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Evaluation of Estrogenic Effects of Phthalate Analogues Using *in vitro* and *in vivo* Screening Assays

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Abstract

Phthalate analogues are a plasticizer and solvent used in industry. Phthalates were classified in the category of "suspected" endocrine disruptors. The purpose of our study was to screen and elucidate the endocrine disrupting activity of seven phthalate analogues. E-screen assay was performed in MCF-7 human breast cancer cells with seven phthalate analogues. In this cell proliferation assay, benzyl butyl phthalate (BBP) and dibutyl phthalate (DBP) showed high estrogenic activity. Their relative proliferation efficiencies (RPE) were 109 and 106%, respectively. *In vitro* estrogen receptor (ER) binding assay, BBP, di-n-octyl phthalate (DOP) and dinonyl phthalate (DNP) showed weak relative binding affinity (RBA: 0.02%) compared to 17β-estradiol (E2) (RBA: 100%). In uterotrophic assay, E2 produced a significant increase, whereas four tested phthalate analogues had potential estrogenic effects *in vitro* did not increased in uterus weight in immature rats. From these results, we demonstrated that phthalate analogues exhibit weak estrogenic activity *in vitro* assays at high concentrations. Although phthalates induced an increase in MCF-7 cell proliferation by an estrogenic effect, they could not induce a uterus weight increase *in vivo*. From these, we may suggest that these phthalate analogues are easily metabolized to inactive forms *in vivo*. Further investigation in other *in vitro* and *in vivo* experimental systems might be required.

Keywords: Phthalate analogues, endocrine disruptors, E-screen assay, *in vitro* estrogen receptor (ER) binding assay, *in vivo* uterotrophic assay

Phthalate analogues (esters of 1, 2-benzenedicarboxylic 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 per a year[1]. Phthalates are also used in plastic goods (e.g., in children's toys, paints, lacquers, cosmetics, as well as food wrappings[2,3]. 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 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[4-6]. Many phthalate analogues have long been known to be reproductive toxicants when animals are dosed as juveniles or adults, and their teratogenicity is well established[7,8]. They have been reported to have estrogenic activities which mimic endogenous estrogen[9,10]. Previous studies have demonstrated that endocrine disrupters, e.g. n-butyl benzyl phthalate, are capable of interacting with estrogen receptors (ERs) and induce ER-mediated response, suggesting that estrogenic or anti-estrogenic effects elicited by these substances may be receptor-mediated[11-14]. Di-n-alkyl phthalates such as n-butyl benzyl phthalate (BBP), di (n-butyl) phthalate (DBP) and di (n-ethylhexyl) phthalate (DEHP) have estrogenic activity that mimics the steroid A ring of receptor binding modes[14].

In this study, we investigated the ability of several phthalate analogues to produce an endocrine disrupting response *in vitro* E-screen assay, competitive receptor binding assay and *in vivo* uterotrophic assay. The E-screen assay was optimized and validated for the sensitive quantitative determination of the total estrogenicity in test compounds. MCF-7 BUS cells showed the highest proliferative response to 17β-estradiol (E2). This quantitative assay compares the cell number of MCF-7 cells in the absence of estrogens (negative control) and in the presence of E2 (positive control) or a range of concentrations of chemicals suspected to be estrogenic. Traditional

estrogen receptor (ER) binding assays are useful for characterizing a chemical's potential to be an estrogen-acting EDC, but they involve displacement of a radioactive ligand from crude receptor preparations at low temperatures. We have utilized a validated (standardized) ER competitive assay to determine the ER affinity for a structurally diverse group of chemicals. Test chemicals that exhibited affinity for the ER in the first tier were subsequently assayed using a wide range of concentrations to characterize the binding curve and to determine each chemica's IC_{50} and relative binding affinity (RBA) values. We also performed the 3 day uterotrophic assay in immature female S.D. rats for detecting real estrogenic activity of four phthalate analogues *in vivo*.

The aim of this study is to compare results obtained by three assays—*in vitro* ER competitive binding assay (ER binding assay), the MCF-7 proliferation assay (E-screen assay) and *in vivo* uterotrophic assay—to evaluate estrogen-like actions of phthalate analogues that were weakly estrogenic.

Estrogenic Effect of Phthalate Analogues on MCF-7 Cells

Seven phthalate analogues were tested for estrogenic activity by the MCF-7 cell proliferation. Their relative proliferative effect (RPE) and relative proliferative potency (RPP) ratio are listed in Table 1. BBP and DBP revealed high values of RPE (109 and 106%, respectively). However, the rest of phthalate analogues used in E-screen assay revealed low RPE values. All of the RPP values of seven phthalate analogues were less than 0.001%. The relative potencies

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

Compound	Concentration	RPE (%)[a]	RPP (%)[b]
17β-estradiol (E2)	0.1 nM	100	100
Benzyl butyl phthalate (BBP)	10 μM	109	0.001
Dibutyl phthalate (DBP)	100 μM	106	0.001
Diallyl phthlate (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.

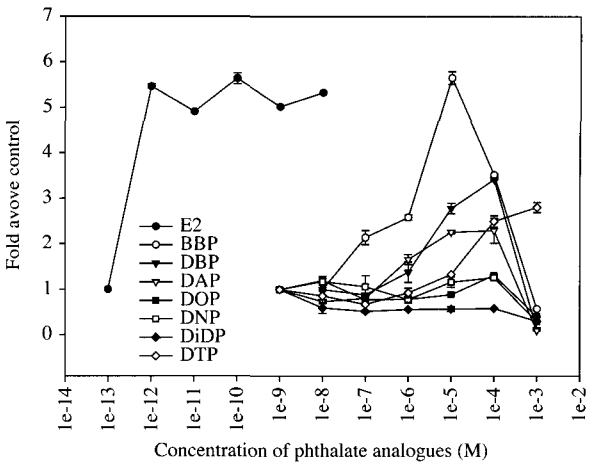

Fig. 1. Effects of 17β-estradiol and phthalate analogues on proliferation of MCF-7 cells. Data are expressed as fold increase in cell proliferation above hormone-free control. X-axis represent the concentration of 17β-estradiol, phthalate analogues in medium supplemented with 5% CD-treated FBS. Abbreviations: E2, 17β-estradiol; DES, diethylstilbestrol; BBP, benzyl buthyl phthalate; DBP, dibenzyl phthalate; DAP, diallyl phthalate; DOP, diotyl phthalate; DNP, dinonyl phthalate; DiDP, diisodecyl phthalate; DTP, ditrydecyl phthalate.

of these descended in order benzyl butyl phthalate (BBP)=dibutyl phthalate (DBP)=diallyl phthalate (DAP)>di-n-octyl phthalate (DOP)>dinonyl phthalate (DNP)=diisodecyl phthalate (DiDP)=ditridecyl phthalate (DTP). Potencies ranged from approximately 1×10^6 to 1×10^7 times less than E2. As shown in Fig. 1, DBP at concentration of 10^{-4} M and BBP at concentration of 10^{-5} M significantly stimulated the growth of MCF-7 cell, but rest of them did not stimulate the growth at concentration between 10^{-9} M and 10^{-3} M. E2 showed the highest proliferative responses as positive control cell yield increased up to six-fold over those of non-treated cells in 144 hr period.

Competitive Inhibition of [^{3}H] 17β-estradiol (E2) Binding to Human Estrogen Receptor (hER) by Phthalate Analogues

Seven phthalate analogues were examined for its ability to displace bound [^{3}H] E2 from hER *in vitro*. Binding was determined indirectly by measuring displacement of bound [^{3}H] E2 following addition of unlabeled competitor. Relative binding affinity (RBA) curves for E2, diethylstilbestrol (DES) and seven phthalate analogues were shown in Fig. 2. RBA and IC_{50} values of seven phthalate analogues were shown in Table 2. E2 and DES displaced bound [^{3}H] E2 in a concentration-dependent manner with IC_{50}

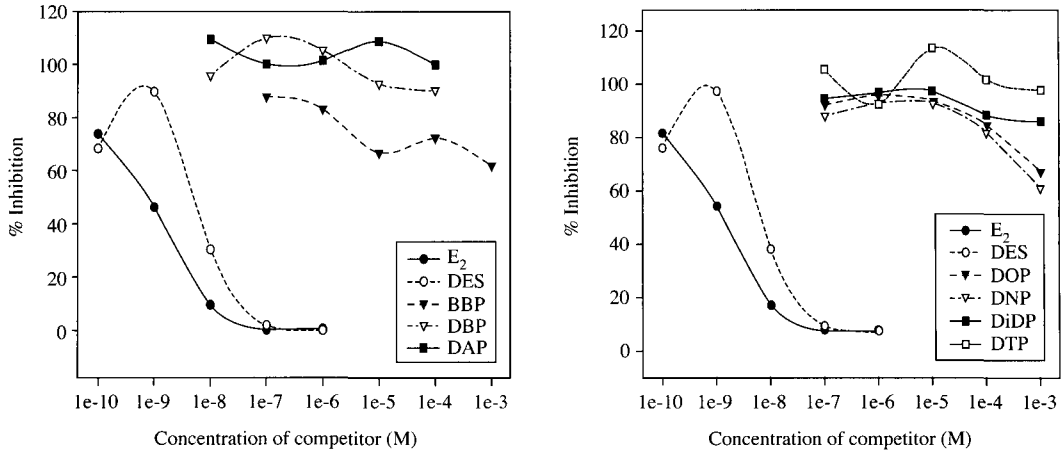

Fig. 2. Competitive inhibition of [^{3}H]-17β-estradiol binding to a human recombinant estrogen receptor α by unlabeled E2, DES and phthalate analogues. Abbreviations: E2, 17β-estradiol; DES, diethylstilbestrol; BBP, benzyl butyl phthalate; DBP, dibenzyl phthalate; DAP, diallyl phthalate; DOP, diotyl phthalate; DNP, dinonyl phthalate; DiDP, diisodecyl phthalate; DTP, ditrydecyl phthalate.

Table 2. Relative binding affinity of seven phthalate analogues for recombinant hER-α.

Compound	RBA (%)[a]	Equilibrium binding constant IC_{50} (nM)
17β-estradiol (E2)	100	1.14
Diethylstilbestrol (DES)	84.4	1.35
Benzyl butyl phthalate (BBP)	0.02	5,670
Dibutyl phthalate (DBP)	0.0041 (inactive)	27,500
Diallyl phthlate (DAP)	0.00002 (inactive)	650,000
Di-n-octyl phthalate (DOP)	0.02	6,600
Dinonyl phthalate (DNP)	0.02 ·	5,760
Diisodecyl phthalate (DiDP)	0.009 (inactive)	12,900
Ditridecyl phthalate (DTP)	0.003 (inactive)	33,000

[a]RBA (relative binding affinity) of each competitor was calculated as ration of concentrations of E2 and competitor required to reduce the specific radioligand binding by 50% (=ratio of IC_{50} values). RBA value for E2 was arbitrarily set at 100.

values of 1.14 nM and 1.35 nM, respectively (Fig. 2, Table 2). But seven phthalate analogues shown very weak binding affinity compared with positive controls (E2 and DES). In ligand competition experiments, the binding affinity decreased in the order E2 > DES ≫ BBP = DOP = DNP > DTP > DBP > DiDP > DAP. Positive control chemicals, E2 and DES showed a higher binding affinity about 100 and 84% respectively. Some of the tested compounds, such as BBP, DOP and DNP exhibited weak competitive binding activity based on their IC_{50} and RBA values (all RBAs were determined compared to E2, which was equal to 100%). And the rest of test chemicals were not shown remarkable changes of RBA.

Effects of Seven Phthalate Analogues on *in vivo* Uterotrophic Assay

When administered to female Sprague-Dawley (SD) rats (21 days old) by subcutaneous (s.c.) injection, the positive control agent for the present experiment, E2, gave a positive uterotrophic response by route of subcutaneous injection. Normalized uterine wet weight increased 2.5-3.5 fold following treatment with 3 μg/kg of E2. E2 represented reproducible and dose-dependent effects on uterine wet weight to vehicle control following s.c. injection of 0.3, 3 and 30 μg/kg of E2. However, four phthalate analogues (BBP, DBP, DAP and DTP) represent negative uterotrophic response (Table 3, Fig. 3).

Discussion

It has been reported that there is a relationship between the structure of a chemical and its estrogenic activity, and estrogenic effect results from a phenolic ring[16]. However, it is unclear what kind of chemical structure is needed for binding to ERs. In the present study, we investigated the possible estrogenic behaviour of seven of phthalate analogues *in vitro* and *in vivo*.

Seven phthalate analogues were tested for estro-

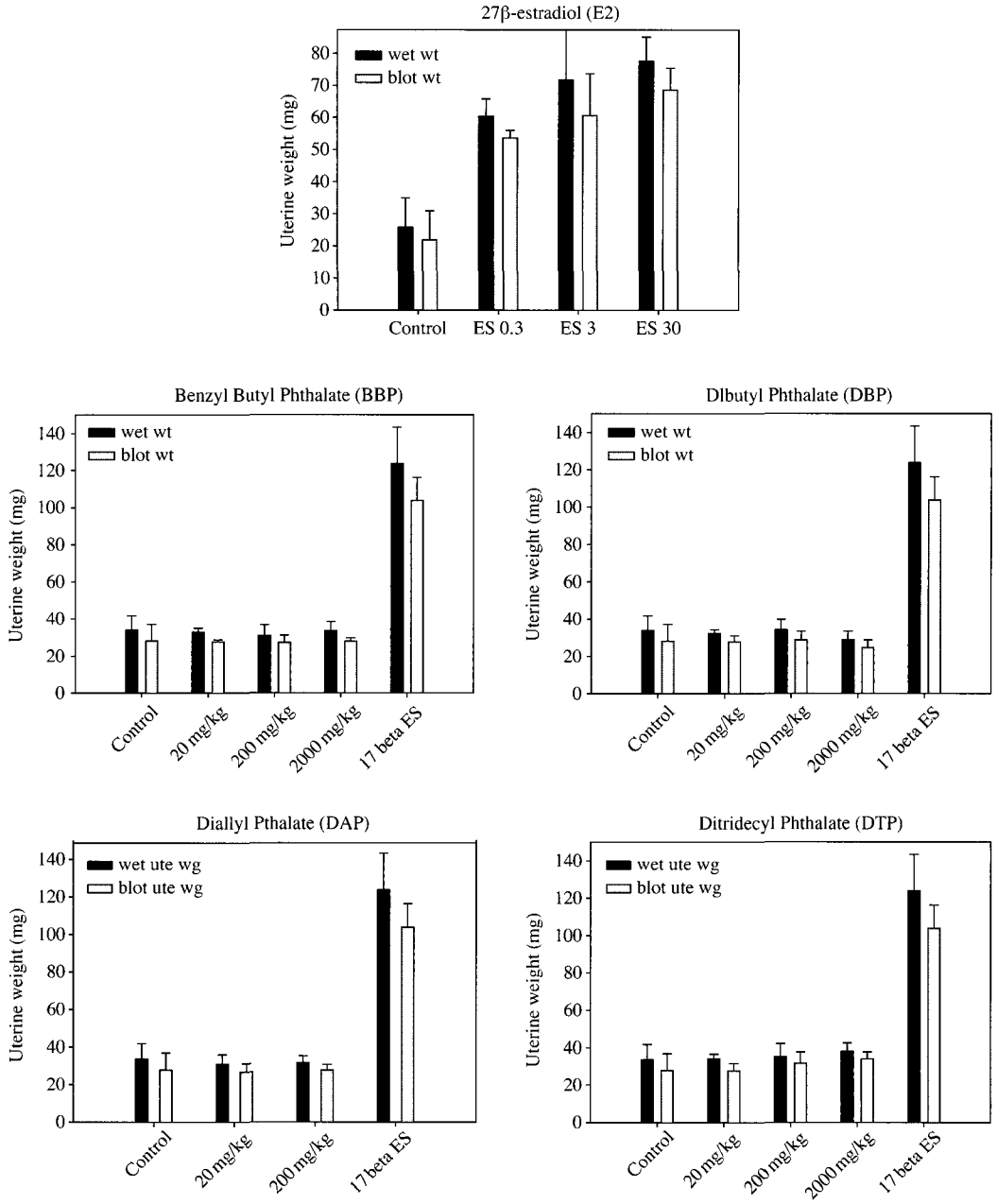

Fig. 3. Effects of 17β-estradiol (E2), BBP, DBP, DAP and DTP on uterine weight of immature rats. Wet uterine weight expressed as percent of control following exposure to each compounds by sc injection. Control uterine weights [means±S.D. (n=5)]. All experiments were performed in triplicate. Each column represents the mean and S.D.

genic activity in E-screen assay. As expected, E2 increased the proliferation of MCF-7 cells, in addition, among seven phthalate analogues, 10^{-4} M DBP and 10^{-5} M BBP revealed high values of RPE. However, most of the phthalate analogues used in this study revealed very low RPE values; they have a weak influence on cell proliferation of MCF-7 cells. This suggests that DBP and BBP have the activity of a partial agonist although concentrations are higher compared with E2 (10^{-10} M) in E-screen assay. DBP[11] and BBP[17,18] was weakly estrogenic in the cell proliferation assay with MCF-7 cells, whereas Soto *et al.*[17] found that DBP was inactive with MCF-7 cells. There might be some differences in the response to estrogenic stimuli between cell lines.

We showed whether phthalate analogues mimic the

Table 3. Effect of Phthalate analogues Uterine Wet weight in Immature Sprague-Dawley Rats.

Treatment	Dose (mg/kg)	Body weight		Uterine wet weight (mg)	Uterine wet weight (mg) /Body weight (g) ratio (mean ± SD)
		Initial	Final		
Corn oil	0.003	46±3	58±3	34±8	0.00058±0.0001
17β-estradiol (E2)		46±6	57±9	124±20**	0.00220±0.0002
Benzyl butyl phthalate (BBP)	20	45±5	54±7	33±2	0.00061±0.0001
	200	46±2	50±2	31±6	0.00062±0.0001
	2000	45±2	54±6	34±5	0.00063±0.00007
Dibutyl phthalate (DBP)	20	42±4	50±11	32±5	0.00066±0.0001
	200	48±4	58±5	34±7	0.00059±0.0001
	2000	45±4	54±3	29±2	0.00053±0.00002
Diallyl phthlate (DAP)	20	44±2	53±5	31±5	0.00059±0.00004
	200	44±5	51±7	28±3	0.00062±0.00005
Ditridecyl phthalate (DTP)	20	44±7	58±9	28±4	0.00064±0.00006
	200	44±5	53±4	35±7	0.00067±0.00012
	2000	44±4	54±5	38±4	0.0007±0.00002

Animals were exposed to Phthalate once daily for 3 consecutive days via subcutaneous injection (SC).
17β-estradiol was used as the positive control agent. Data on the effect of uterine wet weight represent the mean±SE from three experiments.
**; Significantly different from control group ($p<0.01$).

Table 4. Comparison of the assays used to determine estrogenic activity.

Compound	E-screen assay		hERα binding assay		Uterotrophic assay
	Concentration	RPE (%)[a]	IC_{50} (nM)	RBA (%)[b]	(Fold)
17β-estradiol	0.1 nM	100	1.14	100	2.5-3.5
Diethylstilbestrol (DES)			1.35	84.4	-
Benzyl butyl phthalate (BBP)	10 μM	109	5670	0.02	No effect
Dibutyl phthalate (DBP)	100 μM	106	27500	0.0041	No effect
Diallyl phthlate (DAP)	10 μM	80	650000	0.00002	No effect
Di-n-octyl phthalate (DOP)	100 μM	35	6600	0.017	-
Dinonyl phthalate (DNP)	100 μM	35	5760	0.02	-
Diisodecyl phthalate (DiDP)	100 μM	19	12900	0.009	-
Ditridecyl phthalate (DTP)	100 μM	67	33000	0.003	No effect

[a]RPE (relative proliferative effect) is calculated as 100 × proliferative effect (PE). [b]RBA (relative binding affinity) of each competitor was calculated as ratio of concentrations of 17β-estradiol and competitor required to reduce the specific radio ligand binding by 50% (=ratio of IC_{50} values).

effects of E2 by binding to ER. Although there was poor evidence for the mechanism of proliferation, it is likely that estrogenic response is caused by not only ER, but also other unknown factors[19]. The ability of a variety of phthalate analogues to compete with radio-labelled estradiol was investigated in human recombinant ER-α. For [^{3}H] E2 binding to hER, E2 and DES showed significantly competition at concentration about 10^{-12}-10^{-8} M (RPA approximately 80-100%). But all other compounds (BBP, DBP, DAP, DOP, DNP, DiDP and DTP) showed only very weak or no competition (RBA≪0.02%). In recombinant yeast screen assay, BBP[3,20-23] and DBP[3] had a weakly positive result, but DEHP was negative[3]. DBP and BBP weakly competed with E2 for binding to the rat uterine ER[20] and rainbow trout ER[11] in competitive ligand binding assays. On the other hand, in the binding competition to human uterine ER, no activity was observed with DBP and DEHP[24] similar to our results. The capacity of BBP, DEHP, DBP and DOP, to bind rat uterine ER was too weak to obtain IC_{50} values[25]. These results suggest that environmental phthalate analogues are unlikely to produce biological effects by concerns displacing endogenous estradiols from estrogen receptors unless they are present in very high.

In this study, a small number of commercially available phthalate analogues (BBP, DBP, DAP and DTP) are capable of acting as weak estrogens *in vitro* based on RPE value and RPP values (Table 4). So, we selected these phthalates for further *in vivo* rodent 3-day uterotrophic assay. According to the findings of

in vivo studies by Ema *et al.*, BBP[26] and DBP[27] induced embryonic loss, which is mediated, at least in part, by the impairment of uterine function. However, four phthalate analogues tested in the present study represent negative uterotrophic response. This results is similar with data of Coldham *et al.*[22] obtained a negative result with BBP in uterotrophic bioassay in mice.

Not all of the molecular events underlying endocrine disruption by xenochemicals have been delineated. Several environmental estrogens are also anti-estrogens, and hormone-mimicking chemicals can have multiple hormonal activities, and it may be difficult to interpret their mechanisms of action *in vivo*[23]. Di-alkyl phthalate analogues metabolized to mono-phthalate esters *in vivo*. In the case of BBP, it breaks down within 48 hr into the mono-esters, mono-n-butyl phthalate, mono-n-benzyl phthalate, phthalic acid and benzoic acid[19]. None of these metabolites have shown estrogenic activity in the E screen assay and the recombinant yeast assay[3,28].

Phthalates are ubiquitous in the environment and it is possible that humans are continuously exposed to them. It is still unclear that the physiological effects of exposure to phthalates are estrogenic. These questions need to be addressed, and their findings will help to assess the impact of human exposure to estrogenic xenobiotics. In the present study, we demonstrated that phthalate analogues exhibit weak estrogenic activity *in vitro* assays at high concentrations. Although phthalates induced an increase in MCF-7 cell proliferation by an estrogenic effect, they could not induce a uterus weight increase *in vivo*. From these, we may infer that these phthalate analogues are easily metabolized to inactive forms *in vivo*. Further investigation in other *in vitro* and *in vivo* experimental systems might be required. The long-term effects of phthalate esters on humans remain to be clarified.

Methods

E-Screen Assay

Chemicals. DAP (CAS No. 131-17-9), BBP (85-68-7), DOP (117-84-0), DTP (119-06-2) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DiDP (26761-40-0), DNP (84-76-4) were obtained from Merck (Darmstadt, Germany). DBP (CAS No. 84-74-2) and E2 (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%.

Cell line and cell culture conditions. Human breast cancer estrogen receptor-sensitive MCF-7 cell line was kindly donated by Dr. Soto in Tufts University School of Medicine. For routine maintenance, cells were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 5% CO_2/ 95% air under saturating humidity[29]. They were maintained at 37°C in our laboratory as monolayer cul-tures in closed cell culture plastic flasks (75 cm^2).

Preparation of steroid-removed serum by charcoal-dextran treated. 5% charcoal-0.5% dextran (CD) suspension aliquots of a volume similar to the serum aliquots to be processed were centrifuged at 2,500 rpm for 10 min. Supernatant were aspirated and serum aliquots were mixed with the charcoal pellets. This charcoal-serum mixture was maintained in suspension by rolling at 4 cycle/min at 37°C for 1 hr. This suspension was centrifuged at 2,500 rpm for 20 min. The supernatant was then filtered through a 0.22-μm Nalgene filter. Over 99% of serum sex steroid was removed by this treatment. CD sera were stored at −20°C until needed.

Cell proliferation experiments. MCF-7 cell line was tested with slight modification described by Soto *et al.*[18]. 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, and then 5% charcoal dextran-treated fetal bovine serum (CD-FBS) 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 dimethylsulfoxide (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.

ER Binding Assay

Receptor and instruments. Human recombinant estrogen receptor hER-α (Part. No. P2187) was obtained from Pan Vera Corporation (Madison, WI, USA) which made by ER cDNA PCR-amplification. ER α is a 66 kDa transcription factor that regulates expression of genes involved in tissue growth and differentiation. Radioactive [2, 4, 6, 7-^{3}H] estradiol (88.0 Ci/mmol) was obtained from Amersharm

(Buckinghamshire, England). Charcoal, dithiothreitol (DTT) and bovine serum albumin (BSA) were from Sigma. Ultima Gold scintillation cocktail were from Packard BioScience Company. We used table top centrifuge (GR-6R, Beckman, USA) and liquid scintillation counter (2000CA, Packared Instrumental International Switzerland).

Estrogen receptor (ER-α) binding assay. Buffer used for receptor binding was consist of 10 mM Tris (pH 7.5), 10% glycerol, 1 mM DTT and 1 mg/mL BSA. Estrogen receptor, [2, 4, 6, 7-^{3}H] estradiol (10 nM) and various concentration of test compounds were added to the final volume of 200 μL with the buffer to microcentrifuge tube. 10^{-5} M E2 used for nonspecific binding. Positive controls for competitive receptor binding assay were used E2 and DES. After 3 hr incubation at room temperature (25°C), the reaction mixture was filtered through GF/B glass filters presoaked with 0.05% polyethyeneimine using a brandel cell harvester (Brandel, USA) which removes free ligand [2, 4, 6, 7-^{3}H] estradiol. The dried filter paper transferred into the scintillation vial that was added 4.5 mL scintillation cocktail. Radioactivity was measured by liquid scintillation counter.

Rodent 3 Day Uterotrophic Assay

Animals and maintenance. Immature female SD (21 days old) rats with the range of 40-50 g body weights were used. Diets and water were available *ad libitum*. All animals were acclimatized for 24 hr before used. Each group was five animals.

Animals received three daily doses of the test compound and killed 24 hr after the final dosing. The mean body weight variation per group was within 5 g.

Administration of the test substances. Suitable controls receiving corn oil and E2 (3 mg/kg/6 mL) as reference hormone were maintained. The phthalate analogues (20, 200, 2,000 mg/kg/6 mL) were dissolved in corn oil and administered subcutaneously once a day for three consecutive days. Date and time of each administration were recorded exactly. The amount administered was calculated on body weight of the animal on treatment day. The volume of injection per day did not exceed 4.0 mL/kg.

Measurement of uterus weight. The uterus was excised, trimmed free of fat and then weighed (wet weight). And also, uterus blotted to remove excess fluids and weighed (blot weight).

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