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Herbicide acetochlor interferes with proliferation activity of MCF-7 cells enhanced by estradiol

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It is well documented that pesticides used in agricultural processes may have detrimental effects upon human health. Moreover, many of these compounds have been indicated as potential endocrine and reproductive disruptors. In the present study, the ability of herbicide acetochlor to affect a growth of estrogen-sensitive MCF-7 mammary epithelial carcinoma cells was studied using E-screen test. Acetochlor alone did not affect proliferation of MCF-7 cells. Significant inhibition of estradiol-induced (10^{-14}-10^{-8} M) MCF-7 cell growth by the action of acetochlor (10^{-5} M) and interaction between these chemicals were observed. Estradiol-stimulated MCF-7 cell proliferation was decreased by 41% of positive control (17β-estradiol 10^{-8} M, 100%) in the presence of acetochlor (10^{-5} M). Our results demonstrate that acetochlor might interfere with estradiol signaling conducted to altered proliferation activity of MCF-7 cells and might support endocrine disruptive effects of acetochlor.

Key words: acetochlor, herbicide, estradiol, MCF-7 cells, endocrine disruptors

A wide variety of man-made chemicals released into the ecosystem have the ability to interfere with the hormone-regulated physiological processes in wildlife and humans (Combes 2000; Dickerson and Gore 2007; Diamanti-Kandarakis et al. 2009; Skinner 2014). Among environmental chemicals, many nonsteroidal substances of diverse chemical structure have been shown to mimic or block the estrogen action (Witorsch RJ 2000; Ohyama et al. 2001; Rotroff et al. 2014) and consequently change the proliferation rates of estrogen-sensitive target cells (Kakko et al. 2004; Wang et al. 2010). In recent years, many of the pesticides and herbicides used in crop production have been indicated as potential endocrine and reproductive disruptors.

Acetochlor (ACT), a selective chloroacetanilide herbicide, belongs to the most commonly used herbicides for residential as well as agricultural applications (Arcury et al. 2007). This persistent organic pollutant (Turque et al. 2005) is used worldwide including Slovakia for control of annual grasses and certain annual broadleaves (Arcury et al. 2007; Hiller et al. 2008). It is restricted use pesticide although it is currently labeled by U. S. Environmental Protection Agency only as a potential human carcinogen (US EPA 1984).

ACT is a well-established thyroid disruptor (Crump et al. 2002; Turque et al. 2005; Mnif et al. 2011). In rats, ACT exposure has been found to decrease litter size and increase prostate weight, to induce a weak DNA repair response and decrease pregnancy rates (US EPA 1994). In addition, we have demonstrated that ACT [153.6 mg/kg b.w./day - 20% LD_{50} administered orally during 6 days (LD_{50} according to Balinova et al. 1990)] may interfere with binding of estradiol to the uterine nuclear estrogen receptors (ERs) in the uniparous (Rollerova et al. 2000) and ovariectomized female rats (Rollerova et al. 2011). Significantly advanced time of

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vaginal opening and early onset of aberrant estrous cyclicity in adult rats after short time neonatal exposure to ACT (7.68 mg/kg b.w./day - 1% LD_{50} and 15.36 mg/kg b.w./day - 2% LD_{50} administered subcutaneously on postnatal days 4-7) may implicate its potential to alter hypothalamus-pituitary-ovarian axis function (Rollerova et al. 2011).

In the present study, we were interested in possible endocrine disruptive activities ACT in MCF-7 cells, the cell lines widely used to examine estrogen-dependent transactivation (Diamanti-Kandarakis et al. 2009). We performed the modified E-screen bioassay (Soto et al. 1992); being the one of the *in vitro* assays for detection of endocrine-disrupting compounds based on enhancement or inhibition of mitogenesis in estrogen-responsive MCF-7 cells (Gutendorf et al. 2001; Bicchi et al. 2009; Wang et al. 2012). The effect of ACT was compared with that of 17β-estradiol, which is frequently used as a positive control in studying potentially estrogenic, endocrine-disrupting agents in cells that should and should not respond to estrogenic compounds.

Materials and Methods

Materials. Herbicide acetochlor (ACT) used in the present study [2-chloro-(N-ethoxymethyl)-N-(2-methyl phenyl) acetamide, $C_{14}H_{20}ClNO_2$, F.W. 269.8, technical grade 92.2%, CAS no. 34256-82-1] was purchased from Nitrokemia Industries (Fuztogyartelep, Hungary). 17β-Estradiol [E_2, (1,3,5[10]-Estratriene)-3,17β-diol, $C_{18}H_{24}O_2$, F.W. 272.4] obtained from Sigma (Steinheim, Germany) was used as a positive control. Minimal essential Eagle´s medium (MEM) and phenol red-free Dulbecco´s modification of Eagle´s medium (DMEM) were purchased from PAN Systems GMBH (Germany) and Bio Whittaker (Maryland, USA), respectively. Fetal calf serum (FCS) obtained from GIBCO BRL (Scotland) was inactivated at 56°C for 30 min before use.

Cell maintenance. Estrogen receptor-positive MCF-7 human breast adenocarcinoma cells were cultured as a monolayer in Petri dishes in MEM supplemented with 10% heat-inactivated FCS at 37°C in a 5% CO_2 humidified air atmosphere (Soule et al. 1973).

E-screen bioassay. Prior to each experiment, cells were cultured for 6 days in experimental medium, i.e. DMEM supplemented with 10% dextran-coated charcoal-treated FCS (steroid hormone free FCS) (Soto et al. 1992; Berthois et al. 1994; Rasmussen and Nielsen 2002).

The tested compounds (ACT, E_2) were dissolved in absolute ethanol (stock solutions) and diluted further with experimental medium before use. The final solvent concentration in media did not exceed 0.1% (v/v). Control cells were incubated in experimental medium with or without an equivalent amount of the solvent.

MCF-7 cells were plated into 96-well plates at an initial density of 1×10^4 cells per well in experimental medium and allowed to attach for 24 h. After this time period, 10 µl of experimental medium was replaced by 10 µl of experimental medium containing either ACT or E_2, and diluted to final concentrations. Medium was not changed over the course of the experiment. The concentration range of ACT and E_2 used in the experiments were 10^{-10}-10^{-4} M and 10^{-16}-10^{-8} M, respectively. Bioassays were terminated in the late exponential phase after 6 days of incubation, the culture media were removed and the cells were processed according to Yue et al. (1997) with some modifications. The cells were washed with phosphate buffered saline (PBS), fixed with methanol for 20 min, dried and stained with 10% Giemsa solution for 20 min. The stained cells were washed two-times with PBS and lysed with 40% acetic acid for 20 min. Optical density, measured by means of Reader Dynatech MR 7000 at an extinction wavelength of 630 nm, was used as a measure of cell density. Mean cell density from each experiment was normalized to the steroid-free control culture to correct for differences in the initial seeding density. The percentage of proliferation was calculated using the following equation: Percentage of proliferation = (Cell density with tested compound / Cell density of control) x 100. The results represent the average of 4-12 independent experiments. Cell density for each concentration was counted from six wells.

Statistical analysis. Statistical analysis of the E-screen test data was carried out by the multifactor analysis of variance with interactions. Differences between individual values of positive control (E_2) and the mixture of ACT and E_2 were evaluated by *t*-test. The p value ≤ 0.05 was considered as statistically significant.

Results and Discussion

Optical density, as an index of the cell number, for control MCF-7 cells cultured in experimental medium with equivalent amount of solvent (absolute ethanol) was comparable with that for controls grown in medium without solvent (data not show). Based on these results, cell yields of the solvent-treated controls were taken to be 100% (Fig. 1). The addition of E_2 (10^{-16} - 10^{-8}M) to culture medium increased the number of MCF-7 cells

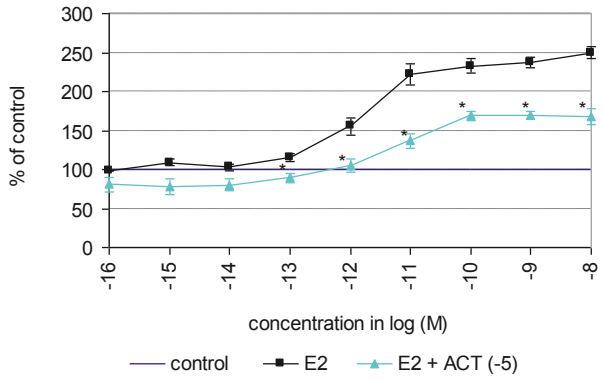

Fig. 1. Effect of acetochlor (ACT) on proliferation of MCF-7 cells induced by 17β-estradiol (E2). MCF-7 cells were grown for 6 days in the culture medium supplemented with different concentrations of E2 (10^{-16} - 10^{-8} M) alone or in combination with ACT (10^{-5} M). The induced cell growth was normalized to 100% of control. Values are expressed as mean ± SE of 4 - 12 independent experiments. The value for each concentration was counted from 6 wells. * p≤0.05 by *t*-test

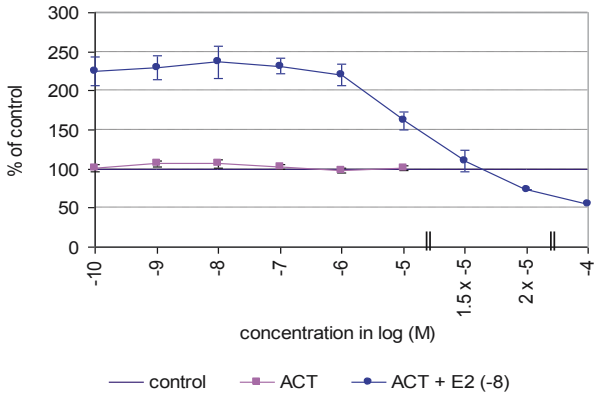

Fig. 2. Effect of acetochlor (ACT) on proliferation of MCF-7 cells. MCF-7 cells were grown for 6 days in the culture medium supplemented with different concentrations of ACT (10^{-10} – 10^{-4} M) alone or in combination with 17β-estradiol (E2, 10^{-8} M). The induced cell growth was normalized to 100% of control. Values are expressed as mean ± SE of 4 - 12 independent experiments. The value for each concentration was counted from 6 wells.

in a dose-dependent manner with maximum proliferation effect observed at concentrations of 10^{-11} - 10^{-8} M (Fig. 1). The maximal cell yield after a 6-day treatment with E_2 (10^{-8} M) was 2.5-fold higher than that seen in the hormone-free control cultures (Fig. 1).

Acetochlor (ACT, 10^{-10} - 10^{-4}M) alone had no effect on proliferation of MCF-7 cells (Fig. 2). The apparent effects of ACT on cell proliferation were observed only in the presence of E_2. Proliferation of MCF-7 cells stimulated by E2 (10^{-8} M) was significantly inhibited in the presence of ACT at 10^{-5} M concentration by 41 % of positive control and decreased to the control level at ACT dose of 1.5 x 10^{-5} M (Fig. 2). Higher concentrations of ACT (2 × 10^{-5} and 10^{-4} M) decreased number of cells under control level (Fig. 2).

Co-treatment of MCF-7 cells with ACT at 10^{-5} M concentration and different concentrations of E_2 (10^{-16}-10^{-8} M) resulted in a significant inhibition of cell proliferation at all tested concentrations of E_2 (Fig. 1). Analysis of variance with interactions confirmed a statistically significant effect of tested compounds (E_2 and combination of E_2+ACT; F=52.646, p ≤ 0.001), their concentrations (10^{-14}-10^{-8} M for E_2 and 10^{-5} M for ACT; F=104.306, p≤0.001), and the interaction between chemicals and their concentrations (F=3.865, p≤0.001).

The response of MCF-7 cells to different types of exposure is summarized in Table 1. Because of the existence of different MCF-7 subclones and different indices of cell proliferation used, a value of proliferation effect (PE) for E_2 at 10^{-10} M concentration was determined in our study (PE = 2.33) and was similar to those seen with subclones MCF-7 BB and MCF-7 BB104 (PE = 2.2 and PE = 2.3; respectively, both for 10^{-10} M of E_2) (Villalobos et al. 1995). The median effective concentration value (EC_{50}) calculated from the ascending part of concentration-response curve for E_2 was equal to 5.3 x 10^{-12} M. This value was comparable to that recorded by Schlumpf et al. (2004) (1.22 x 10^{-12} M) or calculated in a study of Wang et al. (2012) (3.12 x 10^{-12} M). Likewise, highest

Table 1

Response of MCF-7 cells to herbicide acetochlor

Compound	Concentration[a]	PE	RPE (%)
E_2	10^{-10}M	2.33	100
ACT	10^{-5}M[b]	0.96	NA
E_2 + ACT (10^{-5} M)	10^{-10}M	1.69	51.58
ACT + E_2 (10^{-8} M)	10^{-10}M	2.25	93.98

E_2 - 17β-estradiol (10^{-16} - 10^{-8} M), ACT - acetochlor (10^{-10} - 10^{-4} M);
a - the lowest concentration needed for maximal cell yield;
b - the highest concentration tested in culture;
PE - proliferation effect is the ratio between the highest cell yield obtained with the test chemical and hormone - free control;
RPE - relative proliferation effect is calculated as 100 × (PE-1) of the test compound ÷ (PE-1) of E_2. A value of 100 indicates full agonist; a value of 0 indicates that the compound lacks estrogenicity at the doses tested. Intermediate values suggest that the compound is a partial agonist. NA - no activity.

concentrations of ACT tested in our study (2×10^{-5} and 10^{-4} M) were comparable to cytotoxic concentrations determined for ACT in the Chinese hamster ovary cell line ($C_{1/2} = 6 \times 10^{-5}$ M or 4×10^{-5} M) (Soto et al. 1992; Raynburn et al. 2005).

ACT had no effect on proliferation activity of MCF-7 cells above that of the control culture. The proliferative efficiency confirmed no activity of ACT alone in a bioassay (PE = 0.96). Its value was not significantly different from value 1 similarly to pesticide derivatives (chlordane α isomer, chlordane, heptachlor and mirex) tested in the same system, and considered as negative in the bioassay (Soto et al. 1992). The PE for ACT was also comparable with PE identified for industrial chemicals such as bisphenol A ethoxylate (PE = 0.9), bisphenol A propoxylate (PE = 1.0) and bisphenol A diglycidylether dimethacrylate (PE = 1.1) (Perez et al. 1998). Although ACT was inactive in a proliferation assay in different cell lines (Mariotti et al. 1997; Rich et al. 2012), its estrogenic activity was reported *in vivo* using an uterotrophic assay (Mariotti et al. 1997). These findings suggest different sensitivity of test systems and the important role of interaction with factors of the endogenous hormone environment.

To simulate the interaction with endogenous E_2, co-cultivation of MCF-7 cells with ACT and E_2 was performed allowing to more completely characterize the herbicide effects on the cell growth. Under these experimental conditions, ACT in the presence of strong mitogen E_2 (10^{-8} M) exerted inhibitory effects on cell proliferation, similar to those observed for known environmental estrogen bisphenol A (BPA) (Bergeron et al. 1999). No significant proliferation effect of BPA was described in the human uterine carcinoma cell line (ECC-1) in a concentration range of 10^{-8} - 10^{-5} M, though a 2-fold increase of cell yield after E_2 (10^{-8} M) stimulation was observed when compared to the controls on day 4 of incubation. The apparent effect of BPA (10^{-7} M) was recorded in the presence of E_2 (10^{-8} M) only, when BPA significantly abolished stimulation effects of E_2 (Bergeron et al. 1999). Similarly, increased proliferation at low concentrations and cytotoxic effect at higher concentrations were visible in MCF-7 cells co-treated with pyrethroids (pyrethrin and permethrin) and E_2 (10^{-10} M) (Kakko et al. 2004).

Concentrations of ACT that affected E_2-stimulated proliferation of MCF-7 cells were significantly higher than the levels reported in the aquatic environment after application of ACT (0.2 - 4.5 nM at 1-3 months after application) (David et al. 2003). In respect to doses, it could be considered that lower doses of the chemical might be differently effective when compared to the high dose used in our test system, as it was previously observed in the study with ferutinin in MCF-7 cell culture (Lhuillier et al. 2005). In addition, the formation of the active metabolites of ACT in animals and humans is different when compared to metabolites in MCF-7 cell culture with constitutive and inducible cytochrome P450 enzymes (Schlumpf et al. 2004).

In conclusion, based on our results, we could not define ACT as an estrogenic/antiestrogenic agent, but we demonstrated a significant interference of ACT with E_2 effect in estrogen-sensitive test system and might confirm its endocrine disrupting properties. Further experiments are required to exclude possible false negatives of E-screen assay and to elucidate ACT mode of action.

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