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Effect of fenhexamid and cyprodinil on the expression of cell cycle- and metastasis-related genes *via* an estrogen receptor-dependent pathway in cellular and xenografted ovarian cancer models

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ABSTRACT

Fenhexamid and cyprodinil are antifungal agents (pesticides) used for agriculture, and are present at measurable amounts in fruits and vegetables. In the current study, the effects of fenhexamid and cyprodinil on cancer cell proliferation and metastasis were examined. Additionally, the protein expression levels of cyclin D1 and cyclin E as well as cathepsin D were analyzed in BG-1 ovarian cancer cells that express estrogen receptors (ERs). The cells were cultured with 0.1% dimethyl sulfoxide (DMSO; control), 17β-estradiol (E2; 10^{-9} M), and fenhexamid or cyprodinil (10^{-5}–10^{-7} M). Results of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed that fenhexamid and cyprodinil increased BG-1 cell proliferation about 1.5 to 2 times similar to E2 (5 times) compared to the control. When the cells were co-treated with ICI 182,780 (10^{-8} M), an ER antagonist, the proliferation of pesticide-treated BG-1 cells was decreased to the level of the control. A wound healing assay revealed that the pesticides reduced the disrupted area in the BG-1 cell monolayer similar to E2. Protein levels of cyclin D1 and E as well as cathepsin D were increased by fenhexamid and cyprodinil. This effect was reversed by co-treatment with ICI 182,780. In a xenograft mouse model with transplanted BG-1 cells, cyprodinil significantly increased tumor mass formation about 2 times as did E2 (6 times) compared to the vehicle (0.1% DMSO) over an 80-day period. In contrast, fenhexamid did not promote ovarian tumor formation in this mouse model. Cyprodinil also induced cell proliferation along with the expression of proliferating cell nuclear antigen (PCNA) and cathepsin D in tumor tissues similar to E2. Taken together, these results imply that fenhexamid and cyprodinil may have disruptive effects on ER-expressing cancer by altering the cell cycle- and metastasis-related gene expression *via* an ER-dependent pathway.

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1. Introduction

Endocrine-disrupting chemicals (EDCs) are known as environmental compounds that aberrantly regulate naturally circulating steroid hormones *via* their corresponding receptors (Diamanti-Kandarakis et al., 2009). EDCs competitively bind to the receptors of estrogens or androgens since their structures are similar to those of steroid hormones. Thus, EDCs interfere with the actions of endogenous steroid hormones, and disrupt hormonal and homeostatic systems (Raut and Angus, 2010; Kim et al., 2015a). Additionally, these compounds can inhibit endogenous hormones in a noncompetitive manner by inducing distinct conformational change in the tertiary structure of the estrogen receptor (ER), thus affecting the recruitment of cofactors (Yang et al., 2015). Interactions between EDCs and ERs control critical steps in ER-mediated transcriptional regulation and consequently modulate the expression of ER target genes (Celik et al., 2008). Reproductive organs such as the breast, ovary, and endometrium express ERs that are activated by estrogen (E2) which regulates the development of the reproductive system (Hwang et al., 2013). Cancer of estrogen-responsive organs is induced by over-expression of E2 or exogenous chemicals that act like E2 such as bisphenol A, nonylphenol, triclosan, and benzophenone-1 (Park et al., 2013; Lee et al., 2014a; Kim et al., 2015b).

Normal cell proliferation is controlled by the cell cycle that has checkpoints at each phase regulated by various factors such as cyclin D1, p21, p27, and cyclin E (Lee et al., 2014b). These proteins associated with the cell cycle play a critical role in cell proliferation. In cancer cells, the functions of cell cycle checkpoint genes are imbalanced. For example, cyclin D1 and p21 are over-expressed following exposure to EDCs such as bisphenol A or octylphenol *via* the ER pathway in ovarian cancer (Park et al., 2011; Lee et al., 2012). Additionally, benzophenone-1 (BP-1) stimulates the growth of BG-1 ovarian cancer cells through an ER-dependent pathway in the same manner as E2 (Park et al., 2013). These results indicate that the imbalance of cell cycle checkpoints by EDCs causes the cancer due to the over-growth of cells *via* an ER dependent pathway.

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Metastasis is a major problem for the clinical treatment of diverse types of cancer (Mundy, 2002). This process involves the transport of cancer cells from the primary site to a secondary site. It is widely accepted that estrogen plays an important role in cancer metastasis. For example, BP-1 and nonylphenol stimulate MCF-7 breast cancer metastasis by inducing the over-expression of cathepsin D *via* an ER-dependent pathway (In et al., 2015). Cathepsin D is associated with the cancer metastasis in estrogen dependent cancer cells as a lysosomal aspartyl protease (Garcia et al., 1996) and is induced by estrogen (Bretschneider et al., 2008).

Pesticides are widely used to control pests and harmful plants that attack agricultural products (Mofeed and Mosleh, 2013). Fenhexamid (2′,3′-dichloro-4′-hydroxy-1-methylcyclohexanecarboxanilide) inhibits C3-ketoreductase that influences ergosterol biosynthesis. This compound became available in 2000 as a foliar fungicide belonging to the hydroxyanilide class (Fig. 1) and is used to control gray mold in grapes, stone fruits, citrus fruits, vegetables, strawberries, raspberries, black currants, and sweet cherries (Debieu et al., 2001; Hengel et al., 2003; Fillinger et al., 2008). Consequently, fenhexamid has been detected on harvested fruits at concentrations below the legal maximum residue levels (Angioni et al., 2012). Additionally, residual fenhexamid has been frequently found on strawberries and grapes by comprehensive pesticide residue monitoring programs and even in commercial bottled wines (Mercader and Abad-Fuentes, 2009). A previous study demonstrated that fenhexamid changes the expression of ER target genes such as the ones encoding cyclin D1, the progesterone receptor, and nuclear respiratory factor 1 in MCF-7 breast cancer cells (Medjakovic et al., 2013). Moreover, fenhexamid showed a very weak transactivating potential for the androgen receptor (AR) (Medjakovic et al., 2013), stimulated microRNA-21 (miR-21) expression in breast cancer cells (Teng et al., 2013), and regulated invasion and metastasis by tumor repressors, such as programmed cell death 4, phosphatase and tensin homolog, and B-cell lymphoma 2 (Wickramasinghe et al., 2009; Hatley et al., 2010). Cyprodinil (4-cyclopropyl-6-methyl-N-phenylpyrimidin-2-amine) is a broad-spectrum pyrimidinamine fungicide used to control pathogens in fruit plants, vines, cereals, and vegetables (Fig. 1). This reagent inhibits the biosynthesis of methionine and other thionic amino acids in fungi (Kanetis et al., 2008). According to a previous study, the aryl hydrocarbon receptor (AhR) pathway plays a significant role in developmental processes of normal cells and the cell cycle is inappropriately activated by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Kawamura and Yamashita, 2002; Levine-Fridman et al., 2004; Go et al., 2015). Since the aryl hydrocarbon receptor (AhR) and ERs are known to interact in multiple ways (Bemanian et al., 2004; Beischlag and Perdew, 2005; Go et al., 2015), activation of the AhR is potentially linked to the development of hormone-dependent diseases (Safe and McDougal, 2002). Cyprodinil is an activator of the AhR similar to TCDD (Fang et al., 2013; Medjakovic et al., 2013) and enhances dexamethasone-stimulated glucocorticoid response element (GRE)-mediated transcriptional activity (Fang et al., 2013). Furthermore, cyprodinil induces the phosphorylation of the extracellular signaling regulated kinase (ERK) that phosphorylates growth and transcription factors, and regulates various processes including proliferation, differentiation, survival, and migration of mammalian cells (Dunn et al., 2005; Yoon and Seger, 2006). ERK is also generally known to be activated by ER signaling (Huang et al., 2015; Jeon et al., 2015; Liu et al., 2015).

Despite the potential risks of pesticides in promoting the development of diverse diseases, the effects of pesticides in humans have not been thoroughly examined. We therefore investigated the xenoestrogen effects of two pesticides, fenhexamid and cyprodinil, on cell cycle-related mechanisms and metastasis *via* the ER signaling pathway in estrogen-responsive BG-1 human ovarian cancer cells. Additionally, a xenograft mouse model was established to further evaluate the *in vivo* effects of fenhexamid and cyprodinil.

2. Materials and methods

2.1. Reagents and chemicals

17β-Estradiol (E2), fulvestrant (ICI 182,780), fenhexamid, and cyprodinil were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). All chemicals were dissolved in 100% dimethyl sulfoxide (DMSO; Junsei Chemical Co., Tokyo, Japan). The final concentration of DMSO was 0.1% in the cell culture media.

2.2. Cell culture and media

BG-1 human ovarian cancer cells were obtained from Dr. K. S. Korach (National Institute of Environmental Health Sciences, National Institutes of Health, 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), 200 U/mL penicillin, and 200 mg/mL streptomycin (A&E Scientific, Logan, UT, USA) at 37 °C in a humidified atmosphere of 5% CO_2. To suppress the estrogenic effects of compounds in the DMEM and FBS, phenol red-free DMEM (Sigma-Aldrich Corp.) supplemented with 5% charcoal/dextran-treated FBS (CD–FBS) was used to measure the estrogenicity of the EDCs in BG-1 cells. Charcoal/dextran-treated FBS is fetal bovine serum which reduced the levels of many hormones, steroids and growth factors by exposing them to a temperature of 56 °C for 30 min and shaking incubation in 1 h under charcoal/dextran-treated condition. The cells were detached with 0.05% trypsin-EDTA (Life Technologies, Carlsbad, CA, USA).

2.3. Cell proliferation assay

BG-1 cells were seeded at a density of 3×10^3 cells per well in 96-well plates (SPL Life Sciences, Pocheon, Korea). After a 2-day incubation, the culture medium was replaced with new medium containing E2 (10^{-9} M), fenhexamid (10^{-5}–10^{-8} M), or cyprodinil (10^{-5}–10^{-8}) and changed every 3 days. The cells were further incubated for 9 days. Next, the culture medium was removed and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Corp.) was added for 4 h. After the culture medium was removed, 200 μL DMSO (Junsei, Tokyo, Japan) was added to each well. The absorbance was measured at 540 nm using an Epoch ELISA reader (BioTek, Winooski, VT, USA).

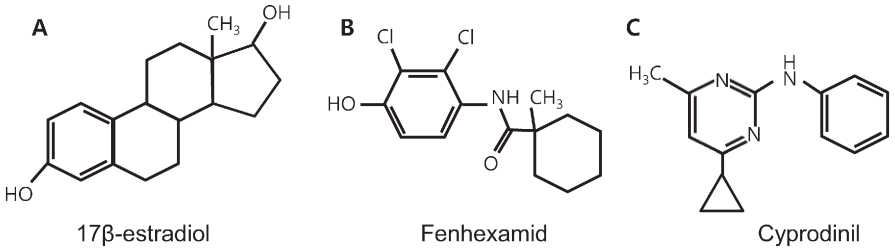

Fig. 1. Chemical structures of 17β-estradiol (E2), fenhexamid, and cyprodinil.

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