# Cell growth of BG-1 ovarian cancer cells is promoted by di-*n*-butyl phthalate and hexabromocyclododecane via upregulation of the cyclin D and cyclin-dependent kinase-4 genes

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**Abstract.** Endocrine-disrupting chemicals (EDCs) are environmentally persistent exogenous compounds released from various industrial products such as plastics, pesticides, drugs, detergents and cosmetics. They can cause a variety of adverse effects to the reproductive, developmental, immune and nervous systems in humans and wildlife. Di-*n*-butyl phthalate (DBP) is the main compound of phthalates and is reported to inhibit estrogen receptor (ER)-mediated gene expression and to interfere with normal fetal development of the male reproductive system. Hexabromocyclododecane (HBCD or HBCDD) is one of the brominated flame retardants (BFRs) which have been widely used in plastic, electronic and textile applications and are known to cause endocrine disruption with toxicity of the nervous system. In the present study, the estrogenic effects of DBP and HBCD were examined in an ovarian cancer cell line, BG-1, expressing high levels of ER via MTT assay and semi-quantitative reverse-transcription PCR. Treatment with DBP ($10^{-8}$-$10^{-5}$ M) or HBCD ($2x10^{-8}$-$2x10^{-6}$ M) resulted in increased cell proliferation of BG-1 cells as observed with 17-β estradiol (E2). In addition, both DBP and HBCD upregulated the expression levels of cell cycle-regulatory genes, such as cyclin D and cyclin-dependent kinase-4 (cdk-4), which are downstream target genes of ER, at 6 h after treatment. However, the expression of the p21 gene was not altered by DBP or HBCD at any time as with E2. Taken together, these results suggest that DBP and HBCD are EDCs which have apparent estrogenic activities by stimulating the cell proliferation of BG-1 cells and by inducing the expression of cyclin D and cdk-4. Our results suggest that DBP and HBCD have sufficient potency to disrupt the endocrine system and to stimulate cell growth in ER-positive cancer cells.

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## Introduction

Endocrine-disrupting chemicals (EDCs) are environmentally persistent exogenous compounds which may have the potential to affect the hormone balance or to disrupt the normal function of the endocrine system in humans and wildlife populations (1-3). In actuality, EDCs threaten the health of living creatures by causing a variety of adverse effects on the reproductive, developmental, immune and nervous processes and by increasing the risk of cancer incidence (4,5). EDCs are released from various industrial products such as plastics, pesticides, drugs, detergents and cosmetics. In general, they have diverse chemical structures similar to that of 17β-estradiol (E2), an endogenous estrogen, and display agonistic and antagonistic effects on steroid receptors such as the estrogen receptor (ER) or the androgen receptor (AR), interfering with the actions of endogenous steroid hormones. Bisphenol-A (BPA), dioxins, dichlorodiphenyltrichloroethane (DDT), alkylphenols, polychlorinated biphenyl (PCBs), and phthalates are well-known EDCs. Among these EDCs, phthalates are chemical compounds that are mainly used as plasticizers, stabilizers, dispersants and emulsifying agents in the manufacture of diverse industrial products (6,7). Di-*n*-butyl phthalate (DBP) is one of the main phthalates, together with di-(2-ethylhexyl) phthalate (DEHP) and benzyl butyl phthalate (BBP) and are often used as a plasticizer and solvent in personal care products including perfumes and hair spray (8,9). Previous studies have shown that DBP and its metabolites suppressed steroid genesis *in vivo*, and the treatment of DBP and DEHP to pregnant rats interfered with normal fetal development of the male reproductive system in multiple generations (10,11).

Hexabromocyclododecane (HBCD or HBCDD) is one of the brominated flame retardants (BFRs) widely used in plastic, electronic and textile applications as a means of lessening the

Table I. Primer sequences and product sizes for the semi-quantitative reverse-transcription PCR.

| Target gene | Sequences | Product size |
|---|---|---|
| Cyclin D | Sense: 5'-TCTAAGATGAAGGAGACCATC-3'<br>Antisense: 5'-GCGGTAGTAGGACAGGAAGTTGTT-3' | 354 bp |
| cdk-4 | Sense: 5'-TCGTGAGGTGGCTTTACTGA-3'<br>Antisense: 5'-AGGCAGAGATTCGCTTGTGT-3' | 698 bp |
| p21 | Sense: 5'-AGGCACCGAGGCACTCAGAG-3'<br>Antisense: 5'-TGACAGGTCCACATGGTCTTCC-3' | 370 bp |
| GAPDH | Sense: 5'-ATGTTCGTCATGGGTGTGAACCA-3'<br>Antisense: 5'-TGGCAGGTTTTTCTAGACGGCAG-3' | 351 bp |

flammability of products. HBCD is the third most commonly used BFR as it is so highly efficient that very low levels are required to reach the desired flame retardancy. However, the toxicity of HBCD and its harmful effects to the environment including lipophilic and bioaccumulating properties are currently being discussed (12,13). In fact, HBCD is widely detected in various environmental samples including air, fresh water, sediments and even in blood, human milk and eggs. It has also been suggested that HBCDs exhibit agonistic effects on thyroid hormonal activity, resulting in endocrine disruption with neurologic toxicity (14-16). At present, the European Chemicals Agency (ECA) has included HBCD in the list of Substances of Very High Concern (SVHC) based on a hazard evaluation of HBCD, which was discussed in the Stockholm Convention (17).

Thus, in the present study, we investigated the estrogenic effect of DBP and HBCD in BG-1 ovarian cancer cells which have a high level of ERs to better understand the cellular mechanisms underlying their endocrine-disrupting effect as reported in previous studies. BG-1 is known to be a highly E2-responsive cancer cell line and is considered to be the most suitable *in vitro* model to detect the estrogenicity of EDCs (2,18). Consequently, we examined the cancer cell proliferation of BG-1 cells via MTT assay and altered expression of genes related to the cell cycle via semi-quantitative reverse-transcription PCR following treatment with DBP or HBCD in comparison with E2 in these cells. Cell proliferation of highly E2-responsive BG-1 cancer cells can be a prominent marker to detect the estrogenicity of DBP and HBCD. Furthermore, identification of altered gene expression may provide the basis of the underlying mechanisms involved in the endocrine-disrupting effects of these two EDCs.

## Materials and methods

*Cell culture and media*. BG-1 human ovarian cancer cells were obtained from Dr K.S. Korach (National Institute of Environmental Health Sciences, NIH, Research Triangle Park, NC, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Inc., Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories), 100 U/ml penicillin G and 100 $\mu$g/ml streptomycin (Life Technologies, Rockville, MD, USA) at 37˚C in a humidified atmosphere of 5% $CO_2$-95% air. To prevent the effects of the estrogenic components of DMEM and FBS, phenol red-free DMEM supplemented with 5% charcoal-dextran treated FBS was used to detect the estrogenicity of EDCs in the BG-1 cells.

*Cell proliferation assay*. Cell growth was demonstrated by MTT assay as previously demonstrated (19,20). BG-1 cells (4,000/well) were plated in 96-well plates in 0.1 ml of phenol red-free DMEM supplemented with 5% charcoal-dextran-treated FBS medium. After incubation for 48 h, the cells were washed and treated with E2 (Sigma-Aldrich Corp., St. Louis, MO, USA), HBCD (Sigma-Aldrich Corp.) and/or DBP (Sigma-Aldrich Corp.) at various concentrations in the medium for 6 days as described above. Dimethyl sulfoxide (DMSO, 0.1%) in the same medium was used as a vehicle. Following treatments, the cells were then treated with 10 $\mu$l of MTT solution (5 mg/ml) and incubated at 37˚C for 4 h. MTT-containing medium was removed and the precipitants were solubilized in DMSO (100 $\mu$l). The absorbance was measured at 540 nm using an ELISA reader (VERSA Max, Molecular Devices, Sunnyvale, CA, USA).

*Total-RNA extraction*. BG-1 cells ($3x10^5$/well) were cultured in 6-well plates and treated with E2, HBCD, DBP, and/or DMSO. Total-RNA was extracted at various time points (0, 6 and 24 h) using TRIzol reagents (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of total-RNAs was measured by a spectrophotometer (Optizen, Mecasys, Dea-jeon, Korea) at 260/280 nm. One microgram of total-RNA was dissolved in diethyl pyrocarbonate-deionized water for cDNA synthesis.

*Semi-quantitative reverse-transcription PCR*. To synthesize cDNAs from total-RNAs for reverse transcription PCR, the reaction mixture consisted of murine leukemia virus reverse transcriptase (M-MLV RT), nonamer random primer, dNTPs, RNase inhibitor and RT buffer (all from iNtRON Biotechnology, Sungnam, Kyeonggido, Korea). The cDNA synthesis was performed at 37˚C for 1 h and 95˚C for 5 min. Cyclin D, cdk-4, p21 and GAPDH mRNAs were amplified by using each forward and reverse primer, Taq polymerase, PCR buffer, dNTP mixture and each cDNA template via PCR as previously conducted (2,19,20). The forward and reverse primer and the expected size of RT-PCR products are shown

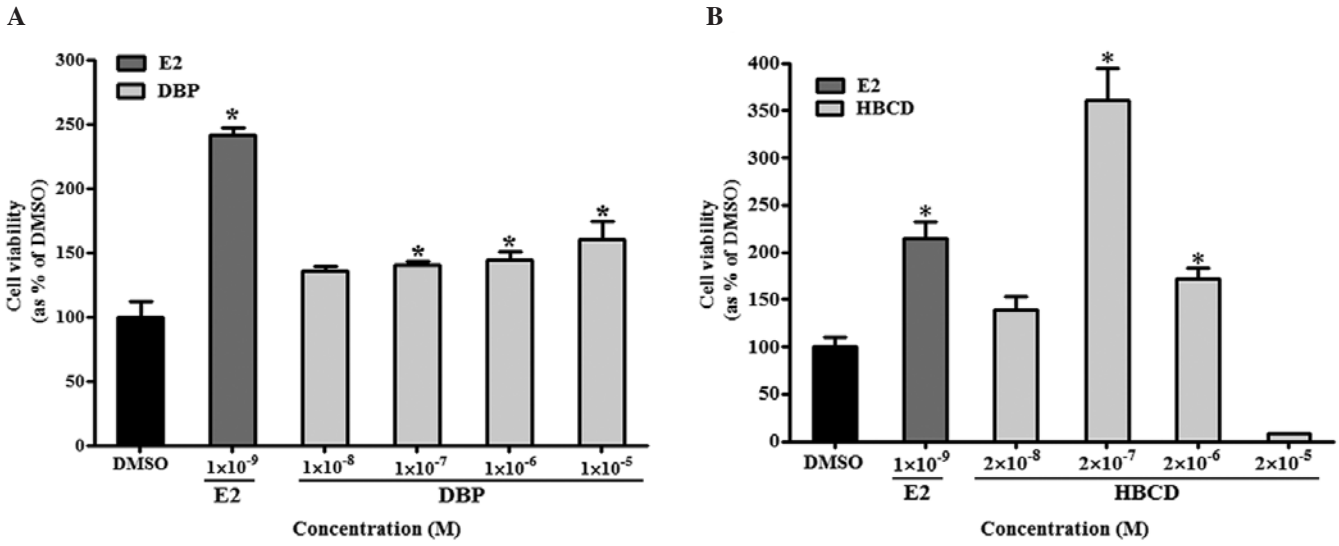


Figure 1. EDC-induced cell growth following treatment with E2, DBP or HBCD in BG-1 cells. Cells were treated with DMSO as a vehicle, E2 ($10^{-9}$ M), DBP ($10^{-8}$-$10^{-5}$ M) or HBCD ($2x10^{-8}$-$2x10^{-5}$ M) for 6 days, and the number of viable cells was measured using MTT assay at 540 nm. (A) Cell proliferation of BG-1 cells after treatment with E2 or DBP. (B) Cell proliferation of BG-1 cells after treatment with E2 or HBCD. Data represent the means ± SD of triplicate experiments. *P<0.05 compared to a vehicle treated with DMSO.

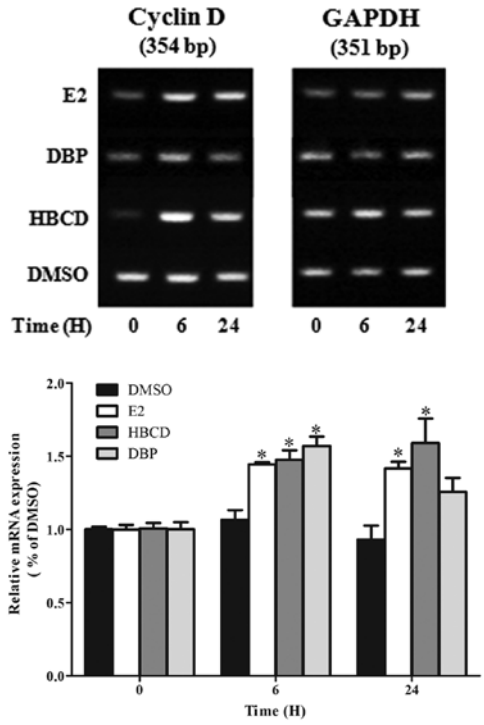


Figure 2. Altered expression levels of the cyclin D gene in BG-1 cells following treatment with E2, DBP or HBCD. BG-1 cells were seeded in 6-well plates and treated with E2 ($10^{-9}$ M), DBP ($10^{-7}$ M) or HBCD ($2x10^{-7}$ M). Total-RNAs were extracted in a time-dependent manner (0, 6 and 24 h). Expression level of cyclin D was detected using semi-quantitative reverse-transcription PCR. PCR products were run on a 1.5% agarose gel, bands were scanned and the density of the bands on the gel was quantified using Gel Doc 2000 as described in Materials and methods. Data represent the means ± SD of triplicate experiments. *P<0.05 compared to a vehicle treated with DMSO.

in Table I. PCR products were run on a 1.5% agarose gel and the bands were compared to 100-bp ladders. The gels were scanned, and the density of the bands on the gel was quantified using Gel Doc 2000 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

*Data analysis*. Data are expressed as the mean ± SD. A statistical analysis was performed using the Student t-test, two-pair comparisons. P<0.05 was considered to denote statistical significance.

## Results

*Effects of DBP and HBCD on the cell proliferation of BG-1 cells*. To evaluate the effects of DBP and HBCD on cell proliferation, BG-1 cells were cultured with treatment of vehicle (DMSO 0.1%), E2 ($1x10^{-9}$ M), DBP ($1x10^{-5}$-$1x10^{-8}$ M) and/or HBCD ($2x10^{-5}$-$2x10^{-8}$ M) for 6 days. The results demonstrated that E2 as a positive control markedly increased the BG-1 cell proliferation in comparison with DMSO as shown in Fig. 1A and B (P<0.05). DBP treatment gradually increased the proliferation of BG-1 cells in a dose-responsive manner (Fig. 1A, P<0.05). HBCD also stimulated the growth of BG-1 cells up to a concentration of $2x10^{-6}$ M (Fig. 1B) (P<0.05). In particular, HBCD exhibited a potent cell proliferation activity at $2x10^{-7}$ M, which greatly exceeded the E2 effect. On the other hand, HBCD appeared to confer strong cytotoxicity at $2x10^{-5}$ M as noted in Fig. 1B (P<0.05).

*Cyclin D gene expression in BF-1 cells treated with DBP and HBCD*. To evaluate the effects of DBP and HBCD on the expression levels of genes related to the cell cycle such as cyclin D, cdk-4 and p21 in BG-1 cells, we treated the cells with increasing concentrations of DBP and HBCD at $1x10^{-7}$ and $2x10^{-7}$ M, respectively, based on the cell proliferation assay. In the semi-quantitative RT-PCR experiment, the gene expression of cyclin D was significantly enhanced by treatment of E2, DBP, or HBCD compared with a vehicle treated with DMSO for 6 h as shown in Fig. 2 (P<0.05). At 24 h, treatment with

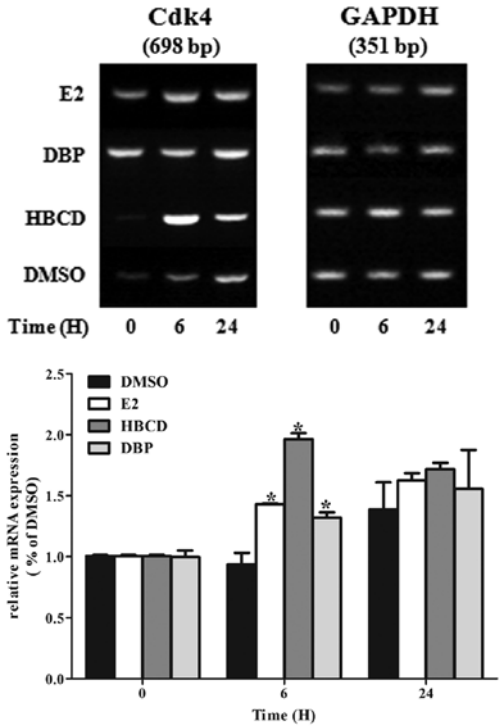


Figure 3. Altered expression levels of the cdk-4 gene following treatment with E2, DBP or HBCD. BG-1 cells were seeded in 6-well plates and treated with E2 ($10^{-9}$ M), DBP ($10^{-7}$ M) or HBCD ($2x10^{-7}$ M). Total-RNAs were extracted in a time-dependent manner (0, 6 and 24 h). The expression level of cdk-4 was detected using semi-quantitative reverse-transcription PCR. Data represent the means ± SD of triplicate experiments. *P<0.05 compared to a vehicle treated with DMSO.

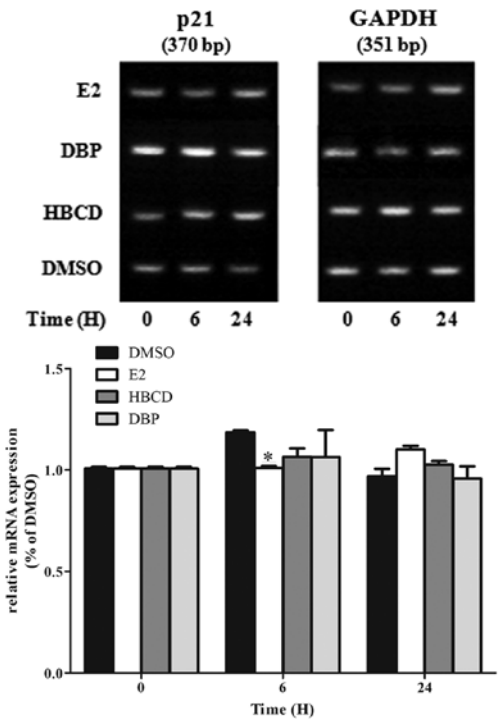


Figure 4. Altered expression levels of the p21 gene following treatment with E2, DBP or HBCD. BG-1 cells were seeded in 6-well plates and treated with E2 ($10^{-9}$ M), DBP ($10^{-7}$ M) or HBCD ($2x10^{-7}$ M). Total-RNAs were extracted in a time-dependent manner (0, 6 and 24 h). Expression level of p21 was detected by using semi-quantitative reverse-transcription PCR. Data represent the means ± SD of triplicate experiments. *P<0.05 compared to a vehicle treated with DMSO.

E2, HBCD or DBP significantly stimulated the expression of Cdk-4 as well in ER-positive BG-1 ovarian cancer cells.

*Cdk-4 gene expression in BF-1 cells treated with DBP and HBCD.* In parallel with an increase in the expression level of cyclin D by HBCD and DBP, the expression of the cdk-4 gene was also increased (Fig. 3). In particular, cdk-4 gene expression was significantly increased by HBCD or DBP treatment at 6 h compared to a vehicle (DMSO treatment, P<0.05) as shown in Fig. 3. However, the difference in the expression level of the Cdk-4 gene was apparently decreased at 24 h between the vehicle and EDC treatment.

*p21 expression in BG-1 cells treated with DBP and HBCD.* The expression level of p21, a cyclin D-cdk-4 complex inhibitor gene, was further examined following treatment with E2, HBCD and DBP. The expression of p21 was suppressed by E2 compared to DMSO treatment at 6 h as noted in Fig. 4 (P<0.05). However, its expression in BG-1 ovarian cancer cells was not significantly altered by HBCD or DBP treatment compared to a vehicle of DMSO treatment at all time points (Fig. 4).

## Discussion

Previous studies have reported that EDCs pose a high human health risk by disturbing the hormone balance in the endocrine system of body organs. Moreover, the cell transformation caused by consistent exposure to EDCs may increase the risk of cancer incidence (21,22). EDCs are known to induce a hormone imbalance by directly interacting with various hormone receptors such as ER, progesterone receptor (PR), AR and thyroid receptor (ThR) as their agonists and antagonists. The main property and harmful factor of many EDCs are their estrogenic effects as they often interfere with endogenous estrogen by having chemical structures similar to E2. In addition, mimicking estrogen function results in fatal damaging responses, i.e., endocrine disruption or tumor promotion (23-25). E2, an endogenous estrogen, is known to mediate cellular responses via two ERs, ERα and ERβ. Therefore, the estrogenic effect of EDCs can be categorized according to their mediating pathway via ERα or ERβ. For instance, diethylstilbesterol (DES), one of the EDCs and a synthetic estrogen, has been reported to inhibit follicle formation and development via ERα. On the other hand, it induces polyovular follicles via ERβ (26). In the present study, we selected BG-1, a human ovarian cancer cell line, to detect the estrogenicity of EDCs as it is a highly estrogen-responsive cell line due to its strong expression of both ERα and ERβ (2,18). We also confirmed its estrogen dependence in the cell proliferation assay, in which the growth of BG-1 cells were increased more than two-fold by a low-dose treatment of E2 ($1x10^{-9}$ M).

In the present study, we examined the estrogenicity of two EDCs, DBP and HBCD, for which harmful effects have been recently disclosed. DBP has reported to disrupt the endocrine

system and generate transformations in the growth and development of male reproductive organs by causing Leydig cell proliferation, germ cell degeneration and testosterone decrease in the fetal testis (27-30). Additionally, HBCD is known to increase cell proliferation of MCF-7 cells and the expression of TFF1, an estrogen-dependent gene, in breast cancer cells. Furthermore, it appears to transform the expression level of genes associated with cell proliferation and DNA methylation as does E2 (31-33). We also examined whether DBP and HBCD have an estrogenic effect by stimulating the cell proliferation of E2-responsive BG-1 ovarian cancer cells. Our data showed that the treatment of cells with DBP ($10^{-8}$-$10^{-5}$ M) or HBCD ($2x10^{-8}$-$2x10^{-6}$ M) obviously increased the cell proliferation as did E2.

Accordingly, we determined whether these EDCs promote cell proliferation by altering the expression level of cell cycle-regulating genes such as cyclin D, cdk-4 and p21, which are known downstream targets of E2 and ER. In cell cycling pathways, cyclin D and cdk-4 form a complex and promote cell cycle progression through the G1 phase. p21, the cdk inhibitor, disrupts the formation of the cyclin-cdk complex and inhibits G1/S transition (34,35). E2 is reported to stimulate cellular proliferation, PR expression and morphological differentiation in the endometrial Ishikawa cancer cell line by stimulating the transition of cell cycle via upregulation of cyclin D and cyclin A and downregulation of p21, p27 and p53 (36,37). Our previous results also showed that the expression levels of the cyclin D and cdk-4 genes in the BG-1 cell line were significantly increased with E2 ($10^{-7}$ M) or BPA ($10^{-5}$ M) treatment for 24 h (2). In this study, DBP and HBCD actually upregulated the expression levels of cyclin D and cdk-4 but had no effect on the expression of p21.

In conclusion, DBP and HBCD are EDCs which exhibited estrogenic activities in an estrogen-dependent cancer cell line, BG-1, by stimulating cell proliferation and by upregulating the expression of cell cycle-regulating genes, cyclin D and cdk-4. In addition, our results support that DBP and HBCD may have sufficient potency to disrupt the endocrine system and to stimulate tumorigenesis as previously reported by the risk assessment of EDCs. A further study is required to elucidate the specific mechanisms of DBP and HBCD in the disruption of the endocrine and reproductive systems *in vitro* and *in vivo*.

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