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Toxicity of diuron in human cancer cells

Marjo Huovinen [a,*], Jarkko Loikkanen [a], Jonne Naarala [b], Kirsi Vähäkangas [a]

[a] University of Eastern Finland, Faculty of Health Sciences, School of Pharmacy/Toxicology, Yliopistonranta 1 C, 70210 Kuopio, Finland
[b] University of Eastern Finland, Department of Environmental Science, Kuopio, Finland

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ABSTRACT

Diuron is a substituted phenylurea used as a herbicide to control broadleaf and grass weeds and as a biocidal antifouling agent. Diuron is carcinogenic in rat urinary bladder and toxic to the reproductive system of oysters, sea urchins and lizards. The few studies carried out in human cells do not include the genotoxicity of diuron. We have investigated the toxicity of diuron in human breast adenocarcinoma (MCF-7) and human placental choriocarcinoma (BeWo) cells. The production of reactive oxygen species (ROS) was statistically significantly increased in both cell lines but only at the highest 200 μM concentration. Diuron clearly reduced the viability of BeWo, but not MCF-7 cells. The relative cell number was decreased in both cell lines indicative of inhibition of cell proliferation. In the Comet assay, diuron increased DNA fragmentation in MCF-7 but not in BeWo cells. The expressions of p53 protein, a marker for cell stress, and p21 protein, a transcriptional target of p53, were increased, but only in MCF-7 cells. In conclusion, our results suggest that diuron is cytotoxic and potentially genotoxic in a tissue-specific manner and that ROS play a role in its toxicity. Thus, exposure to diuron may exert harmful effects on fetal development and damage human health.

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1. Introduction

Diuron is a herbicide and a suspected human carcinogen (US Environmental Protection Agency, USEPA, 1997), widely used around the world although its use is banned or restricted in some countries e.g. in Sweden, Denmark, Germany and UK due to its harmful effects on the environment and human health (Australian Pesticides and Veterinary Medicine Authority, APVMA, 2011). Although accepted as an active substance as a herbicide in plant protection products in Europe (EU pesticides database), diuron is not used for this purpose in Finland (Finnish Safety and Chemicals Agency, 2013). It was first introduced onto the US market in 1954 and is mainly used as a herbicide to control broadleaf and grass weeds and as an antifouling biocide (APVMA, 2011). Diuron is a substituted phenylurea and it acts by inhibiting plant photosynthesis, specifically blocking the Hill reaction which produces energy (ATP) (USEPA, 1997). Since diuron degrades slowly in water, it is quite persistent in the environment. The half-life of diuron in soil is about 100 days (APVMA, 2011) and about two weeks in water (for a recent review, see Guardiola et al., 2012). Thus, the parent compound and its degradation products can be present in soil, surface and ground water, and sediment (for a review, see Giacomazzi and Cochet, 2004). Diuron is regulated in Europe under chemical legislation (REACH and classification, labeling and packaging of substances, CLP) and also under plant protection product and biocide legislation (Finnish Safety and Chemicals Agency, 2013).

The US Environmental Protection Agency has listed diuron as a likely human carcinogen (USEPA, 1997). This statement is based on findings of carcinomas in bladder (rat), kidney (rat) and mammary gland (mouse) after exposure to diuron. In addition, it increased urothelial cell proliferation and has caused hyperplasia of the rat urothelium (Nascimento et al., 2006; Cardoso et al., 2013). Furthermore, diuron has triggered urinary bladder mucosal necrosis in rats (Da Rocha et al., 2010; Cardoso et al., 2013). Diuron is metabolized and excreted into urine (for a recent review, see Da Rocha et al., 2014). It has been demonstrated that the cytotoxicity or hyperplastic urothelial lesions caused by diuron are not due to its precipitation and crystal formation in bladder (Da Rocha et al., 2010). This raises concerns that other mechanisms, including potential genotoxicity, may be involved in the formation of these lesions.

In animals, (USEPA, 2011) and the Ames test (APVMA, 2011) diuron is not mutagenic. In the Comet assay, diuron does not cause DNA damage in urinary bladder cells or peripheral blood leukocytes of male Wistar rats (Nascimento et al., 2006) or Chinese hamster ovary cells (Da Rocha et al., 2010). Furthermore, Grassi and

* Corresponding author.
E-mail address: Marjo.Huovinen@uef.fi (M. Huovinen).

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coworkers did not find any evidence that diuron would possess initiating or promoting properties in their rat liver carcinogenesis bioassay (Grassi et al., 2007), or initiating potential for mammary carcinogenesis in female SD rats (Grassi et al., 2011a). However, in oysters, diuron is genotoxic according to the Comet assay (Akcha et al., 2012; Barranger et al., 2014). No genotoxicity studies have been carried out in human cells.

Diuron is suspected to be an endocrine disruptor and it may produce adverse developmental and reproductive effects (Iyer, 2002) although there have been some discrepant results. Diuron did not induce any signs of ER-mediated effects in an in vitro assay using Chinese hamster ovary (CHO) cells transfected with human ER-receptors (Kojima et al., 2004). However, in the same study, diuron inhibited transcriptional activity of the androgen receptor (AR) induced by dihydrotestosterone in AR transfected CHO cells (Kojima et al., 2004). Orton et al. (2009) found that several phenylurea pesticides including diuron could prevent the binding of the natural ligands (estradiol or dihydrotestosterone) to their receptors in a yeast screen. However, in in vivo tests in female rats, perinatal and juvenile diuron exposure did not have any effect on vaginal opening, estrous cycle or mammary morphology (Grassi et al., 2011b). On the other hand, diuron did reduce the weights of ovaries and *corpora lutea* suggesting that diuron may be toxic to the female reproductive organs (Grassi et al., 2011b).

Exposure of adult male rats to diuron (Fernandes et al., 2007), or perinatal and juvenile diuron-exposure of male rats (Fernandes et al., 2012) did not alter plasma testosterone levels, daily sperm production or sperm morphology and motility. In the sea urchin, exposure to diuron caused malformations of the embryos and inhibited the fertilization rate (Manzo et al., 2006), whereas in lizards, testosterone levels were significantly decreased (Cardone et al., 2008). According to these results, it does seem that diuron can exert hormonal effects, but their exact nature needs to be clarified. Very few studies have been carried out in human cells. In the study of Vinggaard et al. (1999) conducted in MCF-7 breast cancer cells, diuron did not induce cell proliferation. In addition, diuron did not alter CYP19 aromatase activity in human placental microsomes (Vinggaard et al., 2000). However, diuron was able to prevent ovulation in an in vitro ovulation assay as well as reducing the levels of testosterone in ovarian tissue (Orton et al., 2009).

Reactive oxygen species (ROS) are formed both endogenously and exogenously (for a review, see Xue and Warshawsky, 2005). ROS may be carcinogenic not only by causing oxidative DNA damage or DNA strand breaks but also through non-genotoxic mechanisms e.g. by stimulating the cellular signal transduction processes that promote carcinogenesis (Goetz and Luch, 2008). Da Rocha et al. (2013) showed in human urothelial 1T1 cells that diuron metabolites could alter signaling pathways e.g. oxidative stress response, matrix remodeling and inflammation. According to the gene expression analysis in rat urothelial cells conducted by Ihlaseh et al. (2011), diuron may be involved in the Nrf2 (nuclear factor erythroid 2-related factor 2)-mediated oxidative stress response as well as changing the expression levels of genes involved in amino acid, lipid and nitrogen metabolism. Geoffroy et al. (2002) pointed out that diuron could induce two antioxidative exzymes (glutathione reductase and glutathione S-transferase) in microalgae *Scenedesmus obliquus*, and as a consequence ROS production increased.

The mechanism of action (MOA) of diuron has only been clarified in vegetable organisms (e.g. plants and algae). The suggested carcinogenic MOA in rats is "metabolite-induced urothelial cytotoxicity with necrosis and cell exfoliation, consequent regenerative hyperplasia and eventually tumors" (for a recent review, see Da Rocha et al., 2014). Although some hypotheses have been proposed to explain how diuron may induce cancer-related changes in rats and rodent cells, human cells have never been studied from this point of view and responses can be different in human cells. For instance, we have previously shown (Tampio et al., 2008) that responses to benzo(a)pyrene in human MCF-7 cells differed from those in mouse Hepa1c1c7 cells. In this study, we have used two human cancer cell lines, MCF-7 breast cancer cells and BeWo choriocarcinoma cells, to elucidate the molecular toxicity of diuron. BeWo cells represent human placenta which is an important organ between mother and fetus, being the primary route for fetal exposure (Vähäkangas et al., 2014).

2. Materials and methods

2.1. Cell culture and treatments

MCF-7 breast cancer cells were cultured as described previously (Tampio et al., 2008) at 37 °C in a cell culture incubator with a humidified atmosphere containing 95% air and 5% CO_2. BeWo choriocarcinoma cells were cultured in RPMI 1640 without L-glutamine and Phenol Red (BioWhittaker, Belgium) and supplemented with 9% heat inactivated fetal bovine serum (FBS, Gibco, UK), 1 mM Na-pyruvate (BioWhittaker, Belgium), 1x non-essential amino acids (NEAA, BioWhittaker, Belgium), 2 mM L-glutamine (BioWhittaker, Belgium) and Penicillin–Streptomycin (BioWhittaker, Belgium) in the same atmosphere as the MCF-7 cells. Cells were exposed to 100 nM, 10 μM, 50 μM, 100 μM, 150 μM or 200 μM diuron (Sigma, dissolved in dimethyl sulfoxide, DMSO, Sigma). Control cells were exposed to 0.1% DMSO. Cells for protein extraction and Comet assay experiments were exposed on 6-well plates, as single measurements only. Cells for viability and ROS experiments were exposed on 48-well plates, with four replicates per concentration. All the experiments at the different time points and concentrations were repeated independently at least four times (except for the Comet assay with BeWo cells, $n = 2$).

2.2. Viability

2.2.1. Propidium iodide–digitonin assay

The propidium iodide–digitonin cell viability measurement was first described by Sarafian et al. (1994) and carried out as described previously (Huovinen et al., 2011). This method is based on the fluorescence of propidium iodide (PI) that can only enter cells and nuclei with damaged membranes, while the membranes of viable cells are impermeable to PI. After binding to nucleic acids, the fluorescence (F) of PI is increased 20- to 30-fold. Briefly, at the end of the exposure to diuron, PI was added to each well, incubated and the fluorescence was measured. In order to obtain the maximal fluorescence value, which reflects the total number of cells, the cells were then treated with digitonin, a drug known to damage the cell wall and the nuclear membrane, making them permeable to PI. After the incubation, the fluorescence (F_{MAX}) was measured.

2.2.2. MTT-test

Cell viability was measured also by the MTT test where an exogenously administered MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] is converted into the colored formazan by functioning mitochondria inside the cells (Mosmann, 1983). The test was done as previously described by Pesonen et al. (2012) with the results expressed as a percentage of the controls exposed to DMSO that was used as the solvent for diuron. Four separate experiments, containing 4 replicates at every exposure concentration were performed.

2.3. Reactive oxygen species

The production of ROS was measured with a method that uses H_2DCFDA as the substrate (described earlier in Loikkanen et al.,

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