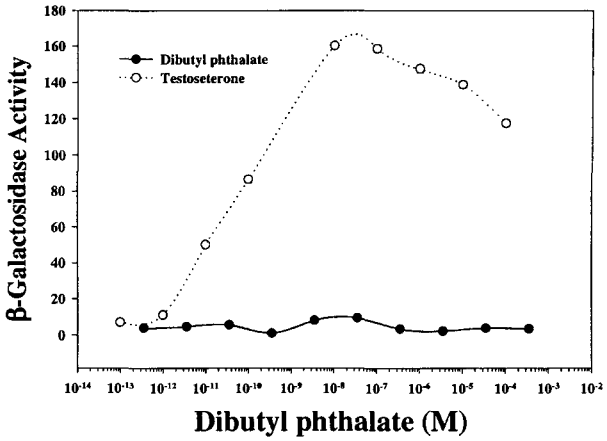

Fig. 2. Yeast assay for androgen receptor activity. DBP was added to yeast cultures in doses ranging from 3.5910^{-13} to 3.5910^{-4} M. Following overnight incubation the cultures were then assayed for β-galactosidase activity. Values represent the mean±SE of three experiments for DBP and are presented as the rate of β-galactosidase activity (V_{max} at $OD_{420\ nm}$) divided by the cell density ($OD_{590\ nm}$).

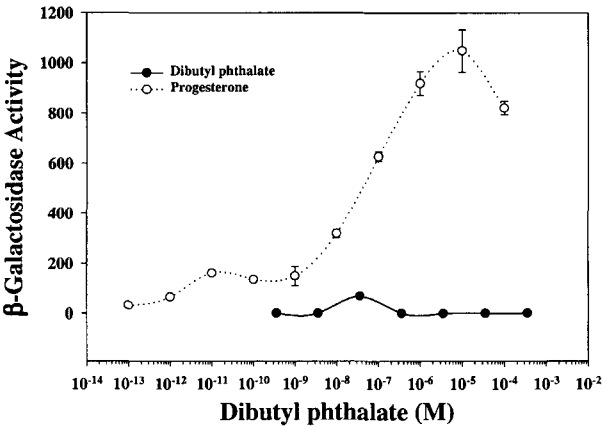

Fig. 3. Yeast assay for progesterone receptor activity. DBP was added to yeast cultures in doses ranging from 3.5910^{-10} to 3.5910^{-4} M. Following overnight incubation the cultures were then assayed for β-galactosidase activity. Values represent the mean±SE of three experiments for DBP and are presented as the rate of β-galactosidase activity (V_{max} at $OD_{420\ nm}$) divided by the cell density ($OD_{590\ nm}$).

modulating activity of DBP. Yeast cells are excellent heterologous hosts to investigate the functionality of nuclear receptors in the presence various agonists and antagonists (Arnold *et al.*, 1996; Berry *et al.*, 1990; Heery *et al.*, 1993; McDonnell *et al.*, 1989; Metzger *et al.* 1988; Ohashi *et al.*, 1991; Purvis *et al.*, 1991; Zysk *et al.*, 1995). The estrogenic activity of DBP was assessed by measuring β-galactosidase activity in triplicate. In estrogen receptor transcriptional assay, DBP in the concentration ranges of 10^{-17}~10^{-10} M revealed similar β-galactosidase activity with 17β-estradiol. Decreasing of β-galactosidase activity was observed at the high concentrations (10^{-9} 10^{-6} M), possibly being due to cytotoxicity of DBP (Fig. 1). However, no significant increase of β-galactosidase activity of DBP in the yeast androgen (Fig. 2) and progesterone screening (Fig. 3) was observed compared with testosterone and progesterone, respectively, in the concentrations used. This data shows that DBP did not interact directly with the androgen and progesterone receptors for mimicking or blocking physiological roles of these steroids. This result is consistent with a recently published study examining the estrogenic activity of a large number of phthalates using a recombinant yeast screen (Harris *et al.*, 1997).

In summary, three phthalates, namely, DBP, BBP and DAP, exhibited weak ER-mediated estrogenic activity in vitro E-screen assay. Especially, estrogenicity data for DBP in E-screen assay and yeast transcription assay indicate that DBP can be classified as the category of weak and/or suspected endocrine disrupting chemicals. BBP and DAP also now under investigating in the yeast transcription assay.

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