Toxicity of diuron metabolites in human cells

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Toxicity of diuron metabolites in human cells.

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Highlights

- Diuron metabolites were toxic in human cancer cell lines BeWo, MCF-7 and Caco-2.
- Clear toxicity by MTT assay indicates mitochondrial-mediated toxicity.
- In comparison between the cell lines, MCF-7 was the most resistant.
- Increased ROS may not explain cytotoxicity of diuron metabolites.

Abstract

Diuron, a highly used herbicide worldwide, is metabolized into several toxic metabolites. DCA (3,4-dichloraniline), DCPU [3-(3, 4-dichlorophenyl)urea] and DCPMU [3-(3,4-dichlorophenyl)-1-methyl urea] reduced viability of human placental choriocarcinoma BeWo, human breast adenocarcinoma MCF-7 and human colon adenocarcinoma Caco-2 cells as judged by the MTT assay, where color formation is dependent on functional mitochondria in viable cells. Based on the IC_{50} values in BeWo cells the order of cytotoxicity was DCA>DCPU>diuron>DCPMU, and in Caco-2 cells DCPMU>DCPU>DCA, diuron. In MCF-7 cells, only DCPU had an IC_{50} within the range of the concentrations used. In the PI-digitonin viability assay, only the highest concentration (200 µM) of DCPU caused a statistically

significant decrease in viability in any cell line. There was no correlation between cytotoxicity and ROS production. This indicates that diuron metabolites are toxic in cells of human origin with mitochondria as the target, but ROS not the likely mechanism.

Key words: MCF-7, BeWo, Caco-2, ROS, MTT assay

1. Introduction

Diuron is a herbicide being one of the most effective and highly used antifouling agents worldwide. It controls vegetation by impairing the photosynthesis related growth of unwanted weeds. Diuron is a phenylurea derivative classified as possibly carcinogenic according to the US-EPA (1997). It is highly stable and persistent, particularly in aquatic environment. Thus, exposure to diuron appears to be inevitable, which poses a threat to aquatic organisms and probably also to highly sensitive individuals, such as developing fetus. Diuron is slowly degraded in the environment by microbes or hydrolysis in water into an equally or more toxic byproduct 3,4-dichloroaniline (DCA), which in lower organisms is also a metabolite of the well-known pesticides propranil (Williams and Jacobson, 1966) and linuron (Engelhardt et al., 1971). This is the reason why there are more studies on DCA than on the other diuron metabolites.

In rats and dogs, diuron has been found to be metabolized primarily to 3-(3, 4-dichlorophenyl)urea (DCPU) followed by 3-(3,4-dichlorophenyl)-1-methyl urea (DCPMU), DCA and 3,4-dichlorophenol (Hodge et al., 1967). However, in a more recent study DCPMU was the only diuron metabolite detected in incubations with various mammalian liver microsomal preparations, with the ranking order of DCPMU formation being dog> monkey> rabbit> mouse> human> minipig> rat (Abass et al., 2007). In the only existing human study, describing analysis of diuron and its metabolites in postmortem urine and blood of one person, van Boven and coworkers (1990) found DCPMU and DCPU. Traces of DCA and 6-OH-DCPU were also detected but only in urine.

Although the toxicity of diuron and DCA have been studied in vivo in animals, there is no toxicity data in human *in vivo*. Furthermore, the toxicity of DCPU and DCPMU have not been studied in vivo in animals. In vitro only one study on all the three main diuron metabolites (DCPU, DCPMU, DCA) exists (Da Rocha et al., 2013), but DCA has been studied more. It is believed that diuron metabolites, mainly DCPU as the main metabolite, are responsible for the

development of urothelial cancer *in vivo* in rats (Da Rocha et al., 2013). According to a viability assay conducted in MYP3 rat urothelial cells, all diuron metabolites were cytotoxic with DCPMU being the most potent, whereas DCA was the most cytotoxic in 1T1, human urothelial cells. Similarly, microarray analysis by Da Rocha and coworkers (2013) showed that DCA alters the largest number of probe-sets in human urothelial cells. If a person is exposed to diuron, DCA is also formed, but only in minute amounts (van Boven et al., 1990).

DCA has been cytotoxic in isolated rat renal cortical cells (Rankin et al., 2008) and in hepatic and renal tissue slices (Rankin et al., 2008; Valentovic et al., 1995). DCA is also toxic *in vivo* in rats with kidney, liver, and urinary bladder as the target organs of acute toxicity (Valentovic et al., 1997). Additionally, DCA has been found hemotoxic in rats, characterized by reductions in platelet count, total number of leukocytes and the percentage of lymphocytes, as well as dose-dependent induction of methemoglobin formation (Guilhermino et al., 1998). *In vivo* toxicity studies in animals indicate that DCA is immunotoxic (Barnett et al., 1992), and nephrotoxic (Valentovic et al., 1997). In V29 Chinese hamster cells, and also in cultured human lymphocytes, DCA has induced aneuploidy (Bauchinger et al., 1989). Moreover, Zhang and Lin (2009) have described that in rats DCA alters important testicular enzymes involved in spermatogenesis indicating reproductive toxicity.

We have shown that diuron can easily cross human placenta in human placental perfusion implicating fetal exposure and fetotoxicity (Mohammed et al., 2018). Furthermore, diuron was metabolized to the toxic metabolite DCPMU in human placenta during the perfusions. This metabolite was found both in maternal and fetal circulations and traces in placental tissue after the perfusions. *In vivo,* fetus is most probably also exposed to diuron metabolites formed in maternal liver and probably in fetal liver that also has xenobiotic metabolizing capacity (for a review, see Myllynen et al., 2009). In human cells, so far, the toxicity of diuron metabolites have been studied in blood cells (Bauchinger et al., 1989; Malerba et al., 2002) and in urothelial cells (Da Rocha et al., 2013).

In the literature, human cancer cell lines differ in their sensitivity to various toxic compounds. The robustness of the cell lines differs depending on the compound studied and on the test system applied (Coleman et al., 2007; Xu et al., 2016; El-Zahabi et al., 2019; Silva et al., 2019). In our previous study using human cancer cell lines, diuron decreased viability of human placental choriocarcinoma BeWo cells, which are placental trophoblastic cells representing cells of fetal origin, but did not affect viability of human breast adenocarcinoma cells (MCF-

7) (Huovinen et al., 2015). Increased production of ROS by diuron was observed in both types of cells which could explain part of the toxicity of diuron. In this study, we used the same human cell lines (MCF-7 and BeWo), as well as human colon adenocarcinoma cells Caco-2 in order to gain more insight into potential human toxicity of the main diuron metabolites.

2. Materials and methods

2.1.Cell culture and chemical treatments

Human placental choriocarcinoma BeWo and human breast adenocarcinoma MCF-7 cells were cultured as described earlier (Huovinen et al., 2015). Human colon adenocarcinoma cells (Caco-2) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, BioWhittaker, Belgium) containing 4.5 g/l glucose and supplemented with 9% fetal bovine serum, 1 mM sodium pyruvate, 1 x non-essential amino acids, 2 mM L-glutamine, and a combination of penicillin and streptomycin. The cells (Caco-2, BeWo and MCF-7) were seeded on 48 well-plates at a density of 30 000-40 000 cells/ml and treated with the concentrations of 25-200 µM of DCPMU, DCA or DCPU for 48 hours. Caco-2 cells were also treated with 50, 100, 150 or 200 µM of diuron. Control cells were treated with equivalent volumes of DMSO (0.1%, Sigma-Aldrich, USA).

2.2. Cell Viability

2.2.1. MTT assay

The experiments were carried out on 48-well plates as described earlier (Huovinen et al., 2015). MTT assay is based on the conversion of MTT [3-(4,5-dimethylthiazo l-2-yl)-2,5-diphenyl-tetrazolium bromide] into a colored compound known as formazan by functioning mitochondria in viable cells. Decrease of absorbance indicates cytotoxicity. Four to five individual experiments (plates) were carried out for each compound with four replicates per concentration in every experiment. Viability was expressed as percent from control.

2.2.2. Propidium Iodide-digitonin assay

Analyses were carried out on 48-well plates according to the method of Sarafian et al. (1994), as described previously (Huovinen et al., 2011; Loikkanen et al., 2003). Briefly, at the end of the treatment with diuron or its metabolites, propidium iodide (PI) was added into the cell

cultures, incubated for 20 minutes at room temperature (RT), and the fluorescence was analyzed. If the cell membrane is damaged, PI will bind to the nucleic acids and the fluorescence of PI is increased by approximately 20-30-fold. In order to count the total number of cells, the cultures were additionally treated with digitonin (for 20 minutes at RT), which causes damage to the cell membranes allowing PI to enter all the cells. After incubation, the maximum fluorescence was analyzed. Viability is expressed as percent fluorescence related to the maximum fluorescence of the corresponding sample, and the relative cell number is expressed as percentage from control values (Huovinen et al., 2011). Four to five independent experiments (plates) were carried out for each concentration of the compounds with four replicates on each plate.

2.2.3. Reactive oxygen species.

The production of reactive oxygen species (ROS) was analyzed on 48-well plates using the substrate 2`7`-dichlorodihydrofluorescein diacetate (H_2DCFDA) as described previously (Tampio et al., 2009). Three to seven individual experiments (plates) were carried out for each compound with four replicates per concentration in every experiment. Production of ROS was expressed as percent from control.

2.2.4. Liquid chromatography- mass spectrometry

Cell cultures were analyzed for diuron and its metabolites, DCPMU, DCPU and DCA, using Agilent 6410 Triple Quadruple LC-MS (G6410A) (Agilent Technologies, USA). The method is described in detail by Mohammed et al. (2018).

2.3.Calculations and statistical analysis.

The 50% inhibitory concentration (IC_{50})-values were calculated from MTT data using non-linear regression analysis. Also, IC_{50} values of diuron cytotoxicity in BeWo and MCF-7 were calculated from our earlier MTT data (Huovinen et al., 2015) in order to compare the toxicity of diuron and its metabolites.

One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test were used for statistical calculations in all comparisons. $P < 0.05$ was considered as statistically significant. Results of viability assays, relative cell numbers and ROS were expressed as a mean ± standard deviation. Graph-Pad Prism 5.05 was used to compute the IC_{50}-values and for statistics.

3. Results

3.1.Metabolism of diuron

CaCo-2 and MCF-7 cells were treated with up to 200µM of diuron for 48 hours. DCPMU was the only metabolite formed after exposure to ≥100µM in both types of cells. No DCPU was found. As to DCA, a peak with a similar retention time to that of the standard was obtained, but the peak did not identically match the molecular ion of DCA standard and thus it could not be identified with certainty.

3.2 Toxicity of diuron metabolites in human cells

DCPMU and DCPU reduced the viability of cells of all the cell lines as judged by the MTT assay (Fig 1). DCA reduced the viability of BeWo and Caco-2 cells but not MCF-7 cells. According to IC_{50}-values in BeWo cells, the order of cytotoxicity was DCA>DCPU>diuron>DCPMU, and in Caco-2 cells DCPMU, DCPU>DCA, diuron. In MCF-7 cells only DCPU had an IC_{50} within the range of the concentrations used (Table 1).

With the other viability assay, PI-digitonin, only DCPU showed a statistically significant decrease in viability in all the three cell lines (Table 2; Fig S1). By the PI-digitonin assay, DCPMU decreased cell viability statistically significantly only in Caco-2 cells. IC_{50} values could not be calculated accurately from this data, because the computed IC_{50} values were higher than the highest concentration used.

Relative cell number, which indicates a decrease in viability, and/or inhibition of proliferation, decreased statistically significantly only with DCPMU in Caco-2 cells and with DCA in BeWo cells (Table 2; Fig S2). ROS increased statistically significantly in all the cell lines only by DCA (Fig. 2). DCPMU increased ROS in BeWo and MCF-7 cells, and DCPU only in BeWo cells (Fig 2).

3.3 Sensitivity of the cell lines to diuron metabolites

There were differences in the responses to the diuron metabolites between the cell-lines depending on the analysis carried out. In MTT test indicating damage to mitochondrial function, BeWo cells were the most sensitive, so that DCA decreased the viability already at the concentration of 25 µM, and DCPU at 50 µM (Fig. 1). Caco-2 cells were the most sensitive

to DCPMU, viability decreasing at the concentrations of 50 µM or higher (Fig 1). In MTT assay MCF-7 cells were the most resistant to the cytotoxicity by diuron metabolites.

In the other assay for viability, the PI-digitonin assay, which indicates cell membrane damage, there were no big differences between the cell lines and the cells were quite resistant to all the used diuron metabolites (Table 2; Fig S1). As to the relative cell number, only BeWo and CaCo-2 were somewhat sensitive to the decrease (Table 1; Fig. S2). In Caco-2 cells the concentrations above 100 µM of DCPMU, and in BeWo cells 200 µM decreased the number of cells statistically significantly.

ROS production was most prominent in MCF-7 cells, where DCA induced a statistically significant increase in ROS already at 50 µM concentration (Fig 2). However, as to the other cell lines, statistically significant increases were noted only by the highest (200 µM) concentration. BeWo cells were the only ones where all three metabolites increased ROS statistically significantly.

3.4 Comparison of diuron toxicity in cell lines

We have earlier studied toxicity of diuron in BeWo and MCF-7 cells (Huovinen et al., 2015). To complete the picture, toxicity of diuron in Caco-2 cells was also studied. IC_{50}-values were calculated from current MTT data for Caco-2 cells, and earlier MTT data for BeWo and MCF-7 cells from Huovinen et al. (2015) in order to compare the toxicity of diuron among the cell lines and to compare diuron with its metabolites. The IC_{50} of diuron in BeWo cells was the only one within the range of the concentrations used (Table 1).

Viability of Caco-2 cells by MTT assay and the relative cell number decreased statistically significantly (Fig 3A and 3D). However, no decrease in viability by PI-digitonin (Fig 3C) or increase in ROS production (Fig 3D) was noticed.

4. Discussion

This study demonstrates toxicity of diuron metabolites in three human cancer cell lines. This is in line with the study by Da Rocha and coworkers (2013), the only earlier study on cytotoxicity of diuron metabolites in cells of human origin. They showed cytotoxicity by diuron metabolites in human urothelial 1T1 cells derived from normal human ureter epithelium and immortalized by transfection of the HPV-16 E6 and E7 genes (Tamatani et al., 1999). Our

study and that by Rocha and coworkers provide basic toxicity data for further molecular studies to pursue mechanisms of toxicity of diuron and its metabolites.

We found differences in the toxicity among the three metabolites in the used cell lines. Interestingly, the order of toxicity of diuron metabolites based on IC_{50} values in BeWo cells was similar to that observed in human urothelial cells; DCA>DCPU>DCPMU (Da Rocha et al., 2013). The IC_{50} value of DCA (73 µM) in BeWo cells was similar to that seen in human urothelial cells (72 µM). Toxicity of DCA to human cells is in line with a study in mice which showed that tetrachloroazobenzene, structurally related to aromatic amines and DCA, induces urethral carcinoma (Singh et al., 2010). It is known that exposure to aromatic amines is associated with bladder cancer in workers in chemical industry (Ferris et al., 2013). In Caco-2 cells, on the other hand, the order of toxicity was similar to that seen in rat urothelial cells with DCPMU and DCPU being more cytotoxic than DCA (Da Rocha et al., 2013). Our study is the first comparative study of toxicity between diuron and its metabolites in more than one human cell line. We show here that in all the three used cell lines, at least some of the diuron metabolites were more toxic than diuron. Overall, according to the IC_{50} values, BeWo cells were the most sensitive to both diuron and its metabolites. The most resistant to diuron and its metabolites were the MCF-7 cells where DCPU was the only metabolite with an IC_{50} value below the highest tested concentration.

Cytotoxicity in MTT assay, shown in our study by all diuron metabolites is indicative of mitochondrial-mediated toxicity, because the assay is based on the enzymes in functional mitochondria. We have found similar cytotoxicity earlier by diuron (Huovinen et al. 2015). Our results are also in line with earlier animal studies showing disturbance of mitochondrial functions by diuron. In rat hepatocytes, Owen and Halestrap (1993) have shown that diuron inhibits oxidative phosphorylation. In their study diuron inhibited gluconeogenesis and reduced mitochondrial ATP/ADP ratio, with a positive correlation between them (Owen and Halestrap, 1993). Likewise, in a recent *in vitro* study using isolated rat liver mitochondria, diuron inhibited cellular respiration and mitochondrial membrane bound enzyme activities (da Silva Simoes et al., 2017). In the same study, diuron reduced oxygen uptake, pyruvate production and cellular ATP content in perfused rat liver. A lack of clear toxicity by PI-digitonin assay reflecting cell membrane integrity indicates less toxicity to the cell membrane.

ROS have been hypothesized as a possible mechanism of diuron toxicity (Huovinen et al., 2015; Kao et al., 2019). This is supported by a diuron-induced, dose-dependent increase of

ROS production in human immortalized liver HepG2 cells (Kao et al., 2019). In our study, increased ROS production was seen by all diuron metabolites in at least some of the cell lines used, with most prominent ROS production in MCF-7 cells. Because MFC-7 cells were more resistant to the toxicity of the metabolites, this may reflect better condition of the MCF-7 cells after the treatment. In gene expression analysis Da Rocha and coworkers (2013) found the signaling pathways of oxidative stress response to be among the pathways induced by all three diuron metabolites (DCA, DCPU, DCPMU) in rat urothelial cells, but in human urothelial cells only by DCA and DCPU. This all is in line with the studies in lower animals where ROS production has been found as a potential mechanism of toxicity of diuron and its metabolites (Behrens et al., 2016).

However, in our study there was no correlation between the cytotoxicity and ROS production. This is in line with our earlier results showing that ROS production was not as evident in BeWo cells treated with diuron as in MCF-7 cells, more resistant to cytotoxicity by diuron (Huovinen et al., 2015). Among the diuron metabolites, DCPU was the only one altering the gene expression pathways involved in oxidative stress in urothelial cells (Da Rocha et al. 2013). Furthermore, Kao and coworkers (2019) have shown that pretreatment of HepG2 cells with N-acetylcysteine, an antioxidant, did not protect the cells from the cytotoxic effects of diuron suggesting that induction of oxidative stress is probably not the major mechanism of diuron cytotoxicity in the cells. Thus, based both on our results and those by Kao and coworkers (2019) it remains unlikely that increased production of ROS is a major mechanism of cytotoxicity by diuron and its metabolites in human cells.

Of the used cell lines, MCF-7 and BeWo cells were earlier studied for diuron toxicity (Huovinen et al., 2015). However, Caco-2 cells have not been studied for diuron toxicity or metabolism before. In all these cell lines diuron was metabolized into DCPMU (this study, Mohammed et al., 2018). Similarly to MCF-7 and BeWo cells, diuron was cytotoxic in Caco-2 cells analyzed by MTT-test. Also, our findings agree with those from the recent *in vitro* study using human HepG2 cells, in which diuron was cytotoxic in MTT assay (Kao et al., 2019). In our earlier study MCF-7 cells were more resistant to diuron toxicity than the BeWo cells (Huovinen et al., 2015). Similarly, Xu et al. (2016) found that MCF-7 cells were more resistant to triazolo-tetrazine derivatives than BeWo cells. MCF-7 cells seem to be more resistant to diuron also in comparison with Caco-2 cells. This is supported by an earlier study where the cytotoxicity with three hexadione isomers were analyzed (Coleman et al., 2007). On the other

hand, there are studies where MCF-7 cells have been more sensitive to e.g. doxorubicin (El-Zahabi et al., 2019) or epigallocatechin gallate (Silva et al., 2019) compared to Caco-2 cells.

In conclusion, our data confirm that diuron metabolites are toxic in cells of human origin. The degree of toxicity varies among the metabolites. Based on this and our earlier study (Huovinen et al., 2015) there are differences in the sensitivity of the cell lines to the toxicity by diuron and its metabolites, MCF-7 being more resistant than Caco-2 or BeWo. Mitochondrial toxicity is probably one of the major mechanisms behind the cytotoxicity. ROS production varied between the cell lines and metabolites, with no clear relation to cytotoxicity in MTT assay indicating that increase of ROS is not the mechanism of cytotoxicity.

CRediT authorship contribution statement

Ali Mohammed, Marjo Huovinen, Kirsi Vähäkangas: Conceptualization, formal analysis

Ali Mohammed, Marjo Huovinen: Investigation

Ali Mohammed, Marjo Huovinen, Kirsi Vähäkangas: Writing, review and editing

Kirsi Vähäkangas: Supervision

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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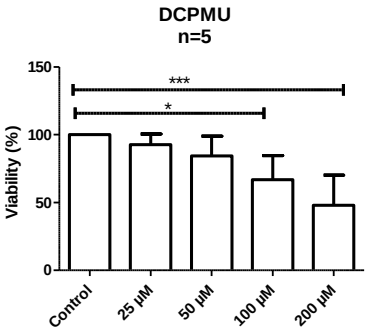
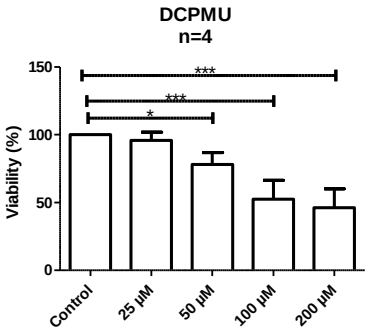
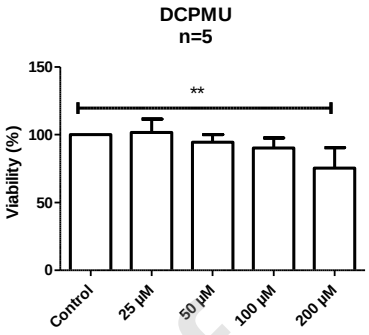
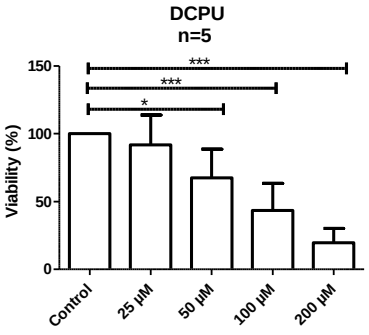
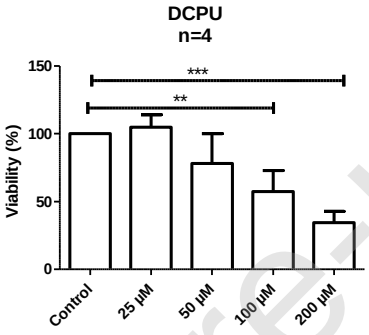
BeWo cells | **Caco-2 cells** | **MCF-7 cells**

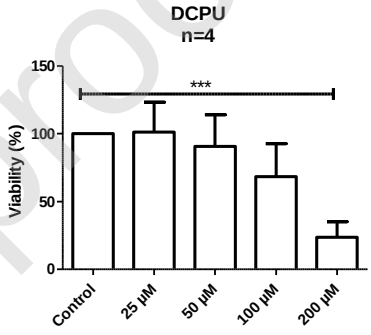


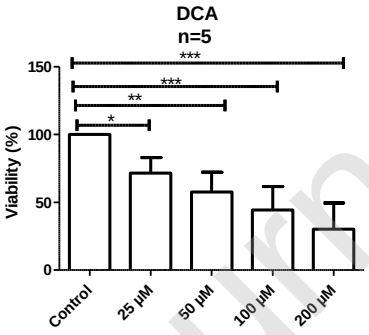


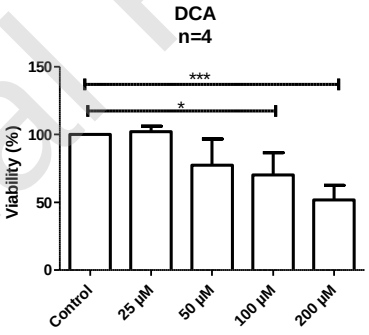












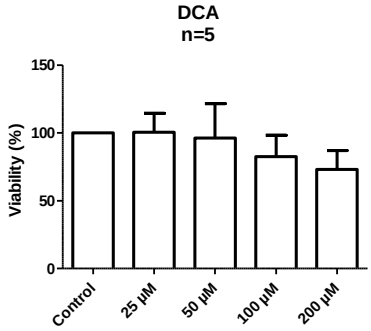
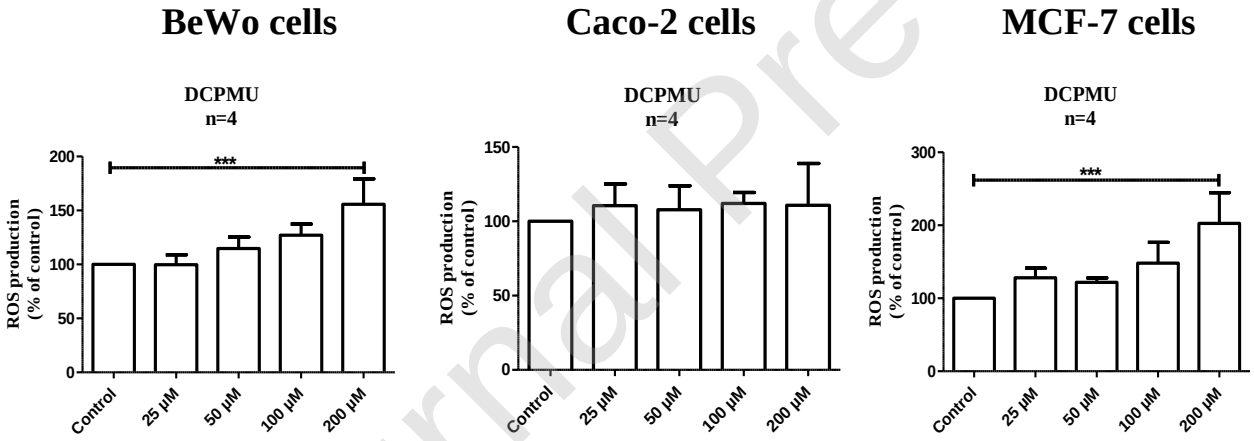


Fig 1. Cell viability, analyzed by MTT assay, of human placental choriocarcinoma BeWo cells (left column), human colon adenocarcinoma Caco-2 cells (middle column) and human breast adenocarcinoma MCF-7 cells (right column) treated with 25-200 µM of 3-(3,4-dichlorophenyl)-1-methyl urea (DCPMU), 3-(3, 4-dichlorophenyl)urea (DCPU) or 3,4-dichloroaniline (DCA) for 48 h. The columns represent the mean ± SD of relative values from 4-5 individual experiments (plates; n = 4 or 5 as indicated in the figure), each a mean of four replicates on the same 48-well plate; *p <0.05, **p < 0.01, ***p < 0.001.



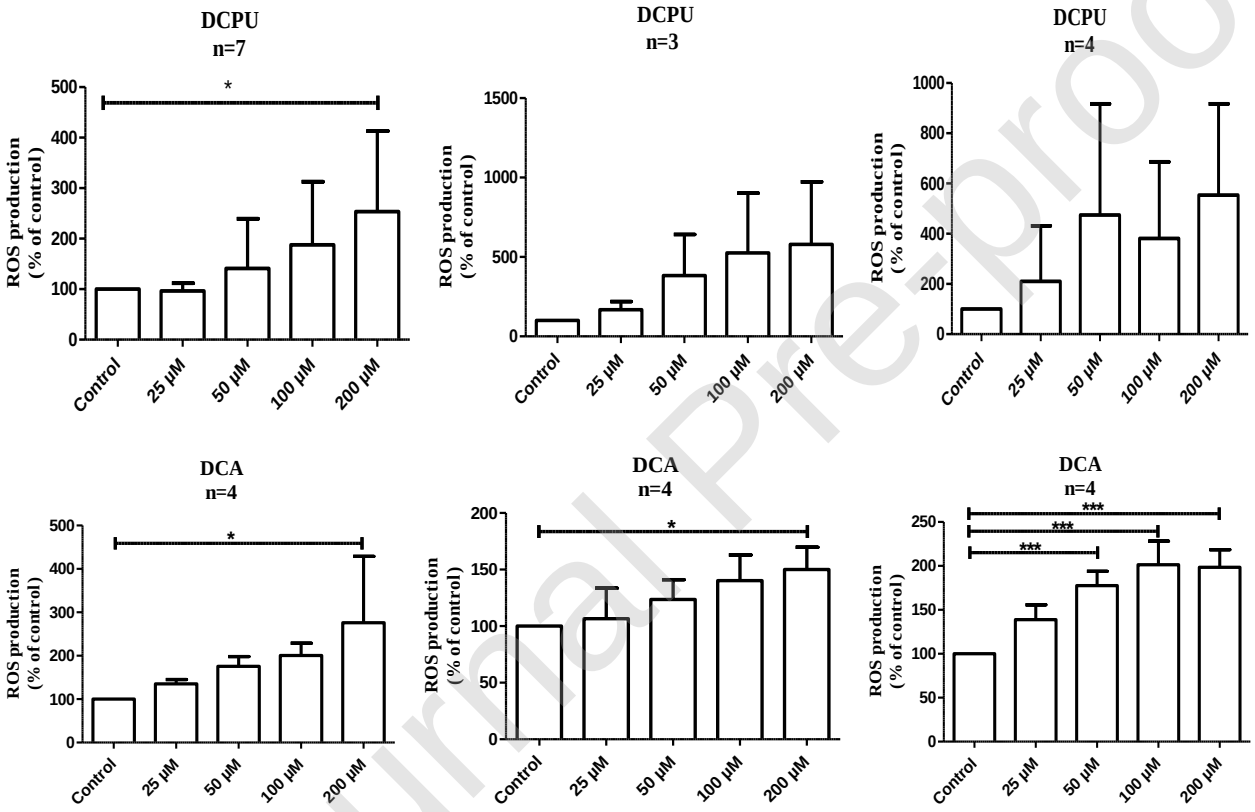
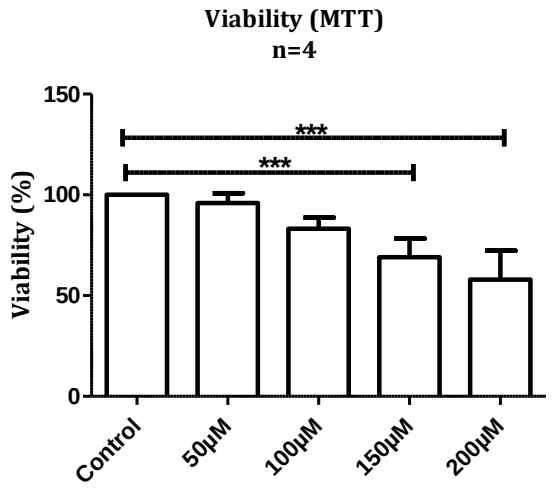


Fig. 2. Production of reactive oxygen species (ROS) in human placental choriocarcinoma Bewo cells (left column), human colon adenocarcinoma Caco-2 cells (middle column) and human breast adenocarcinoma MCF-7 cells (right column) after treatment with 3-(3,4-dichlorophenyl)-1-methyl urea (DCPMU), 3-(3, 4-dichlorophenyl)urea (DCPU) or 3,4-dichloroaniline (DCA) for 48 h. The columns represent a mean ± SD of relative values from 3-7 individual experiments (plates; n= 3-7 as indicated in the figure) each a mean of four replicates on the same 48-well plate. *$p<0.05$, ***$p<0.001$.

A)



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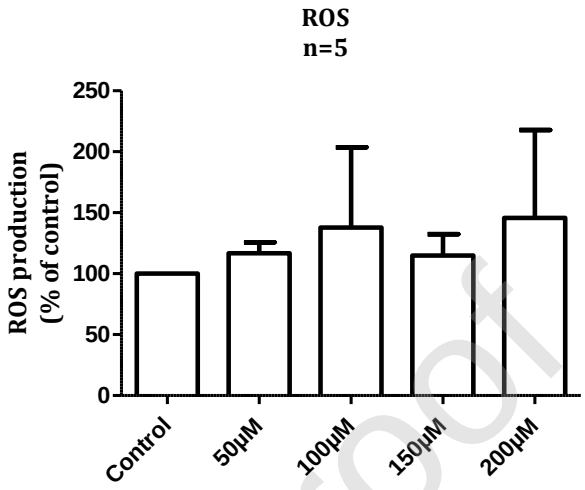

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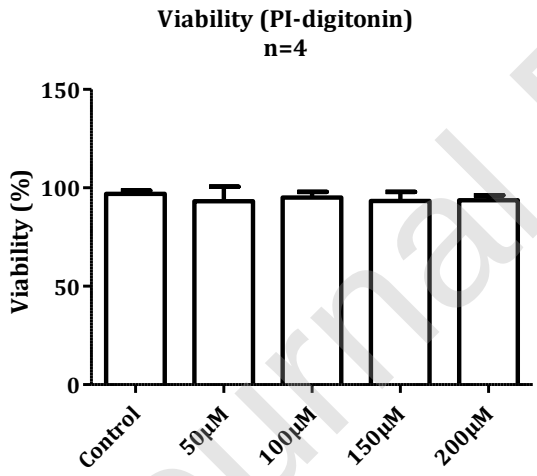

D)

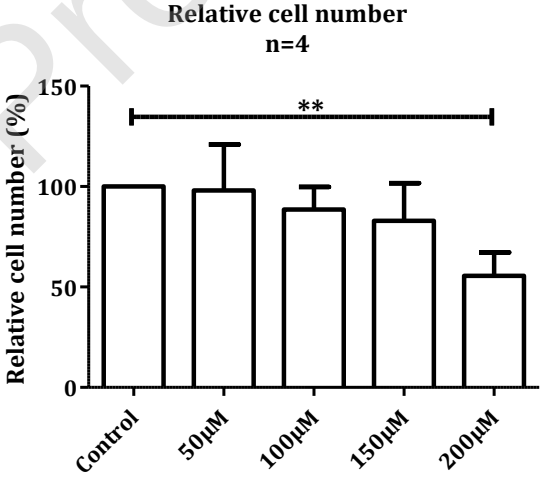

Fig. 3. Toxicity of diuron in human colon adenocarcinoma Caco-2 cells. Cell viability using MTT-assay (A) or Propidium iodide (PI)-digitonin-assay (C), production of reactive oxygen species (ROS) (B) and relative cell number (D) in cells treated with the indicated concentrations of diuron for 48 h. The values are mean ± SD from 4-5 individual experiments (plates; n= 4-5 as indicated in the figure) each a mean of four replicates on the same 48-well plate. **p< 0.01, ***p<0.001.

Table 1. The 50% inhibitory concentration (IC_{50}; µM) of diuron metabolites in human cancer cell lines y at 48 hours after treatment as analyzed by MTT assay

Metabolite	IC_{50}		
	Bewo	**Caco-2**	**MCF-7**
DCPMU	188	158	>200
DCPU	89	152	167
DCA	73	>200	>200
Diuron	152	>200	ND

DCPMU= 3-(3,4-dichlorophenyl)-1-methyl urea; DCPU= 3-(3, 4-dichlorophenyl)urea; DCA= 3,4-dichloroaniline. ND= not determined (no difference in viability between control and treated groups).

Table 2. Concentrations of diuron metabolites inducing a statistically significant reduction of viability and/or relative cell number in human cancer cell lines as analyzed by PI-digitonin assay . Concentrations used were 25, 50, 100 or 200 µM.

Metabolite	Viability			Relative cell number		
	Bewo	**Caco-2**	**MCF-7**	**Bewo**	**Caco-2**	**MCF-7**
DCPMU	NE	200	NE	NE	100 & 200	NE
DCPU	200	200	200	NE	NE	NE
DCA	NE	NE	NE	200	NE	NE

DCPMU= 3-(3,4-dichlorophenyl)-1-methyl urea; DCPU= 3-(3, 4-dichlorophenyl)urea; DCA= 3,4-dichloroaniline. BeWo= human placental choriocarcinoma cells; Caco-2= human colon adenocarcinoma; MCF-7= human breast adenocarcinoma; NE= no effect;