Phthalates are man-made chemicals abundantly found in the environment. Estrogenic activities of phthalate di and monoesters were studied by in vitro assay of human breast cancer MCF-7 cell proliferation. Since phthalate monoesters are formed from diesters by degradation and are found in the environment, we selected some phthalate monoesters in addition to diesters. Among 19 compounds tested, dicyclocetyl phthalate (DCHP), di(2-ethylhexyl) phthalate (DEHP) and butyl benzyl phthalate (BBP) were found to have estrogenic activities, all of which were completely suppressed by the addition of pure anti-estrogen ICI 182,780. DCHP stimulated cell proliferation with maximal cell yield at 5x10^-5 M. Its estrogenic potency was approximately 1700000 times less than that of 17β-estradiol. DEHP and BBP stimulated cell proliferation only slightly at >10^-3 M. No other phthalate diesters or monoesters were tested. Anti-estrogenic activities were also examined by estimating the suppression of cell proliferation in the presence of 10^(-3) 17β-estradiol. Mono-n-pentyl phthalate (MNP), monocyclocetyl phthalate (MCPH), monobenzyl phthalate (MBP), monoisopropyl phthalate (MIIP) and BBP were suggested to have anti-estrogenic activities at higher than 10^-4 M. Among commonly used phthalate esters and those with related structures, some were found to be estrogenic and others were anti-estrogenic in vitro.

Key words MCF-7; phthalate-ester; estrogen; anti-estrogen; dicyclocetyl phthalate (DCHP); monocyclocetyl phthalate (MCHP)

Phthalates are widespread in food and the environment due to their use as plasticizers in consumer products, food packaging materials and biomedical devices. Phthalates are produced industrially in large quantities, mainly to impart flexibility to plastics. More than 400000 tons of phthalate plasticizers, such as di(2-ethylhexyl) phthalate (DEHP), di-n-butyl phthalate (DBP), dimethyl phthalate (DMP), diethyl phthalate (DEP) and butyl benzyl phthalate (BBP), are produced industrially each year in Japan. The ubiquity of these compounds in the aqueous environment is well known, and their presence has been reported in river water, sewage effluent samples, and drinking water. DMP, DBP and DHEP and their corresponding monoesters, monomethyl phthalate (MMP), mono-n-butyl phthalate (MBP) and mono(2-ethylhexyl) phthalate (MEHP), were detected in samples of the Tama river at levels of micrograms per liter. Phthalates are frequently detected in outdoor and indoor air. Measurement of the concentrations of phthalates in a newly built house revealed that those of DEHP and DBP were 1046 and 871 ng/m^3, respectively, and small amounts of DMP, DEP, BBP, diisobutyl phthalate and dicyclocetyl phthalate (DCHP) were also detected.

DEHP has been known to cause liver tumors in rodents and to enhance tumor promotion in skin and liver of mice. However, in 2000 IARC (the International Agency for Research on Cancer) downgraded DEHP from 2B to 3, “not classifiable as to its carcinogenicity to humans.” On the other hand, DEHP and BBP caused adverse effects in male reproductive organs in mice and rats, respectively. Gestational exposure of female rats to BBP and DBP caused malformation of reproductive organs in male offspring. MEHP suppressed proliferation of Sertoli cells in neonatal rats and had toxic effects on testes of prepubertal male rats. DBP is toxic to testes possibly through its metabolite, MBP. Phthalate monoesters such as MEHP are formed as metabolites by various tissue enzymes in mammals from phthalic acid diesters. To examine the human health risks of exposure to dialkyl or aryl phthalate, levels of urinary phthalate monoesters were assessed, and ethyl, benzyl and butyl phthalate were detected, reflecting exposure to high levels of DEP, DBP, and BBP.

Some phthalate diesters have been found to be weakly estrogenic. However, estrogenic activities of phthalate monoesters have not been studied, though they are found in the environment; their anti-estrogenic activities are not fully understood, though they are as important as estrogenic activities. One approach might be an in vitro experimental system that can avoid complex factors of the in vivo system. In the present study, we examined both anti-estrogenic and estrogenic activities of 8 phthalate diesters that are commonly present in the environment, and 11 monoesters, which could be derived from the diesters, using estrogen-dependent human breast cancer MCF-7 cells in vitro, to clarify the characteristics of each compound.

MATERIALS AND METHODS

Chemicals 17β-Estradiol and 4-hydroxytamoxifen (4OH-TAM) were purchased from Sigma. 7α-[9-(4,4,5,5,5-Pentafluoropentylsulfinyl)nonyl]estra-1,3,5(10)-triene-3,17β-diol (ICI 182,780) was from Toctris Cookson, Ltd. DEP, di-n-propyl phthalate (DPP), DBP, di-n-hexyl phthalate (DHP), DCHP, DEHP, and BBP were from Aldrich. Di-n-pentyl phthalate (DPP) was from Wako Pure Chemical Industries, Ltd. All the phthalate monoesters: MMP, monoethyl phthalate (MEP), mono-n-propyl phthalate (MPP), monoisopropyl phthalate (MIIP) and BBP were obtained from Aldrich. Phthalate diesters were obtained from Aldrich. Anti-estrogen (ICI 182,780) was from Tocris Cookson, Ltd.
(MCHP), monobenzyl phthalate (MBZP), mono-n-octyl phthalate (MOP) and MEHP were synthesized by the method of Snell, and their purities were 94.7 to 99.4%.

**Cell Culture and Proliferation Assay** Human breast cancer estrogen-sensitive MCF-7 cells were a generous gift from Drs. A.M. Soto and C. Sonnenschein (Tufts University School of Medicine, U.S.A.). The cell proliferation assay method was described previously. MCF-7 cells were grown and maintained in DMEM supplemented with 5% FBS (growth medium). Briefly, $4 \times 10^5$ cells/well were inoculated into 96-well culture plates, and the medium was replaced by an experimental medium (phenol red-free DMEM supplemented with 5% CDHuS) after 24-h incubation in the growth medium. Chemicals were dissolved in ethanol, diluted with phenol-red free DMEM and added to the well. The culture was continued for 6 d, and the cell number was estimated.

![Fig. 1. Proliferation of MCF-7 Cells](image)

Cell number after treatment of cells for 6 d with the indicated concentrations of chemicals is expressed as a percentage relative to that of cells treated with vehicle (0.1% ethanol) alone (100%). The effect of various concentrations of 17β-estradiol or 4-hydroxytamoxifen (4OH-TAM) on cell proliferation is shown (A). Cell proliferation induced by phthalate diesters (B) and monoesters (C) was examined. Abscissa is logarithmic scale. Each point is the mean±S.D. (n=8).
with a CyQUANT cell proliferation assay kit (Molecular Probes Inc.) by measuring the fluorescence, which parallels the amount of nucleic acids. Experiments were carried out 3–5 times, each time with \( n = 8 \), and the results of one representative experiment was shown in figures. Cell numbers were expressed as percentages, where the control with 0.1% ethanol alone was taken as 100%.

**Inhibition of Cell Proliferation by Pure Anti-estrogen ICI 182,780** The effect of pure anti-estrogen ICI 182,780 on cell proliferation by a compound was examined. Various concentrations of ICI 182,780 were added to the cells in the presence of compounds that exhibited positive cell proliferation at concentrations giving the highest cell yield under the experimental system.

**Anti-estrogen Assay** To examine the anti-estrogenic activity, the effect of various concentrations of phthalate esters on cell proliferation in the presence of \( 10^{-11} \) M \( 17\beta\)-estradiol, which produced a sub-maximal response (90%), was estimated. The ability of chemicals to inhibit the cell proliferation induced by \( 17\beta\)-estradiol was determined. The stimulatory effect of \( 10^{-11} \) M \( 17\beta\)-estradiol in the absence of phthalate esters was taken as 100% and the effects of various concentrations of a chemical were expressed as the percentage of stimulation by \( 17\beta\)-estradiol.

**Cytotoxicity** Cytotoxicity was determined with neutral red uptake assay.\(^{28}\) Briefly, \( 1 \times 10^6 \) cells/well were seeded and cultured for 24 h. Chemicals dissolved in ethanol were added to the wells without changing the growth medium and cells were treated for 24 h. Then, the medium was replaced with that containing 25 \( \mu \)g/ml of neutral red and uptake of dye was carried out for 3 h. The dye was extracted with a solution of 1% acetic acid in 50% ethanol and the absorbance at 540 nm was read.

**RESULTS AND DISCUSSION**

The proliferation of MCF-7 cells was estimated after 6-d treatment of cells with test chemicals (Fig. 1). To examine whether there is a structural modification of a chemical which affects cell proliferative effects in 6-d incubation, medium with a chemical was changed every 2 d. The results were the same whether a fresh chemical was supplied or not (data not shown), suggesting the metabolic conversion is not great in the experimental medium with CDHuS. DCHP stimulated cell proliferation maximally at \( 5 \times 10^{-8} \) M (Fig. 1B), with proliferation reaching about 80% of that of \( 17\beta\)-estradiol at \( 3 \times 10^{-11} \) M (Fig. 1A), whose maximum cell proliferation was more than 400% of control. DCHP had the most potent estrogenic activity among the phthalate esters examined, but its estrogenic potency was 170000 times less than that of \( 17\beta\)-estradiol. DEHP and BBP apparently stimulated the proliferation, though cell yields were not high and the curves were still rising at \( 10^{-3} \) M, the highest concentration examined (Fig. 1B). DEHP gave slightly higher cell yield than BBP. The effect of anti-estrogen ICI 182,780, which inhibits the action of estrogens by competing with \( 17\beta\)-estradiol for estrogen receptor,\(^{29}\) on the cell proliferation with \( 10^{-11} \) M \( 17\beta\)-estradiol, \( 5 \times 10^{-8} \) M DCHP, \( 10^{-3} \) M DEHP or \( 10^{-3} \) M BBP was examined (Fig. 2). It inhibited the cell proliferative effects of all these chemicals in a dose-dependent manner and showed almost complete inhibition at above \( 10^{-8} \) M, a concentration 3 (DCHP) to 5 (DEHP or BBP) orders of magnitude lower than that inducing maximal cell proliferation. It is speculated that phthalate esters interact with estrogen receptor very weakly as compared with ICI 182,780. However, the estrogenic activities of DCHP, DEHP and BBP were thus confirmed by the fact that their effects were suppressed by the pure antagonist. DEP, DPrP, DBP, DPP and DHP had no cell proliferative effects (Fig. 1B), and all the phthalate monoesters, MMP, MEP, MPPr, MIPr, MBP, MIBP, MPP, MCHP, MBZP, MOP and MEHP, were inactive (Fig. 1C).

None of the phthalate esters, including DCHP, DEHP and BBP, had an additive stimulatory effect on cell proliferation by \( 17\beta\)-estradiol (Figs. 3B, C).

There have been no reports of in vitro data on DCHP, in spite of its being detected in indoor air in a newly built house.\(^{30}\) In the in vivo experiments, DCHP is an inducer of some parameters of hepatic xenobiotic metabolism and causes testicular damage detected in histological examination in male rats.\(^{30}\) DEHP\(^{31}\) and BBP\(^{32,33}\) were weakly estrogenic in the cell proliferation assay with MCF-7 cells, while BBP metabolites all lacked such activity.\(^{32}\) The findings in the present study might correspond to these reports, since DEHP and BBP were weakly estrogenic, and MBP and MBZP, both of which may be possible major metabolites of BBP, were negative in a stimulatory effect of cell proliferation. DBP was inactive in the present study with MCF-7 cells and also in the paper by Soto et al.,\(^{22}\) whereas Jobling et al.\(^{25}\) found that DBP as well as BBP exhibited estrogenic activity at \( 10^{-5} \) M by treatment of human breast cancer ZR-75 cells for 10 d. There might be some differences in the response to estrogenic stimuli between cell lines. BBP had a weakly positive result in a yeast two-hybrid assay.\(^{33}\) In recombinant yeast screen assay, BBP,\(^{34,35}\) DBP and DEP\(^{23}\) had a weakly positive result, but DEHP was negative.\(^{23}\) DBP and BBP weakly competed with \( 17\beta\)-estradiol for binding to the rat uterine ER\(^{26}\) and rainbow trout ER\(^{27}\) in competitive ligand binding assays. On the other hand, in the binding competition to human uterine ER, no activity was observed with DEP, DBP, DEHP, MEP, MIBP or MBP.\(^{36}\) The capacity of
the phthalates BBP, DEHP, DBP, DEP, diisobutyl phthalate, diisononyl phthalate, DMP and di-n-octyl phthalate, to bind rat uterine ER was too weak to obtain IC₅₀ values.³⁷) None of eight phthalate esters, DEHP, DBP, BBP, DHP, diiso-heptyl phthalate, di-n-octyl phthalate, diisononyl phthalate or diisodecyl phthalate, induced significant estrogenic responses based upon results obtained from uterotrophic and vaginal cornification assays in rats.²⁴) Coldham et al.³⁴) obtained a negative result with BBP in uterotrophic bioassay in mice.

Cytotoxicity should be taken into consideration to interpret anti-estrogenic activities of the compounds that showed suppressive effects on cell proliferation by 17β-estradiol. Cytotoxicity was determined by neutral red uptake assay in the growth medium (Fig. 3). Marked cytotoxicity was observed
with DCHP, MOP and MEHP. Especially, DCHP inhibited neutral red uptake at the concentration higher than 5 × 10⁻⁵ M, where it gave the maximum stimulatory effect of cell proliferation. Others had little inhibitory effect at the concentration of 10⁻⁴—10⁻³ M.

Sohoni and Sumpter examined estrogenic and anti-estrogenic activities by recombinant yeast screen assays in the absence and presence of 17β-estradiol and they found that BBP was weakly estrogenic, but it did not display pronounced anti-estrogenic activity. Jobling et al. estimated anti-estrogenic activity of DBP and BBP by transfection of ER using MCF-7 cells transiently transfected with reporter plasmid; these compounds were inactive as anti-estrogen, but they stimulated the estrogenic effect of 17β-estradiol. MCF-7 cells, which have characteristics to proliferate depending on estrogens, were effective for the estimation of both estrogenic and anti-estrogenic activities. Anti-estrogenic activities were examined similarly by the MCF-7 cell proliferation assay in the present study. Its activity can be discussed in the range of concentrations where a compound has no cytotoxicity. Estrogenic and anti-estrogenic activities of 40H-TAM, as a reference compound of a partial antagonist against estrogen, were examined. It exhibited a cell proliferative effect with the peak at 10⁻³—10⁻⁴ M (Fig. 1A). In the presence of 10⁻¹ M 17β-estradiol, 40H-TAM at 10⁻³—10⁻⁴ M increased cell yield to higher than that of 17β-estradiol alone (Fig. 3A). Anti-estrogenic activity was observed at 10⁻⁵—10⁻⁶ M, below which concentration it had no cytotoxicity (Fig. 3A).

The anti-estrogenic effect of phthalate esters was examined in the presence of 10⁻¹ M 17β-estradiol (Fig. 3). MPP and MCHP induced a suppression of cell growth stimulated by 17β-estradiol (100%) dose-dependently, and at 10⁻³ M they decreased the cell yield to only 25% of that by 17β-estradiol alone, while they had no inhibitory effect on neutral red uptake assay. MBZP, MIPrP and BBP also suppressed the stimulation of cell proliferation by 17β-estradiol between 10⁻⁴ M and 10⁻³ M, where little cytotoxicity was detected. MBP, MPPrP and DBP also slightly suppressed estrogenic activity (Fig. 3), and alone were not cytotoxic. However, anti-estrogenic activities of phthalate esters were observed at relatively high concentrations and they were less potent than 40H-TAM or ICI 182,780, which had antagonistic effects on 17β-estradiol at 10⁻⁵—10⁻⁶ M and 10⁻⁶—10⁻⁷ M, respectively. Among the compounds exhibiting estrogenic effects, BBP might have anti-estrogenic activity. DEHP had no anti-estrogenic activity or cytotoxicity. DCHP had cell proliferative effect with Cmax of 5 × 10⁻⁵ M, and the cell yield decreased at above 10⁻⁴ M regardless of the presence of 17β-estradiol (Figs. 1B, 3B). Furthermore, these decreasing curves almost coincided with that of cytotoxicity (Fig. 3B). Growth patterns detected by CyQUANT would reflect the sum of stimulatory and suppressive effects on proliferation. Since DCHP, MOP and MEHP had cytotoxicity, their anti-estrogenicity cannot be properly interpreted in the present study. Even if these compounds have anti-estrogenic activities, they are obscured by cytotoxicity.

According to the findings of in vivo studies by Ema et al., BBP, DBP and MBBP induced embryonic loss, which is mediated, at least in part, by the impairment of uterine function. Experimental results that exposure to DEHP caused prolonged estrous cycles and altered natural ovulation times in female rats might indicate the disruption of the physiological function in female reproductive organs. DEHP showed chronic toxicity in rats through a mechanism thought to involve peroxisome proliferation. MEHP derived from DEHP is the peroxisome proliferator. Peroxisome proliferators induce their effects by activating the peroxisome proliferation-activated receptors (PPARs), members of the nuclear receptor superfamily. MEHP, directly or indirectly, alter the transcriptional regulation of aromatase and other genes by acting through a PPAR-mediated pathway, leading to the suppression of estradiol production and the decrease of aromatase mRNA levels in ovarian granulosa cells. However, not all of the molecular events underlying endocrine disruption by xenobiotics 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.

Phthalates are ubiquitous in the environment and it is possible that humans are continuously exposed to them. In the present study, we examined the estrogenic and anti-estrogenic activities of various phthalate esters in MCF-7 cell proliferation assay and anti-estrogenic activities of some phthalate esters were suggested in vitro. Further investigation in other in vitro and in vivo experimental systems, especially to confirm anti-estrogenic activities, might be required. The long-term effects of phthalate esters on humans remain to be clarified. Androgenic and anti-androgenic activities of phthalate esters should also be scrutinized.

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