UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA DE BOTUCATU

DESVENDANDO O MODO DE AÇÃO (MOA) CANCERÍGENO DO DIURON [3-(3,4-DICLOFENIL)-1,1-DIMETILURÉIA] NO UROTÉLIO DE RATOS

MITSCHELI SANCHES DA ROCHA

Tese apresentada ao Programa de Pós- Graduação em Patologia da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista – UNESP para obtenção do título de Doutora em Patologia.

BOTUCATU / SP

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SÃO PAULO STATE UNIVERSITY "JÚLIO DE MESQUITA FILHO"
BOTUCATU MEDICAL SCHOOL

ELUCIDATING THE CARCINOGENIC MODE OF ACTION OF DIURON [3-(3,4-DICHLOROPHENYL)-1,1-DIMETHYLUREA] ON THE RAT UROTHELIUM

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and Oswaldo Moreira da Rocha,
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ABSTRACT

Diuron, a substituted urea herbicide, was carcinogenic to the urinary bladder of Wistar rats at high dietary levels (2500 ppm), with a higher incidence in males. Its proposed carcinogenic mode of action (MOA) includes urothelial cytotoxicity and necrosis followed by sustained regenerative cell proliferation and urothelial hyperplasia. Urothelial cytotoxicity could be induced either by urinary solids or by chemical toxicity by diuron and/or metabolites excreted in the urine. To further elucidate the diuron urothelial MOA in male Wistar rats, this study aimed to: 1) evaluate the possible influence of urinary solids on the development of urothelial lesions; 2) determine the time course and sequence of bladder cytotoxic and proliferative changes; and 3) evaluate the toxicity of the main diuron metabolites on the urothelium. Rats treated with diuron and with NH_4Cl for urinary acidification showed decreased urinary pH and reduced amounts of urinary crystals and precipitates. No difference in the incidence of urothelial lesions was found between diuron and diuron+NH_4Cl treated groups indicating that cytotoxicity is not due to urinary solids. Rats treated with diuron during 1, 3 and 7 days showed urothelial cell swelling beginning on day 1. Swollen cells at day 7 presented degenerative changes such as distention of the cytoplasm, organelles and nuclei characteristic of cytolysis. By day 28, bladder urothelium in the diuron-treated group showed extensive necrosis, exfoliation and piling up of cells suggestive of hyperplasia. At 8 weeks, the bladder urothelium showed necrosis, exfoliation and significantly increased incidence of simple hyperplasia. The metabolite DCPU was found in rat urine at concentrations above the *in vitro* IC50 evaluated in a rat urothelial (MYP3) cell line. Moreover, DCPU induced more alterations of gene expression than the other metabolites in rat urothelial cells. Taken together, these results suggest the urothelial carcinogenic MOA of diuron is metabolism to cytotoxic metabolites, producing urothelial cell degeneration, necrosis and exfoliation, followed by sustained regenerative cell proliferation, leading to hyperplasia and eventually tumors after continued diuron exposure.

RESUMO

Diuron, um herbicida derivado da uréia, foi cancerígeno para a bexiga urinária de ratos Wistar quando administrado via dieta em altas concentrações (2500 ppm), com maior incidência dessa neoplasia em ratos machos. O modo de ação (MOA) cancerígeno proposto envolve citotoxicidade urotelial e necrose, seguido por proliferação celular regenerativa e hiperplasia. Citotoxicidade urotelial pode ser induzida tanto pela presença de sólidos na urina, quanto por toxicidade exercida pelo diuron e/ou seus metabólitos. Para melhor esclarecer o MOA do diuron em ratos Wistar machos, o presente estudo teve como objetivo: 1) Avaliar os efeitos dos sólidos urinários no desenvolvimento das lesões uroteliais; 2) Determinar a temporalidade e sequência das lesões citotóxicidade e proliferativas; 3) Avaliar a citotoxicidade dos principais metabólitos do diuron no urotélio. Ratos tratados com NH_4Cl para acidificação da urina mostraram diminuição do pH e redução na quantidade de cristais e precipitados urinários. Não houve diferença na incidência de lesões uroteliais entre ratos tratados com diuron e ratos tratados com diuron+ NH_4Cl, indicando que a citotoxicidade não ocorre devido a presença de sólidos na urina. Ratos tratados com diuron durante 1, 3 e 7 dias mostraram edema celular sob microscopia de varredura, iniciado logo após o primeiro dia de tratamento. Sob microscopia eletrônica de transmissão, no dia 7 as células uroteliais mostraram degeneração vacuolar e distensão do citoplasma, organelas e núcleo, características de citólise. No dia 28, as bexigas do grupo tratado com diuron mostraram extensas áreas de necrose, exfoliação e empilhamento celular sugestivo de hiperplasia. Após 8 semanas de tratamento, as bexigas mostraram necrose, exfoliação e aumento significativo na incidência de hiperplasias. O metabólito DCPU foi encontrado na urina de ratos tratados em concentrações maiores do que a IC50 verificada *in vitro* com células uroteliais (MYP3) de rato. Além disso, DCPU induziu maiores alterações na expressão gênica de células uroteliais do que os outros metabólitos testados. Nossos resultados sugerem que MOA cancerígeno do diuron ocorre por meio de citotoxidade causada por seus metabólitos, principalmente DCPU, resultando em degeneração, necrose e exfoliação celular, seguido de proliferação celular regenerativa, levando a hiperplasia e eventualmente a tumores após exposição contínua a esse herbicida.

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ORIGINAL RESEARCH ARTICLE

Diuron-Induced Rat Urinary Bladder Tumors: Mode of Action and Human Relevance Evaluation

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ABSTRACT

Diuron, a substituted urea herbicide, was classified as known/likely human carcinogen due to higher incidences of urinary bladder urothelial carcinomas and kidney pelvis papillomas and carcinomas in rats exposed to high doses of diuron (2500 ppm) in a two years bioassay. Diuron is registered for both occupational and residential uses and it's used worldwide for more than 30 different crops. The proposed rat urothelial Mode of Action (MOA) for this herbicide consists of metabolic activation to metabolites that produce cytotoxicity leading to urothelial necrosis and cell exfoliation, regenerative hyperplasia and eventually tumors. In this paper, we describe evidence for this MOA for diuron using the International Programme on Chemical Safety (IPCS) Conceptual Framework for Evaluating a Mode of Action for Chemical Carcinogens and the USEPA and IPCS framework for assessing human relevance.

Key events: Diuron, metabolites, cytotoxicity, proliferation, urothelium, IPCS framework, risk assessment.

INTRODUCTION

During the past 10 years, Brazil's pesticide market grew 190% while the world market grew 93%. Accordingly, in 2008 Brazil reached the highest rank in pesticide sales in the world (Brazilian Institute of Environment - IBAMA, 2010). Herbicides are the most used pesticide type and diuron (3-[3,4-dichlorophenyl]-1,1-dimethylurea), an urea derived herbicide, is the 5th highest-selling in the Brazilian market.

Diuron is registered for both occupational and residential uses. It is used worldwide as both a pre- and post-emergent herbicide to control a wide variety of annual and perennial broadleaf and grassy weeds in crop and non-crop areas (Iyer, 2002; USEPA, 2005). Diuron is used in agricultural areas for more than 30 different crops, including citrus, berries, asparagus, pineapple, cotton, soy bean, sugar cane and others, and in non-agricultural sites such as rights-of-way, commercial and industrial areas. It is also used on ornamental trees, flowers, shrubs, paints and coating, ornamental fish ponds and commercial fish production (APVMA, 2011; USEPA, 2003). Residential exposures are the result of use of diuron as an algaecide in ponds and aquariums and as a mildewcide in paints (USEPA, 2003).

Diuron is applied either by ground or air equipment, and it is broken down in the environment both by biotic and abiotic processes. Its metabolites can be found in the environment, including soil, water, sediments, and groundwater (Giacomazzi and Cochet, 2004; Iyer, 2002). In plants, diuron acts by inhibiting photosynthesis through the Hill reaction, in which the transfer of electrons from water to the electron acceptor is inhibited preventing the formation of ATP and NADPH (APVMA, 2011).

Diuron has low acute toxicity by oral, dermal and inhalation exposure routes (USEPA, 2003). The primary targets of diuron toxicity are the hematopoietic system, as evidenced by erythrocyte damage in rats resulting in hemolytic anemia and

compensatory hematopoiesis, and the urinary bladder (and kidney pelvis), as evidenced by increased incidences of urothelial neoplasms (USEPA, 2003).

In a two year bioassay, rats exposed through the diet to high doses (2500 ppm) of diuron showed urinary bladder carcinomas in both sexes of Wistar rats (males 35/48 and females 13/49), one kidney pelvis papilloma and two carcinomas, and an equivocal increase in the incidence of mammary gland carcinomas in the female NMRI mouse (6/50) (Table 1). Based on these findings diuron was characterized as a "known/likely" human carcinogen by the USEPA in 1996 (USEPA, 2003).

Regarding the mouse mammary gland carcinomas found in the two year bioassay, they were considered not treatment related since the incidence was within the range of the historical controls from the same test facility and other laboratories (APVMA, 2011). Furthermore, in a two-stage carcinogenesis bioassay, female Swiss mice treated with diuron for 13 weeks did not show increased cell proliferation, decreased apoptosis or an increase in the incidence of hyperplastic lesions or neoplasms in the DMBA/BBN initiated groups, suggesting that diuron is not a mammary gland promoting agent (de Moura *et al.*, 2010). In addition, diuron did not show promoting potential in mammary carcinogenesis in female Sprague-Dawley rats initiated with DMBA (Grassi *et al.*, 2011).

In a two-stage mouse skin carcinogenesis assay, the initiating and promoting potentials of diuron were evaluated using twenty-three and twenty-one week protocols (Ferrucio *et al.*, 2010). These studies aimed to re-evaluate the experiment of Antony *et al*. (1989), an initiation-promotion study where topical applications of diuron followed by 12-O-tetradecanoylphorbol 13-acetate (TPA) induced skin papillomas in Swiss albino female mice. However, in the Antony *et al.* (1989) study, no initiating potential was demonstrated when diuron was applied at a single 250 mg/kg b.w. dose, which

could indicate a possible threshold, inconsistent with a supposed initiating potential. Furthermore, diuron dose levels evaluated were not justified in the Antony *et al*. (1989) study, and the levels used were extremely high adding to the questionable relevance of their study (Ferrucio *et al.,* 2010).

The Ferrucio *et al*. (2010) study was especially important as occupational dermal exposure represents a concern about diuron, and the study by Antony *et al.* (1989) suggested a carcinogenic potential of diuron on the skin. In the two protocols developed by Ferrucio *et al* (2010), the first used dimethylsulfoxide (DMSO) as a solvent for the herbicide and the second used acetone as an alternative solvent to determine whether DMSO had an inhibitory influence on potential cutaneous carcinogenic activity. Diuron did not exert either initiating or promoting potential in mouse skin (Ferrucio et al., 2010).

In *in vivo* mid-term studies (15, 25 and 30 weeks), rats had hyperplasia in the urinary bladder and kidney pelvis after high dose (2500 ppm) treatment with diuron (Nascimento *et al.,* 2006; Da Rocha *et al.,* 2010; Ihlaseh *et al.,* 2011*)* which correlates with the neoplastic lesion development in the two year study. Diuron carcinogenicity in two year bioassays is related to the rat urinary bladder and kidney pelvis tumors, and different studies have demonstrated that the urothelium is the target tissue for diuron. Therefore, these observations indicate the need to identify the mode of action (MOA) of this herbicide and the relevance of the rat urothelial tumors for an appropriate human risk assessment.

After treatment for 20 weeks using 2500 ppm, male Wistar rats had significantly increased incidences of bladder and kidney pelvis hyperplasia by light microscopy compared to control (Nascimento *et al.,* 2006; Cardoso *et al., submitted*). Increased incidences of necrosis detected by scanning electron microscopy (SEM) and increased

cell proliferation detected by PCNA labeling index were also present in the urinary bladder suggesting that cytotoxicity, cell death and consequent regenerative hyperplasia, but not direct mitogenesis, is the carcinogenic MOA of this herbicide (Nascimento *et al.*, 2006). Diuron itself is not excreted to a significant extent in the urine, so metabolism to cytotoxic metabolites excreted in the urine must first take place.

Our group conducted different assays to better understand and evaluate the effects of diuron on the urothelium of Wistar rats exposed through the diet and especially to evaluate its MOA (Nascimento *et al.,* 2006; Da Rocha *et al.,* 2010; Ihlaseh *et al.,* 2011; Da Rocha *et al.,* 2012; Cardoso *et al., submitted*). We provide extensive evidence for a carcinogenic MOA that consists of metabolic activation to metabolites that produce cytotoxicity leading to urothelial necrosis and cell exfoliation, regenerative hyperplasia and eventually tumors (Figure 1). In this paper, we describe evidence for this MOA for diuron using the International Programme on Chemical Safety (IPCS) Conceptual Framework for Evaluating a Mode of Action for Chemical Carcinogens (Sonich-Mullin, C. *et al.,* 2001) and the USEPA and IPCS framework for assessing human relevance (Meek *et al.,* 2003; Seed *et al.,* 2005; Boobis *et al.,* 2006; 2008).

1) HYPOTHESIZED MODE OF ACTION (MOA)

The hypothesized MOA for the urothelial carcinogenicity of diuron at high doses involves metabolic activation to reactive metabolites, mainly DCPU, which are excreted and concentrated in the urine, leading to cytotoxicity, starting with urothelial cell degeneration, necrosis and cell exfoliation, followed by sustained regenerative cell proliferation, leading to hyperplasia and eventually tumors.

2) KEY EVENTS

2.1) Absorption, distribution, metabolism and excretion

Diuron is absorbed from the gastrointestinal system and excreted in urine and feces. Oral and intravenous administration showed comparable amounts of radioactivity excreted in the urine, indicating that diuron is completely absorbed after oral administration (APVMA, 2011). Most of the test substance was metabolized within 24 hours and the major excretion occurred in the urine (80-91%). Only small amounts of diuron were detected in feces (0.1-1.6%) indicating extensive degradation/biotransformation of the parent compound (APVMA, 2011).

In Sprague-Dawley rats, N-(3,4-dichlorophenyl) urea (DCPU) and 4,5-dichloro-2-hydroxyphenyl urea (2-OH-DCPU) were the predominant urinary metabolites; lesser metabolites included N-(3,4-dichlorophenyl)-3-methylurea (DCPMU) and trace levels of 3,4-dicloroaniline (DCA). A total of eight metabolites were found in rat urine and four in feces (Figure 2) (APVMA, 2011). DCPU was the major metabolite followed by 2-OH-DCPU in the urine of Wistar rats treated with diuron at high doses (Da Rocha *et al.*, *submitted*). In sub-acute inhalation studies, DCPU was the main urinary metabolite in diuron-treated male and female Wistar rats, corroborating findings in the two oral studies described above. DCPMU, DCA and small amounts of diuron were also detected (APVMA, 2011).

The highest diuron levels are generally found in blood, liver and kidneys after oral doses (APVMA, 2011). Using single or multiple doses of diuron (10 mg/kg body weight), blood levels (male and females) were between 0.13 and 0.29 ppm, kidney levels were between 0.14 and 0.30 ppm, and liver levels were between 0.13 and 0.18 ppm. After single administration of 400 mg/kg bw, blood level was 8.93 and 8.24 ppm, kidneys were oral 7.12 and 8.94 ppm, and liver was 5.57 and 6.80 ppm in males and females, respectively. No apparent tissue accumulation was observed (APVMA, 2011;

Hodge *et al*, 1967). However, a different study suggested low levels of bioaccumulation after oral doses, with the highest residue levels found in erythrocytes, followed by kidney, liver, spleen, adrenal gland and lungs (APVMA, 2011).

Kinoshita and Dubois (1970) showed that diuron is able to cause induction of hepatic microsomal enzymes, with males showing more susceptibility than females after oral administration in the diet (100, 250, 500, 1000 and 2000 ppm) for 13 weeks. In another study, repeated diuron exposures decreased the plasma half-life of antipyrine indicating that hepatic cytochrome P450 isozymes were also induced (Liu, 2001).

In humans, DCPMU and DCPU were found in the urine in a case of product abuse (Boven *et al.,* 1990). It has been suggested that the metabolic scheme for humans involves cytochrome P450s (Abass *et al*., 2007) (Figure 3). Diuron also showed relatively potent inhibition of CYP1A1/2 (Abass *et al*., 2007).

2.2) Cytotoxicity

There is considerable evidence demonstrating urothelial cytotoxicity in rodents administered diuron (Table 2), especially regarding bladder cytotoxicity in male Wistar rats, the same species and strain of animals used in the two years bioassay that developed a carcinogenic response at 2500 ppm (Table 2) (USEPA, 2003). No evidence of carcinogenicity or preneoplastic changes was found in dogs after two years dietary administration at 0, 125, 125, 250 and 2500 ppm diuron concentrations (Hodge *et al*., 1967). In the same study, a two year bioassay with rats was also conducted with the same diuron exposures doses (0, 25, 125, 250 and 2500 ppm) mixed with Purina diet. Surprisingly, no evidence of carcinogenicity was detected in any tissue. However, the study colony suffered from a severe epidemic of pneumonitis-peritonitis resulting in high mortality rates. The parasite *Trichosomoides crassicuda*, known to produce

urothelial hyperplasia (Antonakopoulos *et al*., 1991), was also detected in the bladder of several rats (Hodge *et al.*, 1967). Thus, this study was not of acceptable quality and should not be considered for regulatory proposes (APVMA, 2005).

In a short-term study, the earliest urothelial changes were observed by SEM after dietary exposure for 1 day to 2500 ppm diuron, as evidenced by the presence of swollen cells protruding into the lumen of the bladder epithelium (Figure 1A). The swollen cells increased in incidence and in number by study day 3 and persisted through study day 7 (Da Rocha *et al.,* 2012).

TEM analysis of these swollen cells on study day 7 showed cytoplasmic and nuclear swelling typical of degeneration, proceeding to necrosis and, in the most affected areas, cell loss in some foci. Minimal changes comprised distention of the cytoplasm, organelles, and nuclei characteristic of cytolysis which resulted in enlargement of the superficial cells and protrusion of the superficial cells into the lumen, corresponding to the swelling of the cells observed by SEM. Occasionally, there was also loss of the luminal cytoplasmic membrane and loss of ultrastructural detail of the cytoplasmic organelles, representing severe degenerative changes and cell death (Da Rocha *et al.*, 2012).

SEM analysis also showed cytotoxic alterations of necrosis and exfoliation in the bladder epithelium at different time points in various studies. SEM bladder classification used by Nascimento *et al*. (2006), Da Rocha *et al*. (2010/2012) and Ihlaseh *et al*. (2011) followed the methodology of Cohen *et al.*, (1990). Briefly, class 1 bladders have flat, polygonal superficial urothelial cells; class 2 bladders have occasional small foci of superficial urothelial cell death; class 3 bladders have numerous small foci of superficial urothelial cell death; class 4 bladders have extensive superficial urothelial cell death, especially in the dome of the bladder; and class 5 bladders have

extensive cell death and piling up (hyperplasia) of rounded urothelial cells. Normal rodent urinary bladders are usually class 1 or 2, or occasionally class 3.

SEM classes considered altered (4 and 5) were present on day 28 of diuron exposure (3/10). No bladder in the control groups was classified as classes 4 or 5. After 8 weeks diuron exposure, cytotoxicity was present in 5/10 bladders as evidenced by altered classes in the SEM evaluation (Da Rocha, *et al.*, 2012).

In studies with a longer duration of diuron exposure, the presence of an altered epithelium detected by SEM in bladders of rats treated with 2500 ppm was a common finding. After 15, 20 and 30 weeks 4/5, 3/5 and 5/6 bladders, respectively, showed an increase in necrosis, exfoliation and hyperplasia (Nascimento *et al.*, 2006; Da Rocha *et al.*, 2010) (Table 2).

Cytotoxicity was evident not only in rats administered diuron *in vivo*, but also in *in vitro* in assays utilizing an immortalized urothelial cell line (MYP3). Diuron is extensively metabolized and the metabolites excreted in the urine are the main chemicals in contact with the urothelium after diuron exposure. Alterations of urinary pH and the presence of urinary solids were ruled out as the cause of diuron cytotoxicity, so the possibility remained that diuron itself and/or its metabolites were the predominant toxicants to the urothelium (Nascimento *et al.*, 2006; Da Rocha *et al.*, 2010). Metabolite cytotoxicity was evaluated in MYP3 cells, a non-tumorigenic urothelial cell line derived from a small nodule that developed in a heterotopically transplanted rat urinary bladder after treatment with N-methyl-N-nitrosourea (MNU) (Kawamata *et al.*, 1993). The MYP3 cells were exposed for 72 hours to diuron metabolites DCPU, OH-DCPU, DCPMU and DCA. The IC_{50} for DCPU, 2-OH-DCPU, DCPMU and DCA was 185, 230, 104 and 213 µM, respectively. Diuron could not be evaluated as it has low solubility in water (42mg/L), and it precipitated after a few

minutes in solution or the solvent concentrations that kept diuron in solution were too high and cytotoxic to the cells. (Da Rocha *et al., submitted*). Quantitation of metabolites in the urine from rats treated with diuron showed that N-(3,4-dichlorophenyl) urea (DCPU) was present in rat urine at concentrations higher than the IC_{50} in *in vitro* cytotoxicity testing in a rat urothelial cell line, suggesting that DCPU is the primary metabolite responsible for the rat urothelial cytotoxicity (Da Rocha *et al., submitted*). Furthermore, genomic analysis of the MYP3 cells treated with metabolites demonstrated that DCPU induced the highest number of altered probe sets of all metabolites tested compared to control (Da Rocha *et al., submitted*).

At the genomic level, Ihlaseh *et al*. (2011) evaluated urothelial toxicity with gene expression analysis using microarray gene chips. Male Wistar rats exposed through the diet to different doses of diuron for 20 weeks showed various changes at the high doses (1250 and 2500 ppm) such as oxidative stress, increased cellular metabolism and enhanced cell death. Taken together, these studies provide strong evidence for cytotoxicity in the urothelium of rats exposed to diuron.

2.3) Regenerative cell proliferation

Different studies showed that oral administration of diuron at 2500 ppm to male rats increased the incidences of urothelial proliferative changes. Bladder simple hyperplasia was detected by light microscopy as early as 7 days after the start of diuron exposure (1/10) and after 28 days (2/10). After 8 weeks, a significant difference in bladder hyperplasia incidences was detected in the treated rats (6/10) compared to control (0/10) (Da Rocha *et al.,* 2012). Hyperplasia was a common finding after longer diuron exposures: 8/10 bladders at 15 weeks (Da Rocha *et al*. 2010), 7/10 bladders at 20 weeks (Nascimento *et al.,* 2006) and 9/10 bladders at 25 and 30 weeks (Da Rocha *et al*, 2010).

In another study, after exposures to 2500 ppm of diuron for 2, 4, 12 and 26 weeks, bladder hyperplasia was also observed by light microscopy (APVMA, 2011). In the two year study, at a 12 month interim sacrifice, hyperplasia was observed in the bladder of 10 of 10 Wistar rats treated with 2500 ppm diuron in the diet. No neoplasms were detected at this time point. By the end of the two year bioassay, male Wistar rats showed a high incidence of bladder carcinomas (35/48) (APVMA, 2011).

Reversibility studies were conducted to evaluate the proliferative lesions after the stimulus was removed. Male Wistar rats that received 2500 ppm diuron for 15 weeks followed by basal diet for 15 weeks showed decreased incidences of bladder hyperplasia (3/10) compared to diuron-fed rats for 15 weeks (8/10) and 30 weeks (9/10) (Da Rocha *et al.*, 2010). In another study, no proliferative lesions were observed after 4 weeks diuron exposure followed by 4 weeks of basal diet (7/10) when compared to 10 of 10 bladders with proliferative lesions after 4 weeks of consecutive diuron exposure. In another study, hyperplasia was observed in 7 of 10 bladders after 26 weeks of diuron followed by 8 weeks of basal diet compared to hyperplasia detected in 9 of 9 bladders after 26 weeks diuron exposure (APVMA, 2011) (Table 2).

There was no increase in the BrdU labeling index at early time points (1, 3, 7, 28 days and 8 weeks). However, indications of hyperplasia by SEM and light microscopy at 28 days and 8 weeks suggest that increased cell proliferation did occur. The labeling index is a rate determined by the number of labeled cells divided by the total number of cells in that population. It reflects the rate of replication, not the actual number. Hyperplasia represents an increase in cell number. Even without an increase in the rate of cell proliferation at early times, the increase in cell number (hyperplasia) indicates that urothelial cell proliferation (number of DNA replications) is increased (Da Rocha *et al.*, 2012). The main parameter is the number of DNA replications that can either be

increased by decreasing the number of cell deaths or, more commonly in epithelial tissues in response to chemical toxicity, increasing the number of cell births (Cohen, 1998). Moreover, previous studies have also shown that the cell proliferation index varies considerably between individual Wistar rats regardless of treatment (Lina, *et al.*, 1993; Nascimento *et al.*,2006; Shiota *et al.*, 1994), which could mask slight diuron-induced alterations in proliferation rates at early time points. After 20 weeks diuron exposure, the bladder epithelium showed increased Proliferating Cell Nuclear Antigen (PCNA) labeling index at 2500 ppm (Nascimento et al., 2006). Increase in bromodeoxyuridine (BrdU) labeling index was also detected with 1250 ppm diuron exposure dose after 20 weeks (Cardoso *et al., submitted*) (Figure 1C).

Bladder proliferative lesions have not only been described in Wistar rats. Although further details were not given about the incidence of the lesions, Grassy *et al*. (2011) reported urothelial hyperplasia in the urinary bladder and kidney pelvis in female Sprague-Dawley rats that received 2500 ppm of diuron in the diet with or without initiation with 7,12-dimethlybenz(a)anthracene (DMBA). In another initiation-promotion study with diuron using DMBA and N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), female Swiss mice showed urinary bladder hyperplasia in the initiated group (7 weeks) followed by treatment with diuron 2500 ppm (12 weeks) in all animals (11/11) and additionally two transitional cell carcinomas were also detected; initiated control groups showed bladder hyperplasia in 8/14. In the non-initiated group, the urothelial hyperplasia was observed in 5 of 15 mice (De Moura *et al*., 2010). Diuron-induced hyperplasia in mice was reported in the two year bioassay but no urinary bladder neoplasms were detected (APVMA, 2011).

Increase incidences of kidney pelvis hyperplasia with urothelial cytotoxicity and proliferation are frequently present in diuron studies. This is not unusual for chemicals

affecting the urinary bladder since the lining epithelium (the urothelium) is the same from the kidney pelvis to the proximal urethra. Male Wistar rats exposed to diuron for 20 weeks at 500 and 2500 ppm showed significant increased incidences of kidney pelvis simple hyperplasia, 8/10 and 6/9, respectively (Nascimento *et al.*, 2006). No instance of kidney pelvis hyperplasia was present in the control group. In another study, rats exposed for 20 weeks showed significant incidences at 500, 1250 and 2500 ppm (8/8, 9/10 and 10/10, respectively) (Figure 1F) (Cardoso *et al., submitted*). Rats exposed to 2500 ppm diuron for 25 and 30 weeks also showed kidney pelvis hyperplasia (7/11 and 10/10).

After 12 months exposure at 2500 ppm diuron, male Wistar rats showed greater incidences of kidney pelvis hyperplasia (10/10) compared to female rats (4/10). However, in this study, 5 of 10 rats in the male control group also showed kidney pelvis hyperplasia. The basal diet used in this study was Altromin he study which changes urinary pH and can alter the urothelium without additional chemical treatment (APVMA, 2005; Cohen, 1998). In this study, the incidence of kidney pelvis hyperplasia tended to increase with time in male rats, and by 24 months 37 of 50 rats in the control group showed kidney pelvis hyperplasia (APVMA, 2011). It is unclear, however, if this represented true kidney pelvis urothelial hyperplasia or hyperplasia of the lining of the renal papilla and fornices associated with chronic progressive nephropathy (CPN), which commonly occurs in rats, especially males. In many laboratories, hyperplasia associated with CPN and true kidney pelvis urothelial hyperplasia are listed together and not distinguished. In other studies, no changes in the kidney pelvis were observed in control groups after 20, 25 or 30 weeks using Nuvilab diet, but and as described previously, diuron produced cytotoxicity and proliferative responses in the kidney pelvis.

By SEM, piling up of cells is indicative of hyperplasia and was detected in several bladders after 15, 20 and 30 weeks exposure to 2500 ppm diuron in the diet (Nascimento *et al.,* 2006; Da Rocha *et al.,* 2010; Cardoso *et al., submitted*). Regarding other species, female mice also showed increased incidences of BrdU labeling in the urinary bladder after treatment with 2500 ppm diuron with and without initiation. No changes in apoptotic indexes were detected (De Moura *et al.,* 2010).

3) DOSE RESPONSE RELATIONSHIPS

In the 2-year bioassay in rats, doses were evaluated at 0, 25, 250 and 2500 ppm (USEPA, 2003; APVMA 2011). As described above, the only carcinogenic dose was 2500 ppm. Based on this study the no observed effect level (NOEL) for carcinogenicity was 250 ppm based on transitional epithelial (urothelial) carcinoma in the urinary bladder and kidneys at 2500 ppm, and the NOEL for urothelial hyperplasia as a potential precursor of neoplasia was 25 ppm (APVMA, 2011). Since urothelial carcinogenesis in rats occurs through a sequence of morphological changes starting with simple hyperplasia, nodular and papillary hyperplasia, papilloma, and ultimately non-invasive and invasive neoplasms (Cohen *et al.,* 1993; Cohen *et al.,* 2002), urothelial hyperplasia can be considered a predisposing condition for urinary bladder carcinogenesis and is directly related to the dose and duration of the carcinogenic stimulus (Cohen, 1998).

To better characterize the dose-response relationship of diuron on the urinary bladder of male Wistar rats, two studies were conducted with doses intermediate to the ones used in the two years bioassay to better define the response between 25 and 250 ppm. Male Wistar rats were treated through the diet (Nuvilab) at 0, 60, 125, 500, 1250, or 2500 ppm diuron for 20 weeks, and urothelial histological and ultrastructural lesions

and cell proliferation (labeling index) were evaluated. Different from other studies using the same diet (Nascimento *et al.*, 2006; Rocha *et al.*, 2010), this study showed a significant reduction ($p < 0.05$) in the mean body weight (up to 20% lower than control group) and body weight gain (up to 30% lower than control group) in the high dose group, 2500 ppm (101.6 mg/kg/day). By light microscopy, the incidence of urinary bladder urothelial simple hyperplasia was significantly increased in the 1250 and 2500 ppm diuron-fed groups (8/10 and 7/10, respectively). By SEM, the 500 ppm and 1250 ppm diuron groups showed significant incidences of urothelial alterations (5/10 and 7/10) (classes 4 and 5) characterized by areas of necrosis and regenerative cell hyperplasia. The 2500 ppm group showed an incidence of 4 of 10 bladders with urothelial alterations detected by SEM (classes 4 and 5). Moreover, cell proliferation evaluated by the BrdU labeling index was increased at the two highest concentrations, but the increase was only significant at 1250 ppm. The authors suggested that the lower incidence of advanced ultrastructural lesions at the high dose, even with the high incidence of urothelial hyperplasia at the 2500 ppm dose by light microscopy, could be related to the systemic toxicity of the highest dose of diuron. In this study, the decreased food and water consumptions along with a 34% reduction in body weight gain in the 2500 ppm group indicates excessive toxicity [close to or above the maximum tolerated dose (MTD)] and may compromise the data generated (OECD, 2002; 2010). Thus, 1250 ppm seems to be as effective as 2500 ppm in inducing urothelial lesions after 20 weeks of oral administration (Cardoso *et al., submitted*)

In the same study, the kidney pelvis of rats exposed to 500, 1250 and 2500 ppm diuron had significantly higher incidences of simple hyperplasia (8/8, 9/10 and 10/10, respectively) compared to the control group (Cardoso *et al., submitted*). However, most of the rats in all groups showed a dilatation of the kidney pelvis (hydronephrosis), a

common finding in Wistar rats (Burton *et al.*, 1979), which increases the susceptibility of the kidney pelvis urothelium to the effects of lower urinary tract carcinogens (Mori *et al.*, 1994).

Taken together, this study demonstrated a consistent dose-response of diuron on the rat urothelium, with a NOEL of 125 ppm, a lowest observed adverse effect level (LOAEL) of 500 ppm and a MTD of 1250 ppm which was as effective as the 2500 ppm dose for induction of bladder lesions (Cardoso *et al., submitted*). Another study using 20 weeks treatment with diuron at 125 ppm, 500 ppm and 2500 ppm also showed a dose response effect in the bladder urothelium with increased incidences of hyperplasia (2/10, 2/10, 7/10, respectively) (Nascimento *et al*., 2006) (Table 2). However, contrary to the Cardoso *et al*. (*submitted*) findings, this study showed that rats treated with 125 and 500 ppm had the same incidences of urothelial hyperplasia by light microscopy (2/10), suggesting 125 ppm diuron as the LOAEL.

Moreover, gene expression in the bladder urothelium of rats from this same study (Cardoso *et al., submitted*) was analyzed by Affymetrix microarrays after exposure to 0, 60, 125, 1250, and 2500 ppm diuron. Correlating with the histological response, the urothelial gene expression profile also exhibited a dose-response effect. The number of significantly modulated genes increased progressively with the dietary concentration of the herbicide. In the 60, 125, 1250, and 2500 ppm diuron-exposed groups, the number of differentially expressed transcripts was 257, 291, 532, and 997, respectively. The intragroup similarities and the intergroup differences in the Principal Component Analysis (PCA), Venn diagrams and pathway level analyses demonstrated that the doses tested could be combined into two larger groups that maintain clear differences in gene expression: a low-dose group (60 and 125 ppm) and a high-dose group (1250 and 2500 ppm). In addition, the gene expression analyses demonstrate that 1250 and 2500

ppm diuron exposure has the same effect on the urothelium (Ihlaseh *et al.*, 2011). The 1250 ppm diuron dose was not evaluated in the two year carcinogenic study (APVMA, 2011). Ihlaseh *et al*. (2011) suggested 125 ppm as the NOEL for histological and transcriptional changes.

4) TEMPORAL ASSOCIATION

After oral administration, diuron is rapidly absorbed from the gastrointestinal system and metabolized within 24 hours. After 1 day exposure, continued at 3 and 7 days, rat bladder urothelium showed by SEM swollen cells typical of degeneration, also demonstrated by TEM after 7 days. After 28 days diuron exposure, bladders showed extensive necrosis and exfoliation resulting in altered SEM classes (Da Rocha *et al.*, 2012). After 2 and 4 weeks diuron administration at 2500 ppm diuron, rats showed bladder simple hyperplasia by light microscopy (3/10 and 10/10, respectively) (APVMA,2011). After 8 weeks the incidences in male Wistar rats exposed to the same dose were 6/10 (Da Rocha *et al*., 2012). With 12 months exposure, microscopic evaluation by light microcopy showed 10/10 rat bladder with hyperplasia and increase in the severity of the bladder lesions (APVMA, 2011). After 2 years, urinary bladders exposed to 2500 ppm diuron showed an increased incidence of urinary bladder neoplasms, mostly transitional epithelial (urothelial) carcinomas. One papilloma and two carcinomas in the kidney pelvis were also considered treatment related. Taken together, all these studies indicate that diuron urothelial lesions increase in incidence and severity with time of exposure (Table 2).

5) STRENGTH, CONSISTENCY, SPECIFICITY OF THE ASSOCIATION BETWEEN DIURON AND UROTHELIAL ALTERATIONS

Urothelial changes after diuron exposure have been repeatedly demonstrated in different studies from multiple laboratories. In a two year bioassay, rats exposed to diuron in Altromin diet showed increased numbers of urothelial hyperplasia in the bladder after one year exposure and bladder carcinomas after two years (APVMA, 2011). Another study showed that 2500 ppm diuron mixed with Altromin diet induced bladder simple hyperplasia at different time points, starting after two weeks (APVMA, 2011). Both studies were performed at the Bayer Toxicology Institute and followed the principles of Good Laboratory Practices(GLP).

In other studies conducted at São Paulo State University (Botucatu, Brazil) (TOXICAM), male Wistar rats exposed to diuron at different concentrations in Nuvilab diet also showed bladder and kidney pelvis changes using different evaluations including light microscopy and SEM PCNA and BrdU labeling indexes, and gene expression analyses (Nascimento *et al.*, 2006; Da Rocha *et al.*, 2010; Cardoso *et al.*, submitted; Ihlaseh *et al.*, 2011).

Recently, another study from the University of Nebraska Medical Center (UNMC) showed bladder urothelial alterations including degeneration, cytotoxicity and hyperplasia by light microscopy, SEM and TEM in male Wistar rats treated with 2500 ppm diuron mixed with Purina diet (Da Rocha *et al.*, 2012).

Even with differences in diets, which may cause different urinary pH and different grades of responses in the urothelium, various studies using different techniques and conducted in multiple laboratories showed that the urothelium is the target tissue of diuron in rats after high doses exposure and showed changes consistent with the proposed MOA.

6) BIOLOGICAL PLAUSIBILITY AND COHERENCE

Different modes of actions have been identified for bladder carcinogens. Basically, bladder carcinogens can be divided into DNA reactive and non-DNA reactive carcinogens (Cohen, 1998). Based on the weight of evidence, diuron has been considered a non-DNA reactive carcinogen (APVMA, 2005; Iyer, 2002; USEPA, 2003).

For non-genotoxic carcinogens there is an increase in the number of cell replications, which can occur either by increasing cell births or decreasing cell deaths. An increase in cell births can occur through direct mitogenic stimulation or toxicity and regeneration (Cohen, 1998) (Figure 4). Most of the urothelial non-genotoxic chemicals produce tumors through toxicity and regeneration. Just one chemical so far, propoxur, is described in the literature as causing direct mitogenesis (Cohen, 1998; Cohen *et al.*, 2007).

Based on evidence of cytotoxicity and an increase in cell proliferation at different time points in rats exposed to diuron, the hypothesized MOA is toxicity and regeneration and is a common MOA for chemically induced urothelial tumors in multiple species. Furthermore, cytotoxicity and regenerative proliferation is a common MOA for chemically induced tumors for a variety of chemicals involving tumors of several different target tissues, such as chloroform-induced tumors of the liver and kidney (Golden *et al.*, 1997; Andersen et al., 2000; Meek et al., 2003).

Inorganic arsenic is a well-known carcinogen in humans, inducing different types of carcinomas, including urinary bladder carcinomas (NRC, 1999). Dimethylarsenic acid (DMA^V), a urinary metabolite of inorganic arsenic, is carcinogenic to the rat urinary bladder, but not to mice. DMA^V has been shown to increase rat urinary bladder carcinomas after two years at high doses of dietary administration, with a greater response in females (Arnold *et al.*, 2006). In addition, it does not produce changes in

urinary composition, does not produce urinary solids and causes urothelial necrosis followed by regenerative hyperplasia. The carcinogenic mode of action involves reduction of DMA^{V} to the reactive trivalent metabolite (DMA^{III}), causing urothelial cytotoxicity followed by regenerative cell proliferation, leading to tumor induction (Cohen *et al.,* 2006).

In a 2-year bioassay, another chemical, pulegone, slightly increased the incidence of urinary bladder tumors in female F344 rats after oral administration (NTP, 2011). Pulegone, a monoterpene ketone, is used as a flavoring agent in foods, drinks and dental products (NTP, 2011). A study conducted in female F344 rats for 4 and 6 weeks with oral administration of pulegone at different doses showed superficial necrosis and exfoliation detected by SEM and increased BrdU labeling index in the high dose group. *In vitro* studies indicated that pulegone and its metabolites, especially piperitenone, are excreted and concentrated in the urine at cytotoxic levels when pulegone is administered at high doses to female rats. Thus, the hypothesized MOA for pulegone-induced tumorigenicity in female rats involves urothelial cytotoxicity followed by regenerative cell proliferation, ultimately leading to tumors (Da Rocha *et al.,* 2012).

Other chemicals, such as tributyl phosphate (TBP) and transfluthrin, a pyrethoid insecticide, also produce urothelial neoplasms in rats through cytotoxicity and regenerative cell proliferation. Both induced urinary bladder tumors in rats but not in mice and are non-genotoxic chemicals (Arnold *et al.*, 1997; Yokohira *et al.,* 2011). TBP produces ulceration and hemorrhage into the bladder lumen and consequent diffuse papillary and nodular hyperplasia. Transfluthrin produces superficial cytotoxicity and necrosis in the urothelium.

Urothelial toxicity followed by cell proliferation can be produced by three different mechanisms: (1) production of urinary solids (precipitate, crystals, and/or calculi); (2)

extreme alterations in urinary composition, such as extremes of volume, pH or urinary dilution; or (3) production of reactive metabolites (Cohen, *et al.,* 2007) (Figure 4).

Production of urinary solids is another common MOA for nongenotoxic bladder carcinogens in rodents, as demonstrated with sodium saccharin-induced precipitate formation that leads to abrasion of the urothelium and consequent cytotoxicity (Cohen *et al.,* 2000). Changes in the urinary solids and urinary composition were excluded as the cause for the cytotoxicity produced by diuron oral administration (Da Rocha *et al.,* 2010). *In vitro* studies also showed that the diuron metabolite DCPU was concentrated in the urine above the *in vitro* IC_{50} for a rat urothelial cell line (MYP3). Thus, the urothelial carcinogenic MOA involving cytotoxicity and regenerative cell proliferation as described above is common, and it is a plausible biological response in rats exposed to diuron at high doses.

7) ALTERNATIVE MODES OF ACTION

A genotoxic MOA always needs to be considered in chemical carcinogenicity evaluations. Many *in vitro* and *in vivo* studies were performed with diuron at different doses to evaluate a DNA reactive response.

In reverse mutation assays in bacteria, including the Ames assay, diuron was not found to be mutagenic to TA97, TA98, TA100 and TA1535 strains of Salmonella typhimurium either with (+S9: 10, 25, 100 and 250 µg/plate) or without (-S9: 0.5, 1, 2.5, 5 and 10 µg/plate) metabolic activation (Iyer *et al.,* 2002). In a summary of genotoxicity studies published by APVMA (2011), a total of 11 assays encompassing different strains of *S. typhimurium* and *E. coli* exposed at different concentrations of diuron with or without metabolic activation did not show evidence of mutagenicity. Mutagenesis at the HLGPRT locus in CHO cells also showed a negative response. In chromosomal

aberration and sister chromatid exchange tests, diuron was also negative. (APVMA, 2011).

Diuron showed a positive response in an *in vitro* cytogenetic study with human lymphocytes at 500 and 1000 ug/ml with and without metabolic activation. However, in many other *in vivo* assays [cytogenetic assay with mouse (NMRI) germ cells, chromosomal aberration assays and sister chromatid exchange assay in Chinese hamster bone marrow, and in micronucleous tests], diuron showed negative responses. In another positive study, a significantly higher aneuploidy level was observed in diuron treated oysters, and this alteration was also observed in the next generation (Bouilly *et al.,* 2007). In addition, diuron showed equivocal genotoxicity in a Mutatox test (Canna-Michaelou and Nicolau, 1996).

In studies conducted by our group using the standard alkaline version of the single-cell gel (comet) assay, urinary bladder cells and peripheral blood leukocytes from male Wistar rats exposed to different concentrations of diuron demonstrated an absence of genotoxicity (Nascimento *et al.,* 2006). In another study, a modified version of the comet assay developed *in vitro* with Chinese hamster ovary cells indicated that diuron also does not induce DNA cross-links (Da Rocha *et al.,* 2010).

In the literature, there is little information available concerning the genotoxicity of diuron metabolites. The most studied diuron metabolite is 3,4-dichloroaniline (3,4-DCA), and the risk assessment report from the European Union (2006) concluded that: "Although *in vitro* genotoxicity tests were negative for gene and chromosome mutations, there is limited evidence for a mutagenic potential mainly due to a weakly positive SCE test *in vitro* and a positive test for induction of spindle damage *in vitro*. The clearly negative *in vivo* micronucleus tests indicate that this potential is unlikely to be expressed *in vivo*." In addition, DCA was detected at very low levels in rat and

human urine after exposures to diuron (35 µM and 12 µM, respectively) (Da Rocha, *et al, submitted;* Boven *et al*, 1990). Taken together, most of the available genotoxicity data strongly indicate diuron is a non-DNA reactive, nongenotoxic chemical (APVMA, 2011; USEPA, 2003).

Another mode of action that was evaluated is the possibility of a mitogenic effect. As an example, propoxur showed no evidence of toxicity in the bladder epithelium including by SEM evaluation (Cohen, 1998). However, this compound generated an increase in proliferation and increased BrdU labeling index (Cohen, 1998). Although propoxur is the only known bladder carcinogen to cause a mitogenic response, this possibility was evaluated with diuron and was excluded in a time course study with 2500 ppm due to the presence of bladder cytotoxicity detected by SEM as early as 1 day exposure and confirmed by TEM at 7 days. These findings demonstrate cytotoxicity as the first step in the key events of the diuron MOA (Da Rocha *et al.,* 2012).

Bladder cytotoxicity for nongenotoxic compounds can be induced either by urinary solids, extremes in urinary composition (such as pH > 9), or by chemical toxicity (parent compound and/or its metabolites) in the urine (Cohen, 1998). Urinary pH of 6.5 or greater is required for the formation of calcium-containing crystals. Thus, treatment that leads to urine acidification inhibits the formation of the crystals and consequently the formation of tumors. To evaluate a possible role for urinary solids in the development of diuron-induced urothelial lesions, rats were fed with diuron (2500 ppm) either with or without NH_4Cl to acidify the urine. Urinary acidification did not reduce the development of urinary bladder proliferative lesions (hyperplasia) induced by high concentration of diuron, despite decreased amounts of crystals and precipitates in the urine. Thus, diuron cytotoxicity cannot be explained by the presence of urinary solids (Da Rocha *et al.,* 2010).

In the two year bioassay where rats showed a positive carcinogenic response to diuron exposure, the basal diet used was Altromin 1321. Altromin 1321 is known to induce alkaline urine in rats (APVMA, 2005). It was suspected that increased alkaline pH could have enhanced the diuron carcinogenic effect observed after the two year dietary exposure (APVMA, 2011). Urinary pH can influence urothelial cell physiology in multiple ways, such as altering the degree of ionization of xenobiotics and their metabolites, varying the degree of hydrolysis of urinary metabolites and the structure or the charges of urinary compounds, altering the solubility of various chemicals and salts, modifying the degree of affinity of urinary components with macromolecules and possibly altering urothelial cell surface receptors (Clayson *et al.,* 1995; Cohen, 1995). When studies using different diets are compared, male Wistar rats fed with 2500 ppm diuron mixed with Altromin 1321 diet showed higher incidences of bladder urothelial hyperplasia at 4 weeks (10/10) then rats fed with 2500 ppm diuron mixed with Purina diet for 4 weeks (2/10) and 8 weeks (6/10) or mixed with Nuvilab diet for 15 weeks (8/10). Therefore, alkalization of the urine enhanced the urothelial response to diuron. However, although there was no change in urinary pH in rats treated with diuron mixed in Purina or Nuvilab diets, urothelial hyperplasia was observed in the diuron exposed groups, indicating that continued exposure to diuron could lead to urothelial lesions irrespective of the urinary pH (Nascimento *et al.*, 2006, Da Rocha *et al.,* 2010; Ihlaseh *et al.,* 2011).

8) UNCERTAINTIES, INCONSISTENCES AND DATA GAPS

In the two year study, rats were treated with diuron mixed with Altromin diet which alkalinizes urinary pH, and increased incidences of bladder carcinomas were detected in male and female rats at 2500 ppm. Another study (APVMA, 2011) with

2500 ppm diuron in Altromin diet showed a stronger response at 4 weeks (hyperplasia in 10/10 rats) compared to 4 weeks (2/10) and 8 weeks (6/10) in rats treated with diuron in Purina diet (Da Rocha *et al*., 2012) which does not alkalinize the urine. In the two year bioassay, males showed higher incidences of bladder carcinoma incidences than females; no other studies have been conducted to verify if the sex differences are consistent when other diets are used. However, other studies with diets that do not alkalinize the urinary pH also showed significant changes with significant increases of urothelial damage after diuron exposure (Table 2).

Limited information is available concerning the various biologic activities of diuron metabolites. With mitogenicity and changes in urinary composition and urinary solids excluded as possible MOAs (Nascimento *et al.*, 2006; Da Rocha *et al.*, 2010; Da Rocha *et al.*, 2012), the urothelial effects of the metabolites are an important consideration. The most studied urinary diuron metabolite is DCA. However, DCA is present in the urine at negligible levels. No information about the toxicity or genotoxicity of the other metabolites such as DCPU and DCPMU are available. Also, metabolism studies and metabolite quantitation in mouse urine are not available in the literature; a quantitative comparison between rat and mouse urine could explain the species differences in bladder response to diuron. Another interesting but not fundamental piece of information is additional evaluation of the kidney pelvis cytotoxicity and labeling index. The detailed mechanism of cytotoxicity induction by diuron remains unclear.

Nevertheless, there is sufficient data available to demonstrate that the MOA for diuron-induced rat bladder tumors is known and consists of the key events of metabolic activation, excretion and concentration of the active metabolites in the urine, cytotoxicity with necrosis and exfoliation and consequent regenerative cell proliferation.

Furthermore, other MOAs, such as genotoxicity, formation of urinary solids and direct mitogenesis have been excluded.

9) ASSESMENT OF POSTULATED MODE OF ACTION

In summary, several studies conducted in different laboratories provide strong evidence for the sequence of key events of the hypothesized MOA that consists of metabolic activation, cytotoxicity, urothelial necrosis, and cell exfoliation, regenerative hyperplasia and tumors. The evidence provides for a strong degree of confidence in this well-known, biologically plausible MOA.

10) HUMAN RELEVANCE: QUALITATIVE EVALUATION

After oral administration, Sprague Dawley rats that received [^{14}C]-diuron showed complete and rapid absorption. Diuron was excreted as metabolites after 24 hours dosing. The majority of excretion was through the urine (80-91%) and the parent compound was present only in small amounts in the urine (amount) and in the feces (0.1-1.6%) (APVMA, 2011). Radioactive residues in rat tissue were low after 96 hours administration. The highest residue levels were generally found in blood, liver and kidneys. No evidence of tissue accumulation was found after repeat dosing. In another study, enterohepatic circulation was evident using the biliary fistula technique (APVMA, 2005; 2011).

In rats, diuron biotransformation involves ring hydroxylation, N-oxidation, demethylation, dechlorination, and glucuronide conjugation. After oral doses with [^{14}C]-diuron, a total of eight metabolites were found in urine. DCPU followed by OH-DCPU were the predominant metabolites; lesser metabolites included DCPMU. No unchanged diuron or DCA was found in this study (APVMA, 2011). Rats treated with

diuron at 2500 ppm showed DCPU and OH-DCPU in urine as the main metabolites, corroborating previous study findings. Very low levels of DCA, DCPMU and diuron (which may have represented contamination from the diet) were also detected (Da Rocha *et al., submitted*). In rats exposed by aerosol, the predominant metabolite in urine was DCPU; other metabolites included DCPMU, DCA and unchanged diuron (APVMA, 2011). Diuron and its metabolites are almost completely eliminated, mainly by the renal route. In all rat studies, the main metabolite in rat urine was DCPU, either by oral or inhalation exposures to diuron (APVMA, 2005; 2011; Da Rocha *et al., submitted*). In cows exposed to diuron, residues were detected mainly in urine, followed by feces and ultimately blood. No residue was detected in milk. The main metabolite after diuron oral administration was again DCPU (Kalra and Chahal, 1979).

In contrast to these other species, DCPMU is the main urinary metabolite in humans after cases of diuron product abuse. DCPU and trace amounts of DCA were also detected (Boven *et al.,* 1990). In humans, metabolism via demethylation and hydroxylation appears to be major routes (Verheij and Greef, 1989). The metabolic pathways in rats and cows were similar to humans, with demethylation an important route (APVMA, 2005). In another study, the only metabolic pathway detected using human liver homogenates and seven other mammalian species, including human liver microsomes was demethylation at the terminal nitrogen atom. The rank order of N-demethylation formation in liver microsomes based on intrinsic clearance was dog > monkey > rabbit > mouse > human > minipig > rat (Abass, *et al.,* 2007).

In vitro studies (Da Rocha *et al., submitted*) with urothelial rat (MYP3) and human (1T1) cell lines showed differences in the metabolite cytotoxicity response. For rat urothelial cells, DCPMU (IC_{50} of 104 µM) was the most cytotoxic metabolite, followed by DCPU (IC_{50} of 185 µM). DCPU was the metabolite found at the highest

concentration in rat urine, well above the IC_{50} concentration. In addition, microarray analyses showed that DCPU induced the highest number of altered probe sets in the rat urothelial cell line (893) compared with DCPMU (158) and DCA (108). Contrary to the results found with MYP3 cells, DCA was the most cytotoxic metabolite for the 1T1 human urothelial cell line and was also the metabolite which altered the highest number of probe-sets in a human microarray analysis (2296), followed by DCPMU (1242) and DCPU (443). However, as indicated above, DCA is present in human urine only at trace levels, even in product abuse cases.

In humans, a proliferative response is possible in the urothelium. Although no direct data concerning diuron-induced urothelial proliferation was is available in humans, many cases in the literature have documented the proliferative response of the bladder urothelium exposed to a variety of agents (Rosin *et al.*, 1994). Many irritants thought to increase cancer development by stimulation of proliferation are frequently associated with inflammation in the tissue (Rosin *et al.*, 1994). As an example, it is well known that urinary tract chronic infection caused by *Schistosoma hematobium* is associated with increased incidences of bladder cancer, especially in Egypt (Rosin *et al.*, 1994, Cohen *et al.*, 1991). Infection with *S. haematobium* occurs when individuals are exposed to the larval stage of the parasite in contaminated water. The cercariae penetrate the skin and at maturity, male and female worms travel through the blood system to the bladder. The female moves into the venules of the bladder mucosa where the eggs are placed. These eggs either penetrate the mucosa and are released into the urine or become trapped in the tissue. In either case, a chronic inflammatory reaction is initiated and causes chronic inflammation, fibrosis, squamous metaplasia, and sustained, increased urothelial and squamous cell proliferation. Another example is the bladder infection caused by uropathogenic *Escherichia coli* that stimulate the expression of

numerous pro-proliferation and differentiation genes within the bladder epithelium, promoting the rapid repair and regeneration of the bladder epithelium (Rosin *et al.*, 1994).

Cytotoxicity and regenerative cell proliferation are the main key events of the carcinogenic MOA of rats exposed to diuron (Fig 4). The qualitative assessment of the relevance of this MOA to humans showed that these key events could possibly occur in humans. However, it is important to take into account the levels of exposure necessary to initiate these key events. The quantitative risk assessment is discussed in the next section.

11) HUMAN RELEVANCE: QUANTITATIVE EVALUATION

Rat urothelial cells exposed to the diuron metabolites DCPU, OH-DCPU, DCPMU and DCA showed that DCPMU is the most cytotoxic metabolite ($IC_{50=}$104 µM). However, DCPMU was found at low concentrations in rat urine (13 µM) after chronic exposure to 2500 ppm diuron, and in another study with [C^{14}]-diuron, DCPMU was found at 0.3-0.8% of total excretion (Da Rocha *et al., submitted*; APVMA, 2011). In contrast, DCPU was cytotoxic in rat urothelial cells at 185 µM (IC_{50}). In the [C^{14}]-diuron excretion study, DCPU was present at 22-30%, the metabolite with the highest concentration. DCPU was also the metabolite with the highest urinary concentration in rats exposed to 2500 ppm diuron, with concentrations above 488 µM (Da Rocha *et al.*, *submitted*; APVMA, 2011).

DCPU was the main metabolite in rat urine with both oral and inhalation exposures to diuron. Lower amounts of diuron (54.4 µM), DCA (35.4 µM) and DCPMU (13.5 µM) were found in the urine of animals treated with 2500 ppm diuron. In rats, the only metabolite with urinary concentrations above the *in vitro* IC_{50} was DCPU.

So, it is likely that DCPU is the diuron metabolite most responsible for the cytotoxicity of the urothelium.

Contrary to the results found with rat urothelial MYP3 cells, in the human urothelial cell line 1T1, DCA was the most cytotoxic metabolite (IC_{50} of 72 µM), followed by DCPU, DCPMU and OH-DCPU at 157 µM, 224 µM, 329 µM, respectively (Da Rocha *et al., submitted*). In a case of product abuse, DCPMU was present at the highest concentration in the urine (180 mg/L), followed by DCPU (68 mg/L). Unchanged diuron was not detected in human samples and only trace amounts of DCA were detected (0.2 mg/L) (Boven *et al.*, 1990). Therefore, although DCA can cause cytotoxicity in human urothelial cells, it is unlikely that DCA can trigger a cytotoxicity response in real exposure scenarios since even in cases of product abuse, DCA was present only in trace amounts.

The evidence strongly indicates that a minimum (threshold) amount of the critical metabolite must be present in the urine to induce cytotoxicity and ultimately tumors. The U.S. Department of Agriculture`s pesticide Data Program (PDP) did not detect diuron residues in citrus, milk and other commodities (USEPA, 2003). In the Australian Total Diet Surveys (ATDS) diuron was not detected in food (APVMA, 2005). Diuron was present in water, but at low levels concentrations (ppb) (APVMA, 2005). In Florida, 17 of 438 water samples contained diuron, and the highest reported concentration was 1.2 ppb. The drinking water level (DWLOC) determined by USEPA was 28 ppb (USEPA, 2003). Thus, agricultural workers have the greatest exposure to diuron, occurring with application of the herbicide (APVMA, 2005; USEPA, 2003). For humans, there is negligible risk of tumor development at exposures below that required to produce urothelial cytotoxicity. Based on the data available, this will not occur in

humans at environmental or occupational exposure levels not even at exposures associated with diuron abuse.

OVERALL ASSESMENT

Diuron was classified as known/likely human carcinogen due to higher incidences of urinary bladder carcinomas (males 35/48 and females 13/49), and one kidney pelvis papilloma and two carcinomas in rats exposed to high doses of diuron after two years. Studies from multiple laboratories provide strong evidence establishing the MOA in animals: metabolite-induced urothelial cytotoxicity with necrosis and cell exfoliation, consequent regenerative hyperplasia and eventually tumors. It is plausible that the key events in animals occur in humans. However, high doses are required to produce a concentration that will be cytotoxic to the urothelium. Since humans are exposed to levels of diuron that are not expected to produce urinary concentrations of metabolites that would be cytotoxic, we conclude that humans will not have a carcinogenic response at usual occupational or environmental exposures.

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STATEMENT OF INTERESTS:

None.

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TABLES:

TABLE 1: Tumor incidence in male and female rats administered diuron in the diet (Bayer, 1985a). From: Iyer *et al. (*2002).

Dose, ppm (mg/kg-day: males, females)	Urinary bladder epithelial carcinomas (%)[1]		Kidney renal pelvis epithelial carcinomas or papillomas in males (%)[3]
	Males	Females	
0 (0, 0)	1/49 (2)	1/47 (2)	0/49 (0)
25 (1, 1.7)	0/50 (0)	0/49 (0)	0/50 (0)
250 (10, 17)	1/49 (2)	1/50 (2)	0/50 (0)
2500 (111, 203)	35/48 (73)[2]	13/49 (27)[2]	3/48 (6)

[1] Statistically significant increasing trends for both males and females; $p < 0.01$ (Exact trend test).

[2] Statistically significant increased incidence versus controls; $p < 0.01$ (Fisher's Exact test).

[3] Statistically significant increasing trend; $p < 0.05$ (Exact trend test).

TABLE 2: Summary of urothelial findings in Male Wistar rats exposed to diuron by diet[a].

Rat gender	Dose (ppm)	Time	Diet	Light microscopy (hyperplasia)	SEM altered (classes 4 and 5)	Cell proliferation	Renal Pelvis hyperplasia	Reference
Male	125	20 weeks	Nuvilab	2/10	-----	0.44±0.84	2/10	Nascimento *et al.*, 2006
	500			2/10	-----	0.21±0.26	**8/10**	
	2500			**7/10**	3/5	**0.87±1.10**	**6/9**	
	Control			0/10	0/3	0.27±0.63	0/10	
Male	2500	15 weeks	Nuvilab	**8/10**	4/5	----	----	Da Rocha *et al.*, 2010
	Control			0/10	0/6	----	----	
	2500	25 weeks		**9/12**	-----	----	**7/11**	
	Control			0/11	-----	----	0/9	
	2500	30 weeks		**9/10**	5/6	----	**10/10**	
	Reversibility	15+15 weeks		3/10	2/5	----	1/7	
	Control	30 weeks		0/10	1/6	----	0/10	
Male	60	20 weeks	Nuvilab	1/10	1/10	0.73 ± 1.93	0/10	Cardoso *et al., submitted*
	125			0/10	2/10	0.58 ± 1.22	1/10	
	500			2/10	**5/10**	0.44 ± 0.98	**8/8**	
	1250			**8/10**	**7/10**	**4.93 ± 4.14[g]**	**9/10**	
	2500			**7/10**	4/10	1.56 ± 1.81	**10/10**	
	Control			0/10		0.38±0.77	1/10	
Male	2500	1 day	Purina	0/10	0/10	0.14±0.10	----	Da Rocha *et al.*, 2012
	Control			0/10	0/10	0.31±0.31	----	

	2500	3 days		0/10	0/10	0.34±0.16	----	
	Control			0/10	0/10	0.24±0.19	----	
	2500	7 days		1/10	0/10	0.42±0.37	----	
	Control			0/10	0/10	0.24±0.32	----	
	2500	28 days		2/10	3/10	0.13±0.09	----	
	Control			0/7	0/7	0.24±0.32	----	
	2500	8 weeks		6/10	5/10	0.19±0.14	----	
	Control			0/10	0/10	0.21±0.16	----	
Male	25	12 months	Altromin	5/10	-----	-----	4/10	Schimit, 1985 Data accessed from APVMA, 2011
	250			5/10	-----	-----	6/10	
	2500			**10/10**	-----	-----	**10/10**	
	Control			4/10	-----	-----	5/10	
Female	25	12 months	Altromin	3/10	-----	-----	3/10	
	250			**5/10**	-----	-----	4/10	
	2500			**9/10**	-----	-----	4/10	
	Control			0/10	-----	-----	1/10	
Male	2500	2 weeks	Altromin	3/10	-----	-----	-----	Schimit and Karbe, 1986b. Data accessed from APVMA, 2011.
	Control			0/10	-----	-----	-----	
	2500	4 weeks		**10/10**	-----	-----	-----	
	Control			1/10	-----	-----	-----	
	2500	12 weeks		**10/10**	-----	-----	-----	
	Control			2/10	-----	-----	-----	
	2500	26 weeks		**9/9**	-----	-----	-----	
	Control			2/10	-----	-----	-----	
	2500	4+4weeks		7/10	-----	-----	-----	
	Control			3/10	-----	-----	-----	
	2500	26+8weeks		**7/10**	-----	-----	-----	
	Control			1/10	-----	-----	-----	

[a]Bold numbers means significant different from control. ($p<0.05$)

FIGURE 1: Key events of carcinogenic diuron MOA: (A) swollen cells SEM x200; (B) necrosis and exfoliation foci SEM x600; (C) cell proliferation labeled with BrdU x40; (D) regenerative hyperplasia HE x40; (E) regenerative hyperplasia SEM x400; (F) simple hyperplasia in kidney pelvis HE x40.

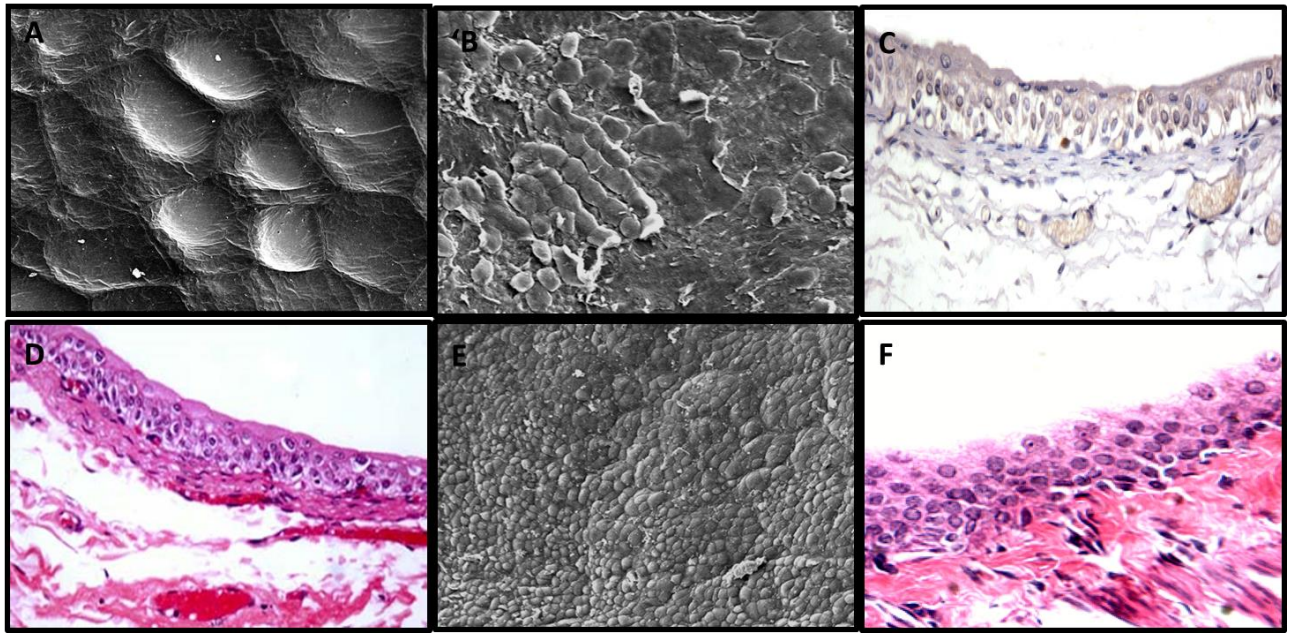
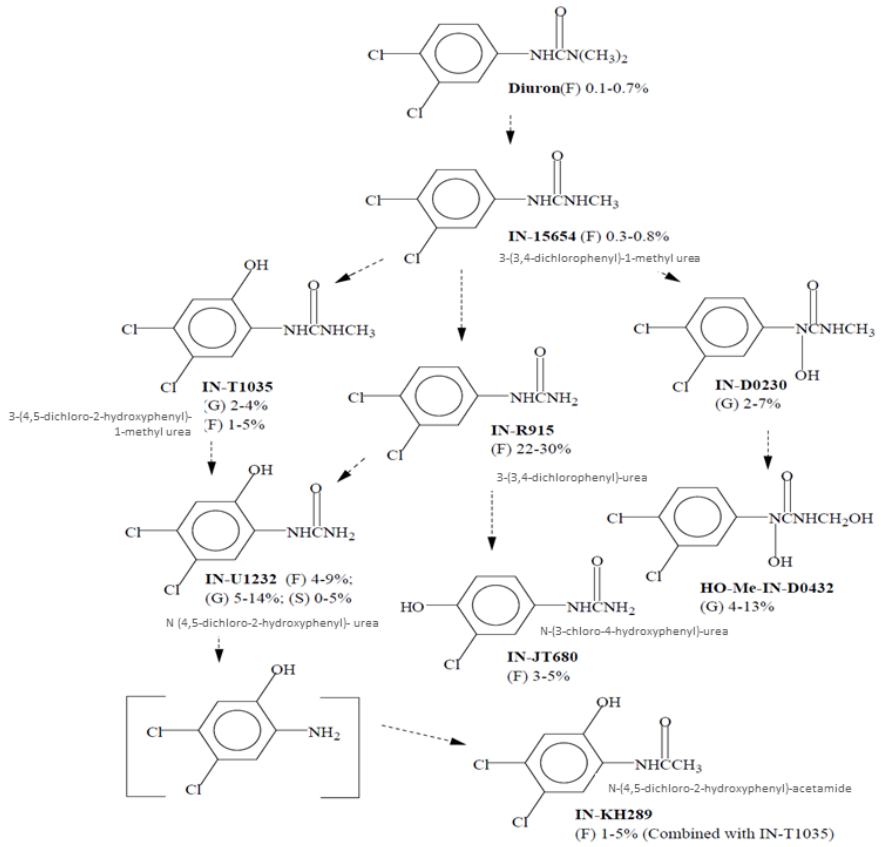


FIGURE 2: Proposed Metabolic Pathways of Diuron in Rats by Wu D (1996).



Metabolite excreted in the free form (F), as a glucoronide (G), or as a sulfate (S).

FIGURE 3: Proposed Metabolic Pathways of Diuron in Human by Abass *et al. (*2007)

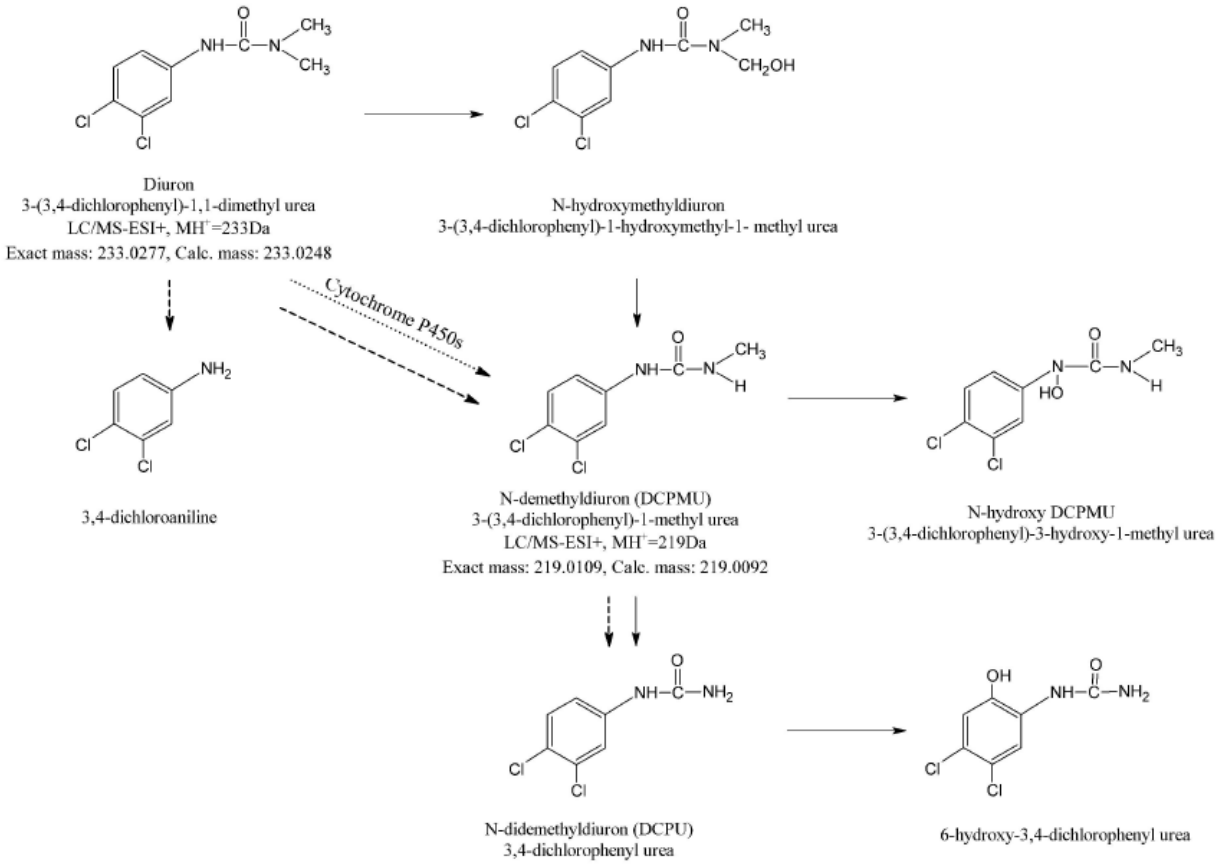
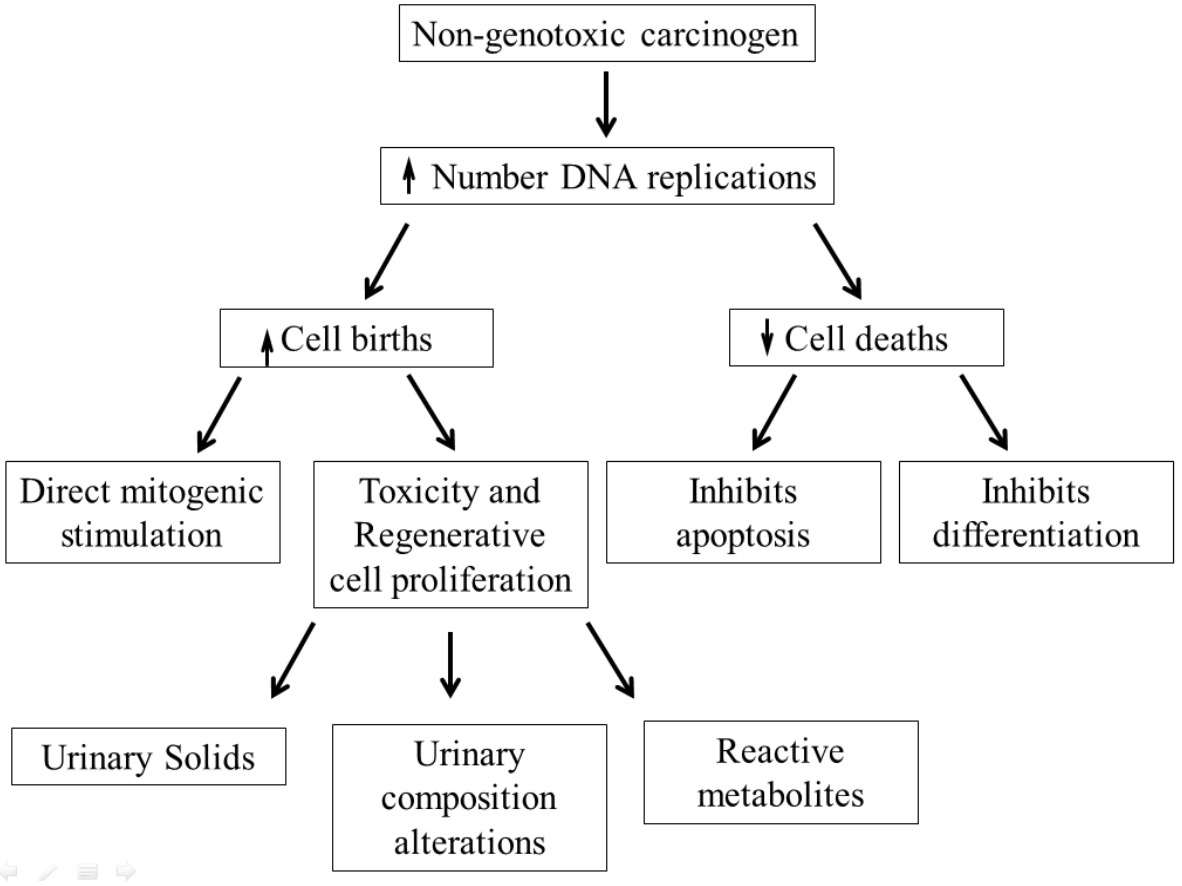


FIGURE 4: Nongenotoxic Mode of Action scheme for urothelial carcinogens - Adapted from Cohen (1998)



OBJECTIVE

The aim of this study is to elucidate the carcinogenic Mode of Action of diuron in rat urothelium.

SPECIFIC OBJECTIVES

1) Evaluate the possible effects of urinary solids on the development of urothelial lesions;

2) Determine the time course and sequence of bladder cytotoxic and proliferative morphological changes;

3) Evaluate the main diuron metabolites for cytotoxicity and gene expression on human and rat urothelial cells exposed to the diuron metabolites DPU, DCPMU and DCA and compare with urinary concentrations of the metabolites after diuron treatment at 2500 ppm.

MANUSCRIPT 02

Authorized by the Pathology Graduation

Program to be part of doctoral avaliation.

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Cytotoxicity and Regenerative Proliferation as the Mode of Action for Diuron-Induced Urothelial Carcinogenesis in the Rat

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Diuron, a substituted urea herbicide, is carcinogenic to the urinary bladder of rats at high dietary levels. Its proposed carcinogenic mode of action (MOA) includes urothelial cytotoxicity and necrosis followed by regenerative cell proliferation and sustained urothelial hyperplasia. Cytotoxicity could be induced either by urinary solids or by chemical toxicity by diuron and/or metabolites excreted in the urine. Diuron was not genotoxic in a previous single-cell gel (comet) assay, but possible cross-linking activity remained to be evaluated. The present study explored the MOA of diuron and the effect of urinary acidification on the development of urothelial lesions. Male Wistar rats were fed diuron (2500 ppm, about 130 mg/kg of body weight) either with or without NH_4Cl 10,000 ppm to acidify the urine. Reversibility of urothelial changes was also examined. The animals were euthanized after 15, 25, or 30 weeks. Diuron-fed rats had urinary amorphous precipitate and magnesium ammonium phosphate crystals similar to control animals. Groups treated with diuron + NH_4Cl showed decreased urinary pH and reduced amounts of urinary crystals and precipitate. Urothelial necrosis and simple hyperplasia were observed by light microscopy and scanning electron microscopy both in diuron- and in diuron + NH_4Cl-treated groups. Cytotoxicity and proliferative changes were mostly reversible. A modified comet assay developed *in vitro* with Chinese hamster ovary cells showed that diuron did not induce DNA cross-links. These data suggest that cytotoxicity with consequent regenerative cell proliferation is the predominant MOA for diuron rat urothelial carcinogenesis, the cytotoxicity being chemically induced and not due to urinary solids.

Key Words: **urinary bladder; urinary pH; urothelial hyperplasia; urinary crystals and precipitates; DNA cross-link; cytotoxicity.**

Diuron (3-[3,4-dichlorophenyl]-1,1-dimethylurea), an urea-derived herbicide, is widely used for weed control in soy, cotton, citrus fruit, and sugar cane cultures. This herbicide has also been used as a mildewcide in paints and stains and algaecide in commercial fish production (Thomas *et al*., 2002; Thurman *et al*., 2000). It exerts its herbicidal action by inhibiting the photosynthesis by preventing oxygen production in plants. Diuron is breakdown in the environment by abiotic (hydrolysis and photolysis) and principally by biotic aerobic and possibly anaerobic processes. However, these processes are relatively slow (a month to a year), and consequently, the herbicide can be found in many environments such as the soil, water, and sediments; 3,4-dichloroaniline (DCA) is considered the most important product of diuron breakdown (Bouilly *et al*., 2007; Giacomazzi and Cochet, 2004).

Diuron has been categorized as a ''known/likely'' human carcinogen by U.S. Environmental Protection Agency (USEPA) mostly based on a 2-year bioassay that indicated increased incidence of urothelial bladder and renal pelvis papillomas and carcinomas after continuous dietary high-concentration (2500 ppm) exposure in both genders of Wistar rats and a trend for increased incidence of mammary gland adenocarcinomas in female NMRI mice (Iyer, 2002; USEPA, 2003, 2004). In a previous study from this laboratory, Wistar rats fed diuron at 2500 ppm for 20 weeks showed increased urothelial cell proliferation demonstrated by the proliferating cell nuclear antigen labeling index and simple hyperplasia (SH) by light microscopy (Nascimento *et al*., 2006), conditions considered to predispose to neoplasia in the rat urinary bladder mucosa (Cohen, 1998). Besides, scanning electron microscopy (SEM) evaluation showed severe urinary bladder mucosal necrosis. Analysis of urinary sediment from control and 2500 ppm diuron-fed animals revealed that both groups had magnesium ammonium phosphate crystals; in the urine of the 2500 ppm diuron-exposed group, these crystals were compacted and accompanied by a greater quantity of amorphous precipitates (Nascimento *et al*., 2006). These precipitates are composed mainly of calcium phosphate and small amounts of potassium, chlorine, sulfur, and silica; they can act as microabrasives to the mucosa causing cellular death and regenerative hyperplasia that can lead to urothelial carcinogenesis (Cohen *et al*., 1990, 1991a).

The characteristics of urinary amorphous precipitates and microcrystals are dependent on various factors, such as urinary

pH, mineral content, and test chemical concentration. Urinary pH can influence urothelial cell physiology in multiple ways, such as altering the degree of ionization of xenobiotics and their metabolites, varying the degree of hydrolysis of urinary metabolites and the structure or the charges of urinary compounds, particularly macromolecules (proteins and mucopolysaccharides), altering the solubility of various chemicals and salts, and modifying the degree of affinity of urinary components with macromolecules. In addition, pH modifications may alter urothelial cell surface receptors (Clayson *et al*., 1995; Cohen, 1995). Exogenous chemicals can induce urinary pH alterations, inhibiting or enhancing urothelial cell proliferation, and consequently modifying the susceptibility to tumor development. Protocols that combine exogenous test chemicals with substances able to control the urinary pH, such as ammonium chloride for acidification or sodium bicarbonate for alkalinization, have been useful to investigate the role of urinary pH on potential mode of action (MOA) of chemicals on urinary bladder carcinogenesis (Cohen *et al*., 1995; Lina and Kuijpers, 2004).

In a previous study, absence of genotoxicity was observed using the standard alkaline version of the single-cell gel (comet) assay conducted with urinary bladder cells and peripheral blood leukocytes of male Wistar rats exposed through the diet for 20 weeks to different concentrations of diuron (Nascimento *et al*., 2006). The standard comet assay allows the detection of diverse kinds of DNA alterations, such as double-strand breaks, single-strand breaks, alkali-labile sites, and incomplete repair (Tice *et al*., 2000; Witte *et al*., 2007). However, cross-linking agents connect DNA with DNA and/or proteins and do not allow DNA migration, thus are not readily detected in DNA eletrophoretic migration on the standard comet assay (Merck and Speit, 1999; Pfuhler and Wolf, 1996). Therefore, the possibility of diuron being a cross-linking inducer cannot be ruled out based only on our previous findings.

Considering previous studies on the effects of diuron and the established knowledge concerning the urinary bladder carcinogenesis process, the present studies were undertaken to better understand the MOA of diuron on the urinary bladder of male Wistar rats with emphasis on the roles of urinary pH acidification, urinary sediments, and the reversibility potential of the lesions caused by this herbicide. In addition, a modified comet assay was developed to evaluate *in vitro* the potential of diuron to induce DNA cross-links using Chinese hamster ovary (CHO) cells.

MATERIALS AND METHODS

In vivo *experiment.* This study was approved by the Committee for Ethics in Animal Experimentation of the UNESP Medical School, São Paulo, Brazil (protocol no. 548). Ninety-six 4-week-old male Wistar rats were purchased from the Multidisciplinary Center for Biological Investigation (CEMIB, Campinas State University, Campinas, São Paulo, Brazil). Diuron (CAS no. 30-54-1; Sigma Chemical Co., St Louis, MO, 97% purity) was mixed with a powdered commercial diet (Nuvilab CR1; Nuvital, PR, Colombo, Brazil) at final concentrations of 2500 ppm with or without of 10,000 ppm ammonium chloride (NH_4Cl) (CAS no. 12125-02-9; Dinamica, Diadema, Brazil). As informed by the supplier, the commercial diet was prepared according to the National Research Council/National Institutes of Health-USA "Recommendations for Mice and Rats Balanced Feed." Ammonium chloride was used for urine acidification. The final concentration of diuron in the diet was analytically confirmed by HPLC/UV (TASQA Ltda., Paulinia, Brazil).

After a 2-week quarantine and acclimation period, rats were weighed and randomized into nine groups that were allocated to either 15-, 25- or 30-week long experiments (Fig. 1). Groups with 10 animals each were assigned to the 15-week experiment: group 1 (G1), diuron 2500 ppm; group 2 (G2), diuron 2500 ppm + NH_4Cl 10,000 ppm; and group 3 (G3), basal diet. Groups for the 25-week experiment consisted of 12 animals each: group 4 (G4), diuron 2500 ppm; group 5 (G5), diuron + NH_4Cl 10,000 ppm; and group 6 (G6), basal diet. Finally, the three 30-week groups consisted of 10 animals each: group 7 (G7), diuron 2500 ppm; group 8 (G8), reversibility group, diuron 2500 ppm during 15 weeks followed by another 15 weeks with diuron-free diet; and group 9 (G9), basal diet. Water and feed were provided *ad libitum*.

Water and feed consumptions and body weights were determined at the commencement and at every other week during the experiment. At the end of each experimental period, the animals were anesthetized with 3% sodium pentobarbital (30 mg/kg ip). The urinary bladder was exposed, injected with Bouin's fixative, removed, and immersed in the same fixative for 4 h. Immediately after removal of the bladder, the animals were euthanized by opening the abdominal cavity and sectioning the inferior vena cava. The kidneys were collected, weighed, and placed in 10% buffered formalin.

Fresh urine collection. Freshly voided urine was individually collected by manual compression in 1.5-ml plastic microtubes between 7:00 A.M. and 9:00 A.M. during the 5th and 13th weeks in the 15-week period groups (G1–3) and at the 6th, 14th, and 22nd weeks in the 25-week period groups (G4–6). Individual determination of pH occurred immediately after collection using special pH indicator paper (Merck KGaA, Darmstadt, Germany), pH 4.0–7.0 and 6.5–10.0. Urine samples collected from G4, G5, and G6 at the 14th week were used to determine the occurrence and incidence of amorphous precipitates and crystals by SEM (Cohen *et al*., 1991b).

Light microscopy and SEM analyses. The urinary bladders were sectioned mid-sagittally and washed three times in 70% alcohol. One half of each bladder was cut longitudinally into four segments, which were embedded in paraffin and stained with hematoxylin and eosin (HE) after sectioning. Urinary bladders of all animals were analyzed for proliferative lesions, including SH and papillary and nodular hyperplasia (PNH). The other half of the urinary bladders collected at the 15- and 30-week periods were processed for SEM in a Philips 515 scanning electron microscope (Philips, Inc.,

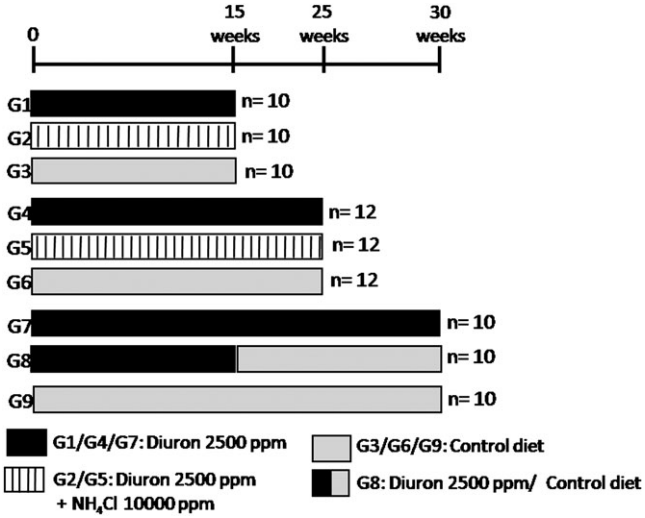

FIG. 1. Experimental design.

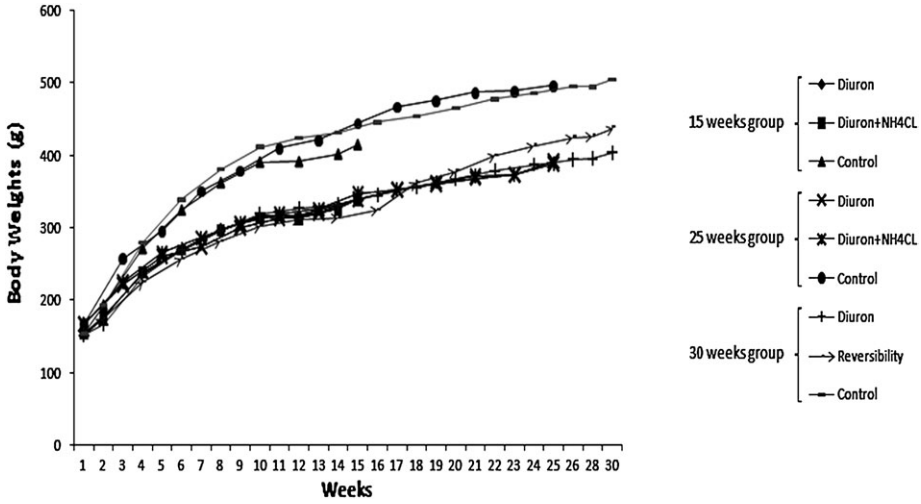

FIG. 2. Mean body weights of rats during 15, 25, and 30 weeks. Diuron was fed at 2500 ppm and NH_4Cl at 10,000 ppm.

Eindhoven, The Netherlands) at the Institute of Biosciences, UNESP, Botucatu, São Paulo. SEM morphological characteristics of the urothelial surface were classified according to Cohen *et al*. (2002): class 1—flat polygonal superficial urothelial cells; class 2—occasional small foci of superficial urothelial necrosis; class 3—numerous small foci of superficial urothelial necrosis; class 4—extensive superficial urothelial necrosis, especially in the urinary bladder dome; and class 5—necrosis and piling up of rounded urothelial cells (urothelial hyperplasia). Classes 1–3 can be found in normal urothelial mucosa. After SEM analysis, the results were grouped as "normal" (classes 1–3) and altered (classes 4 and 5).

In vitro *experiment.* According to Pfuhler and Wolf (1996), the modified alkaline comet assay protocol for cross-link evaluation combines the treatment of the putative cross-linking agent followed by a known genotoxic compound. The cross-link lesion does not allow electrophoretic DNA migration; thus, it is expected that the combined treatment shows comet "tail" length and density lower than the genotoxic compound by itself. Methyl methanesulfonate (MMS), a known genotoxic agent, causes DNA lesions that lead to strand breaks and therefore has been used for standardized induction of this type of DNA damage in the alkaline comet assay. Diuron was combined with MMS (5 μg/ml) up to the limit of its solubility (50, 25, and 12.5 μg/ml) in 1% Dimethylsulphoxide (DMSO). To exclude the possibility of DMSO causing DNA damage at this concentration, one treatment was conducted separately only with 1% DMSO. Treatment with only MMS was used as the positive control. CHO cells were treated with diuron for 3 h and subsequently exposed to MMS for 1 h. Three independent experiments were conducted, and their results were used for data interpretation. Immediately after diuron treatment, a trypan blue dye exclusion assay indicated a higher than 94% cell viability in all experiments (data not shown). Mean values of three slides per treatment were the basis for statistical analysis. The comet tail moment and tail intensity were estimated by the software Comet Assay 2.2 (Perspective Instruments, Suffolk, UK), and the data were compared by the ANOVA followed by the Dunn's test.

RESULTS

During the experiment, food consumption by diuron and diuron + NH_4Cl groups were significantly decreased when compared to the control (data not shown). The mean ingested levels of the herbicide by diuron- and diuron + NH_4Cl-fed groups corresponded to 135 mg/kg/day. In the average, both diuron-fed groups presented body weights 20% below the respective controls at each time point (Fig. 2). After diuron withdrawn from the diet, food consumption by the reversibility group increased significantly and reached the same level of the controls but the body weights increased only slightly and remained significantly different from the control group.

Urinary pH was reduced in all groups treated with diuron + NH_4Cl at every experimental time point (Table 1). Urinary pH of diuron-treated groups did not differ significantly from control groups.

TABLE 1
Urinary pH at Different Times of the 15- and 25-Week Long Experiments

	15-week study		25-week study		
Treatment	Week 5 (*n*)	Week 13 (*n*)	Week 6 (*n*)	Week 14 (*n*)	Week 22 (*n*)
Diuron (2500 ppm)	7.5 ± 0.5 (6)	7.3 ± 0.4 (7)	7.2 ± 0.3 (11)	7.8 ± 0.2 (11)	7.9 ± 0.1 (10)
Diuron (2500 ppm) + NH_4Cl (10,000 ppm)	6.7 ± 0.4* (8)	6.5 ± 0.3* (8)	6.3 ± 0.2* (11)	6.7 ± 0.3* (11)	6.7 ± 0.2* (12)
Control	7.4 ± 0.4 (8)	7.8 ± 0.2 (5)	7.5 ± 0.3 (10)	7.9 ± 0.1 (7)	7.7 ± 0.2 (5)

Note. *n*, number of animals.
*Significantly different from control group ($p < 0.05$).

TABLE 2
Effect of Dietary Diuron on the Urothelial Mucosa and Urinary Sediment of Male Wistar Rats at Different Experimental Periods

		Incidence of urinary bladder mucosal lesions				Incidence of urinary bladder mucosal changes by SEM[a]			Urinary sediment by SEM at 14th week		
Experimental periods	Treatment	*n*	Normal	SH	PNH	*n*	Normal	Altered	*N*	Incidence of crystals (%)	Incidence of precipitates (%)
15 week	Diuron (2500 ppm)	10	2*	8*	0	5	1*	4*	—	—	—
	Diuron + NH_4Cl (10,000 ppm)	10	0*	9*	1	5	1*	4*	—	—	—
	Control	10	10	0	0	6	6	0	—	—	—
25 week	Diuron	12	3*	9*	0	—	—	—	7	6 (86)	7 (100)
	Diuron + NH_4Cl	12	0*	11*	1	—	—	—	7	1 (14)*	2 (29)*
	Control	11	11	0	0	—	—	—	6	5 (83)	5 (83)
30 week	Diuron	10	1*	9*	0	6	1	5	—	—	—
	Reversibility[b]	10	7	3	0	5	3	2	—	—	—
	Control	10	10	0	0	6	5	1	—	—	—

Note. *n*, number of animals; PNH, papillary and nodular hyperplasia.
[a]See ''Light microscopy and SEM analyses'' section.
[b]Group fed with 2500 ppm diuron for 15 weeks followed by 15 weeks of control diet.
*Significantly different from control ($p < 0.05$).

At the end of the 15-, 25-, and 30-week periods, the urinary bladders of the diuron 2500 ppm groups showed significantly ($p < 0.001$) increased incidences of SH: 8/10, 9/12, and 9/10, respectively. No SH was found in control animals (Table 2). Rats exposed to diuron + NH_4Cl also presented increased incidences ($p < 0.05$) of SH: 9/10 at the 15-week and 11/12 at the 25-week periods. At both periods, one animal developed papillary and nodular hyperplasia (PNH). The diuron-treated reversibility group (30 week) showed SH in the urinary bladder of 3/10 rats, not significantly different from the control group but significantly less than the incidences of continuous diuron treatment for 15, 25, or 30 weeks (Table 2).

After 15 weeks, SEM analysis of the urinary bladder of diuron and diuron + NH_4Cl groups revealed similar and significantly higher incidences (four of five) of severe urothelial alterations compared to the control (Table 2). These changes were represented by foci of necrosis and regenerative cell hyperplasia (Fig. 3). After 30 weeks, the diuron group showed SEM alterations in five of six animals compared to one of six in the control group. In the reversibility group, two of five urinary bladders showed SEM alterations (Table 2).

Analysis of urinary sediment by SEM at the 14th week showed the presence of magnesium ammonium phosphate crystals and amorphous precipitates in most of the animals of the control and diuron groups. The incidences of crystals and precipitates were significantly reduced in the diuron + NH_4Cl group (Table 2, Fig. 3).

The renal pelvis was analyzed by light microscopy only at the 25- and 30-week time points. After 25 weeks, diuron and diuron + NH_4Cl groups had 7/11 and 6/11 ($p < 0.05$) rats with SH in the renal pelvis, respectively. At the 30th week, the diuron group showed a significantly higher incidence of SH (10/10) ($p < 0.001$) than the reversibility group (SH in only one of seven rats) (data not shown).

No significant differences were detected by the *in vitro* comet assay for any concentration of diuron. Only the DMSO treatment showed significant difference when compared to the MMS-treated positive control. Significant differences related to DNA migration were clearly observed between the MMS treatment alone and untreated cells ($p < 0.05$) (Fig. 4).

DISCUSSION

The present results add support to the hypothesis that cytotoxicity with consequent regenerative cell proliferation is the predominant MOA for diuron rat urothelial carcinogenesis. Precipitates and magnesium ammonium phosphate crystals were present in the urine of diuron-treated rats in a similar amount as that observed in control animals. Decreasing urinary pH by coadministration with NH_4Cl was accompanied by marked reduction of crystals and precipitates in the urine, but this treatment did not affect the incidence or severity of the urothelial lesions induced by diuron, i.e., cytotoxicity was not influenced by acidification of the urine. These data suggest that urinary solids do not play a role in the MOA of diuron in the development of the cytotoxicity or putative preneoplastic hyperplastic urothelial lesions and consequently are not involved in diuron-induced urothelial carcinogenesis in rats. Thus, the carcinogenic MOA of diuron probably involves cytotoxicity produced by the parental compound and/or its metabolites excreted in the urine.

Degradation steps of diuron appear to follow the general pathways for N,N-dimethyl-substituted phenylureas involving

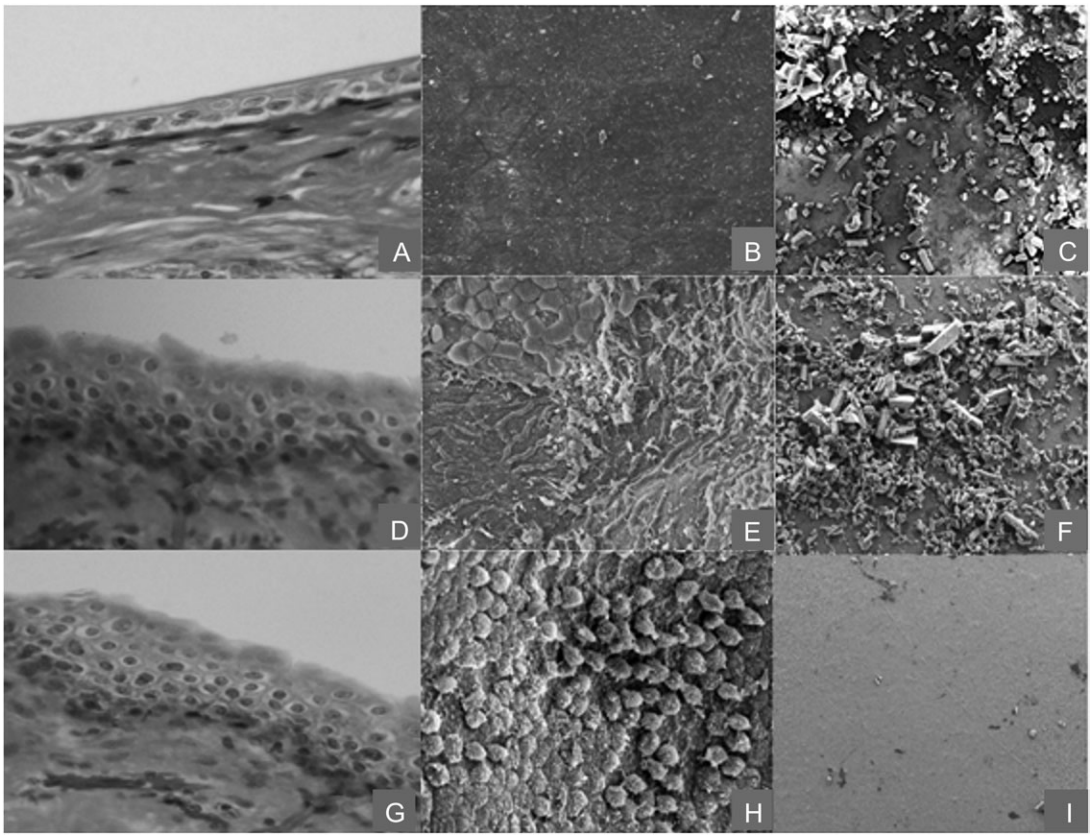

FIG. 3. Urinary bladder under histology (HE) and SEM. Control animals: (A) normal urothelium (HE, ×400), (B) normal urothelium (SEM, ×600), and (C) crystals and precipitates in urinary sediment (SEM, ×400). Diuron-fed animals: (D) hyperplasia (HE, ×400), (E) necrosis and exfoliation (SEM, ×600), and (F) crystals and precipitates in urinary sediment (SEM, ×400). Diuron + NH_4Cl–fed animals: (G) hyperplasia (HE, ×400), (H) hyperplasia (SEM, ×600), and (I) absence of crystals and precipitates in urinary sediment (SEM, ×400).

either an initial demethylation to N-(3,4-dichlorophenyl)-N′-methylurea (DCPMU) or a hydrolysis to DCA (Abbas *et al.*, 2007). The only metabolic pathway detected by liquid chromatography/mass spectrometry in human liver homogenates and seven types of mammalian liver microsomes including human was demethylation at the terminal nitrogen atom. No other phase I or phase II metabolites were observed. The rank order of N-demethyldiuron formation in liver microsomes based on intrinsic clearance (V_{max}/K_m) was dog > monkey > rabbit > mouse > human > minipig > rat (Abbas *et al.*, 2007).

Diuron metabolites have been reported to be more harmful to nontarget organisms than the parental compound itself (Giacomazzi and Cochet, 2004; Tixier *et al.*, 2002). When provided orally to mammals, as in the present study, diuron is absorbed from the gastrointestinal system and excreted in urine and feces. In rats and dogs, N-(3,4-dichlorophenyl)urea was the predominant metabolite in the urine and small amounts of DCPMU, DCA, 3,4-dicholorophenol, and unchanged diuron were also detected (Hodge *et al.*, 1967). In poisoning incidents involving accidental ingestion of diuron, the same metabolites were detected in human blood and/or urine, indicating that the metabolic pathways in humans are similar to those verified in experimental animals (Abbas *et al.*, 2007; Australian Pesticides and Veterinary Medicine Authority [APVMA], 2005). One of the most ubiquitous diuron metabolites, DCA, has been shown to be acutely hemotoxic, causing metahemoglobinemia in male Wistar rats (Guilhermino *et al.*, 1998), the same as registered for diuron itself (Iyer, 2002). Acute toxicity of DCA to the liver, kidney, and urinary bladder of F344 rats after intraperitoneal injections at doses up to 1.0 mmol/kg was also reported, although DCA toxicity to the urinary bladder was characterized by inflammation and not by urothelial damage (Valentovic *et al.*, 1997). The possibility that diuron metabolites are the ultimate cytotoxic agents to the urothelium cannot be ruled out with the little information available about mammalian toxicity of the metabolites.

In the present study, urothelial SH in diuron-treated animals was present at the three different time points studied. Fifteen weeks was adequate for diuron to induce SH by light microscopy and necrosis by SEM in the urinary bladder of Wistar rats. The incidence and severity of the lesions were comparable at 15, 25, and 30 weeks. Urothelial hyperplasia can progress to PNH, papillomas, and carcinomas or can regress

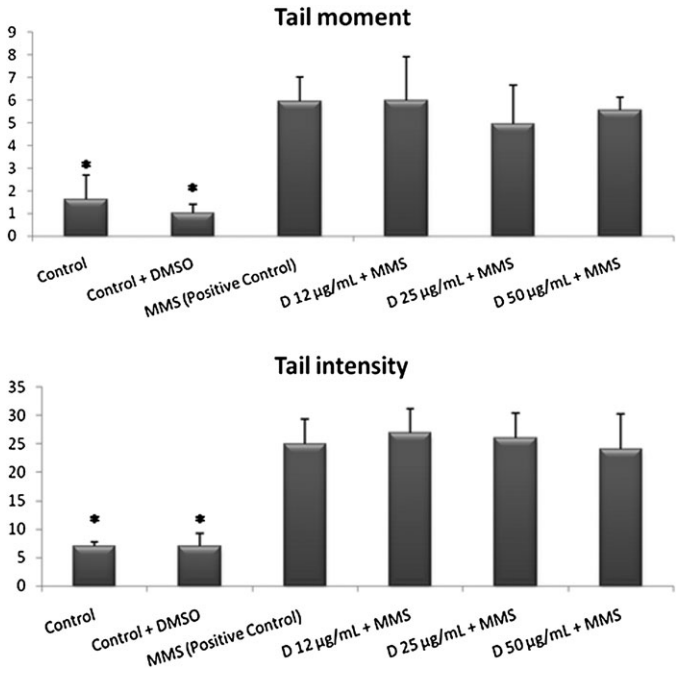

FIG. 4. Effect of the herbicide diuron (D) on MMS (5 µg/ml)-induced DNA migration (comet tail moment and tail intensity) in an *in vitro* alkaline comet assay with CHO cells. *Significantly different from positive control ($p < 0.05$).

with reestablishment of the normal appearance of the urothelium if the noxious stimulus is withdrawn (Cohen, 1998; Fukushima *et al*., 1982; Shibata *et al*., 1994). Reversibility of urothelial hyperplasia was documented at the 30th week in the animals submitted to the regimen of 15 weeks of diuron followed by 15 weeks without diuron: This group presented a lower incidence of urothelial lesions (3/10 rats) detected by light microscopy when compared to the animals that were kept on diuron (9/10 rats). Under SEM, two of five (40%) animals of the reversibility group and five of six (83%) diuron-fed rats showed altered mucosa. These findings indicate that urothelial hyperplastic lesions were reversible in the majority of the animals after diuron withdrawal.

Continuity of urinary bladder lesions in fewer animals after withdrawal of diuron could be explained by the maintenance of some already established SH lesions by other endogenous agents occurring in the rat urine or by a weak genotoxic potential of diuron metabolites. In fact, it has been stated that when urothelial proliferative lesions associated with genotoxic or nongenotoxic chemicals are compared, the former tend not to regress in contrast to the latter (Kagawa *et al*., 1993; Shirai *et al*., 1987; Sugiura *et al*., 2003). Evaluation of the genotoxic potential of diuron in assay protocols with different biological complexities has provided variable results. Although some of these studies suggested that diuron is genotoxic (Agrawal and Mehrota, 1997; Agrawal *et al*., 1996; Bouilly *et al*., 2007; Canna-Michaelidou and Nicolaou, 1996), others report that diuron is a nongenotoxic compound (APVMA, 2005; Gee, 1997; Iyer, 2002; USEPA, 2003). In an initiation-promotion study, topical applications of diuron followed by 12-*O*-tetradecanoylphorbol 13-acetate–induced skin papillomas in the Swiss albino female mice (Antony *et al*., 1989). Since initiation of carcinogenesis by a chemical agent implies that the chemical has genotoxic potential (Pitot and Dragan, 2001), that observation could suggest that diuron has genotoxic potential. However, two studies recently developed in our laboratory attempting to reproduce the experiment of Antony *et al*. (1989) in the same Swiss albino strain indicated that diuron did not exert either initiating or promoting potentials in the mouse skin and, therefore, behaves like a nongenotoxic compound in that assay protocol (Ferrucio *et al*., 2009). This observation, as others reported in the literature indicating that diuron is a nongenotoxic agent, is in line with previous results that have shown that the herbicide does not damage DNA in the *in vivo* alkaline comet assay (Nascimento *et al*., 2006) and in the specialized *in vitro* comet assay protocol designed to detect cross-linking in CHO cells, as presented in this paper. The comet assay is a relatively sensitive technique for the analysis of DNA damage in mammalian cells; it seems especially useful for screening purposes because false positives associated with excessive toxicity appear to occur less frequently than in other tests for genotoxicity (Witte *et al*., 2007). Among the various versions of the comet assay, the alkaline method enables detection of the broadest spectrum of DNA damage and therefore has been recommended for regulatory purposes (Tice *et al*., 2000). In the overall, it can be assumed that the carcinogenic MOA of diuron in the rat urinary bladder is predominantly nongenotoxic, what fits well with the observation that urinary bladder tumors occurred only above a threshold, i.e., in rats submitted to a long-term exposure to 2500 ppm but not to 1250 ppm or lower dietary concentrations of diuron (USEPA, 2004).

Previously, it was demonstrated that diuron induces superficial urothelial cytotoxicity and necrosis, suggesting that cell death and consequent regenerative cell proliferation and not direct mitogenesis is the carcinogenic MOA of this herbicide (Nascimento *et al*., 2006). Herein, documentation provided that neither pH nor urinary solids play a role in the rat urinary bladder carcinogenesis induced by high dietary concentration of diuron. The proposed MOA involves cytotoxicity and regenerative proliferation, the cytotoxicity appearing to be induced by chemical toxicity by diuron and/or metabolites excreted in the urine and not due to the formation of urinary solids.

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Diuron-Induced Rat Bladder Epithelial Cytotoxicity

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Diuron, a substituted urea herbicide, is carcinogenic to the rat urinary bladder at high dietary levels (2500 ppm). To further elucidate the mode of action, this study aimed to determine the time course and sequence of bladder cytotoxic and proliferative changes induced by diuron treatment of male Wistar rats. Rats were randomized into two groups (control and 2500 ppm diuron) and treated for 28 days. Ten rats from each group were terminated on each of study days 1, 3, 7, or 28. Scanning electron microscopy (SEM) showed urothelial cell swelling beginning on day 1, and by day 28, showed extensive necrosis, exfoliation and piling up of cells suggestive of hyperplasia. No difference in the bromodeoxyuridine labeling index was detected. In a second experiment, rats were randomized into control and diuron-treated groups and treated for 7 days or 8 weeks. After 7 days, transmission electron microscopy showed cell degenerative changes and distention of the cytoplasm, organelles, and nuclei characteristic of cytolysis. This resulted in protrusion of the superficial cells into the lumen, corresponding to the cell swelling observed previously by SEM. After 8 weeks, bladders in the diuron-treated group showed an increased incidence of simple hyperplasia by light microscopy (6/10, $p < 0.05$) compared with controls (0/10) and a significantly different SEM classification. In summary, our results support the hypothesis that urothelial cytotoxicity followed by regenerative cell proliferation are the sequential key events that occur with high-dose diuron exposure in rats.

Key Words: **Diuron; urinary bladder; cytotoxicity; proliferation; morphology; electron microscopy.**

Pesticide expenditures totaled more than 39.4 billion dollars in 2007 and the amount used exceeded 5.2 billion pounds worldwide, with herbicides responsible for the largest portion (United States Environmental Protection Agency [USEPA], 2011). The potential toxicity of agricultural pesticides to human health is an important public health issue.

Diuron (3-[3,4-dichlorophenyl]-1,1-dimethylurea) is a substituted urea compound registered as a pre- and postemergent herbicide selective for broadleaf weeds and some annual grasses that can be used alone or in combination with other herbicides. Reported use is on industrial sites, on rights-of-way, around farm buildings, and on irrigation and drainage ditches for general weed control. It is also used with many crops, such as soybeans, cotton, citrus fruits, sugar cane, alfalfa, wheat, grapes, pineapple, cocoa, and coffee. Furthermore, diuron can be used as a mildewcide in plants and stain preventative and as an algaecide in commercial fish production (Australian Pesticides and Veterinary Medicine Authority [APVMA], 2005; USEPA, 2003).

In a 2-year bioassay, continuous dietary exposure to diuron at 2500 ppm was carcinogenic to rats, with an increased incidence of urinary bladder papillomas and carcinomas in both genders of Wistar rats and a renal pelvis papilloma and two carcinomas in male Wistar rats (APVMA, 2011). There was also a marginal (equivocal) increase in mammary gland adenocarcinomas in female NMRI mice (APVMA, 2011) at the same dietary concentration. One study reported that dietary exposure of male Wistar rats to 2500 ppm diuron after 4 weeks led to increases of urothelial hyperplasia (APVMA, 2011). Likewise, 20 weeks of dietary exposure to 2500 ppm diuron in male Wistar rats induced increased urothelial cell proliferation, demonstrated by increased proliferating cell nuclear antigen (PCNA) labeling index and simple hyperplasia of the urothelium by light microscopy (Nascimento *et al*., 2006), conditions considered to predispose rats to neoplasia in the urinary bladder mucosa (Cohen, 1998). In addition, scanning electron microscopy (SEM) evaluation showed urinary bladder mucosal necrosis by 15 and 30 weeks after diuron administration (Da Rocha *et al.*, 2010). Based on these data, cytotoxicity with consequent regenerative hyperplasia of the urothelium has been proposed as the mode of action (MOA) for diuron-induced rat urothelial carcinogenesis. However, whether cytotoxicity actually occurs prior to increased proliferation has not been reported.

Urothelial cytotoxicity can be induced by either the presence of abnormal urinary solids or the toxicity of the parent chemical and/or its metabolites excreted in the urine. Analysis

of urinary sediment from control and 2500 ppm diuron-fed animals revealed that both groups had urinary crystals. In the urine of the 2500 ppm diuron-exposed group, the crystals were compact and accompanied by a greater quantity of amorphous precipitate (Nascimento *et al*., 2006). In a subsequent study, coadministration of 2500 ppm diuron and 10,000 ppm NH_4Cl in the diet decreased urinary pH resulting in a marked reduction of crystals and precipitate in the urine of diuron-treated animals. However, there was no apparent effect on the incidence and/or severity of the urothelial lesions induced by diuron, indicating that urothelial cytotoxicity present in diuron-treated rats was not due to urinary solids (Da Rocha *et al.*, 2010). Therefore, the cause of the cytotoxicity must be chemical induction by diuron itself and/or its metabolites excreted in the urine.

Diuron is considered nongenotoxic. This includes comet assays performed on the target organ, the rat urothelium. Absence of genotoxicity was observed using the standard alkaline version of the single-cell gel (comet) assay conducted with urinary bladder cells and peripheral blood leukocytes of male Wistar rats exposed through the diet for 20 weeks to different concentrations of diuron (Nascimento *et al*., 2006). In addition, a modified version of the comet assay developed *in vitro* with Chinese hamster ovary cells indicated that diuron also does not induce DNA cross-links (Da Rocha *et al.*, 2010). Other mutagenicity studies also support that diuron is nongenotoxic (USEPA, 2003).

The objective of the present studies was to evaluate the proposed MOA of diuron by determining the time course of the cytotoxic and proliferative changes induced by diuron in the urinary bladder epithelium of male Wistar rats. The evaluation was based on light microscopy, labeling index, and both SEM and transmission electron microscopy (TEM).

MATERIALS AND METHODS

Test Material

Diuron (N'-(3,4-dichlorophenyl)-N,N-dimethylurea) was provided by E.I. du Pont de Nemours and Company (Wilmington, DE). The purity of the test material as determined by Advinus Therapeutics Private Ltd. (Bangalore, India) was 98.6%. The diet concentration of 2500 ppm diuron was adjusted based on the purity. The test material was administered in Certified Purina Rodent LabDiet 5002 (PMI Nutrition International, Richmond, IN) at a concentration of 2500 ppm. The test diet was analyzed by DuPont Haskell Global Centers for Health and Environmental Sciences (Newark, DE) for homogeneity/concentration verification and stability by high-performance liquid chromatography with ultraviolet detection. Analytical results from homogeneity/concentration verification testing of the test diet used in this study showed that diuron was homogeneously mixed into the diet at a concentration of 2500 ppm.

Test Animals

Wistar rats were purchased from Charles River Laboratories, Inc. (Raleigh, NC). This is the same species and strain of animal used in the 2-year carcinogenicity study. On arrival, the animals were placed in an animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and quarantined for at least 1 week before starting treatment. The study protocol was approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and the level of care provided to the animals met or exceeded the basic requirements outlined in the Guide for Care and Use of Laboratory Animals (NIH Publication #86-23, revised 1996). The animals were housed two per cage in polycarbonate cages with dry corn-cob bedding in a room with a targeted temperature of 22°C, humidity of 50% and a light/dark cycle of 12 h (0600 lights on/1800 lights off) and fed basal chow (Purina 5002). Diet and water were available *ad libitum* throughout the study.

Experiment 1

Study design. Animals were randomized into two groups (40 rats/group) using a weight stratification method (Martin *et al*., 1986). Following quarantine, Group 1 continued to receive the basal chow (Purina Certified LabDiet 5002) and Group 2 was changed to Purina Certified LabDiet 5002 supplemented with 2500 ppm diuron for up to 28 days. Body weights were measured for all animals prior to randomization on study day 0. Body weights were measured on animals treated for 3 and 7 days at the end of the consumption period, which was also the day of sacrifice. Body weights for animals treated for 28 days were measured weekly. Animals were checked daily for moribundity and mortality, and more detailed clinical observations were performed prior to randomization and sacrifice. Beginning on study day 0, water consumption and diet consumption were measured over a 24-h period for animals treated for 1 day, over a 3-day period for animals treated for 3 days, and over a 7-day period for animals treated for 7 and 28 days. For the remaining treatment period, consumption was measured weekly.

All animals were terminated by an overdose of Nembutal (150 mg/kg body weight; Lundbeck, Inc., Deerfield, IL) after 1, 3, 7, or 28 days of treatment. Ten animals per group were sacrificed at each time point. One hour ± 1 min prior to sacrifice, all animals were injected ip with bromodeoxyuridine (BrdU) (100 mg/kg body weight) (Sigma Aldrich, St Louis, MO). At necropsy, the urinary bladder was inflated *in situ* with Bouin's fixative while the rat was under anesthesia but still alive, removed and placed in the same fixative along with a small section of the duodenum. It was essential to fix the bladder while the animal was alive because autolysis is detectable by SEM within 1 min of death (Cohen *et al*., 1996, 2007). Kidneys were removed after the bladder and duodenum, weighed and fixed in 10% buffered formalin. Following fixation, the bladders and intestinal tissue were rinsed in 70% ethanol, weighed, and observed macroscopically for abnormal findings. One half of each bladder was examined by scanning electron microscopy (SEM) and classified as described previously (Cohen *et al*., 1990). Enlarged urothelial cells protruding into the lumen of the bladder were evaluated as part of the classification for cytotoxicity. Briefly, class 1 bladders have flat, polygonal superficial urothelial cells; class 2 bladders have occasional small foci of superficial urothelial cell death; class 3 bladders have numerous small foci of superficial urothelial cell death; class 4 bladders have extensive superficial urothelial cell death, especially in the dome of the bladder; and class 5 bladders have extensive cell death and piling up (hyperplasia) of rounded urothelial cells. Normal rodent urinary bladders are usually class 1 or 2, or occasionally class 3. The other half of the bladder was cut longitudinally into strips and the intestinal tissue and sections from both kidneys were embedded in paraffin. Approximately 4–5 µm sections were stained with hematoxylin and eosin (H&E) and examined histopathologically (Cohen, 1983; Cohen *et al*., 1990). A diagnosis of mild simple hyperplasia was made when there were four to five cell layers in the bladder epithelium.

Unstained slides of the bladder and intestinal tissue from all animals were used for immunohistochemical detection of BrdU (Cohen *et al*., 2007). The duodenal tissue served as a positive staining control and to assess the adequacy of the BrdU injection. Also, slides from three control rats and five diuron-treated rats sacrificed on study day 7 were used for immunohistochemical detection of cleaved caspase-3 for assessment of apoptosis. The duodenal tissue served as positive control. Anti-BrdU (Millipore Corporation, Temecula, CA) was used at a dilution of 1:200. Anti-cleaved caspase-3 (Asp175) (Cell Signaling Technology, Beverly, MA) was used at a dilution of 1:10. For both assays, if the duodenum showed positive staining of the mucosa, at least 3000 urothelial cells (all layers) in the urinary bladder sections were counted to determine the number of labeled cells and expressed as the percent of labeled cells to total number of cells.

Experiment 2

Study design. The rats were randomized into two groups: Group 1 with 13 animals and Group 2 with 15 animals, using a weight stratification method (Martin *et al*., 1986). Following quarantine, Group 1 continued to receive the basal chow (Purina 5002) and Group 2 was changed to Purina 5002 supplemented with 2500 ppm diuron. Body weights were measured the day after arrival, just prior to randomization, and on days 7, 35, and 42 and the day of sacrifice. Animals were checked daily for moribundity and mortality. Water consumption and diet consumption were measured over a 7-day period starting on days 0 and 35. Detailed clinical observations were performed prior to randomization and at the end of each consumption period.

After 7 days of treatment, the three animals from the control group and five animals from the 2500 ppm diuron group were terminated by an overdose of Nembutal (150 mg/kg body weight; Lundbeck, Inc.) and the bladders removed. Half of each bladder was processed for and analyzed by TEM to further evaluate the diuron-induced swelling of the urothelial cells observed by SEM in the first experiment. The urinary bladders were inflated *in situ* with and placed in MPG fixative (2% paraformaldehyde/2.5% glutaraldehyde in phosphate buffer) for 4 h. The bladder tissue was then longitudinally bisected. One half was processed for examination by TEM in the Electron Microscopy Laboratory (Department of Anatomic Pathology, The Nebraska Medical Center). The other half was washed in PBS after fixation and processed for examination by SEM following the procedure and classification described in the first experiment (Cohen *et al*., 2007). For TEM, after bladder fixation, multiple 1 × 3 mm strips were dissected and fixed in MPG electron microscopy fixative (pH 7.38) for 2 h at 4°C. EM blocks were washed in 0.1M phosphate buffer (pH 7.4, 2 × 5 min), processed through a 6-h electron microscopy processing schedule and infiltrated with epon resin (Electron Microscopy Sciences, Fort Washington, PA) on a Leica EM TP automatic tissue processor. Tissues were embedded, oriented longitudinally and polymerized at 60°C for 24 h. Multiple 1-µm thick sections were stained with 1% toluidine blue in 1% borax and evaluated by light microscopy. Representative areas were selected, ultrasectioned at 70 nm (silver sections), and mounted on 300 mesh copper grids, double stained with Reynolds lead citrate and uranyl acetate and examined with a JEOL 1320 electron microscope.

After 8 weeks, the remaining 10 animals in each group were terminated and the bladder analyzed by light microscopy, SEM and immunohistochemical detection of BrdU as described in experiment 1. One hour ± 1 min prior to sacrifice, all animals were injected ip with BrdU (150 mg/kg body weight) (Sigma Aldrich). The necropsy, histopathology, immunohistochemical procedure, and evaluation followed the same methodology described in experiment 1. The presence of numerous swollen cells was included in the SEM analysis and classification, because it was recognized as a test substance-induced effect.

Statistics for Experiments 1 and 2

All values are reported as the mean ± SD. Group means for body and tissue weights, food and water consumption, and the BrdU and caspase labeling indices were evaluated using ANOVA, followed by Duncan's multiple range test for group-wise comparisons. Histopathology was compared using the two-tailed Fisher's Exact test. SEM data were analyzed using one-way nonparametric procedures followed by a chi-square test. P values less than 0.05 were considered significant. The statistical analyses were performed using SAS for Windows (Version 9.1).

RESULTS

Experiment 1

Clinical observation, body weight, water consumption, food consumption, and organ weight. There were no instances of animal morbidity or mortality, and none of the animals had any visible abnormalities. However, during bladder SEM evaluations, bacteria from an unknown source were observed on the bladder surface of three animals sacrificed on study day 28 (control group). The animals had no visible abnormalities during the course of the experiment, and water and food consumption and body weights were similar to other animals in the control group. SEM classification of the bladders from these animals was class 1 or 2 which is considered normal, and no abnormalities were observed during light microscopic examination of H&E slides of the bladder and kidney tissues, suggesting that the contamination occurred after the bladders were removed from the rats. However, due to the presence of the bacteria on the urothelial surface, all results for bladder and kidney histopathological diagnosis, SEM classification, and BrdU labeling index for these three animals were excluded from data analysis.

The animals treated with 2500 ppm diuron gained less weight during the experiment. Beginning on study day 7 and continuing throughout the study, the mean body weights of the 2500 ppm diuron group were significantly reduced compared with the control group (Supplementary Table 1). At all time points, the food consumption was decreased in the 2500 ppm diuron group compared with control. No difference in water consumption was detected (data not shown). Absolute and relative bladder weights were increased in the 2500 ppm diuron group compared with controls at all time points; however, the increase was significant only on study days 7 and 28. On study days 1 and 28, relative and absolute kidneys weights, respectively, were significantly decreased in the 2500 ppm diuron group compared with controls (Supplementary Table 2).

Histopathological examination of urinary bladder and kidneys. There was no significant incidence of changes in the bladder epithelium of rats treated with 2500 ppm diuron at any time point (Table 1). There were no treatment-related changes observed in the kidneys except for changes in the papilla and medulla in one diuron-treated rat suggestive of retrograde nephropathy.

SEM examination of the bladder epithelium. On study day 1, the SEM classification showed evidence of an effect on the epithelium due to the presence of swollen cells protruding into the lumen of three bladders (two bladders, occasional swollen cells; one bladder, numerous swollen cells) in the 2500 ppm diuron group. The presence of swollen cells was increased in the bladders from diuron-treated rats on study days 3 (6/10) and 7 (6/10) (Fig. 1). The SEM classification was significantly different compared with control only on study day 7; however, over the time course of the study, SEM analysis showed an increase in the number and size of foci of urothelial necrosis in the 2500 ppm diuron group resulting in a gradual increase in the class of the bladder surface. On study day 28 in the diuron-treated group, there were two rats with class 4 bladders showing extensive necrosis and exfoliation and one rat with a class 5 bladder with the same necrotic changes and, in addition, piling up of round cells indicative of hyperplasia (Table 1). These round cells showed ropy microridges and occasional uniform microvilli.

TABLE 1
Effects of Dietary Administration of Diuron at Early Time Points on the Urinary Bladder of Male Wistar Rats

		Histopathology			SEM classification				
Group	Treatment	Normal	Simple hyperplasia	BrdU labeling index (%) Mean ± SE (*n*)	1	2	3	4	5
Day 1									
1	0 ppm diuron	10	—	0.31 ± 0.31 (9)	5	3	2[a]	—	—
2	2500 ppm diuron	10	—	0.14 ± 0.10 (10)	6[b]	2[c]	2[b]	—	—
Day 3									
1	0 ppm diuron	10	—	0.24 ± 0.19 (9)	6	3[a]	1	—	—
2	2500 ppm diuron	10	—	0.34 ± 0.16 (10)	3[a,c]	4[c,d]	3[d]	—	—
Day 7									
1	0 ppm diuron	10	—	0.24 ± 0.32 (9)	7	2	1	—	—
2	2500 ppm diuron[e]	9	1	0.42 ± 0.37 (7)	2[f]	4[a,d,g]	4	—	—
Day 28									
1	0 ppm diuron[h]	7	—	0.24 ± 0.32 (7)	4[i]	2[j]	1	—	—
2	2500 ppm diuron	8	2	0.13 ± 0.09 (10)	2[k]	3	2	2	1

[a]Surface of one bladder covered by glycocalyx.
[b]Occasional swollen prenecrotic cells present on the surface of one bladder.
[c]Numerous swollen prenecrotic cells present on the surface of one bladder.
[d]Occasional swollen prenecrotic cells present on the surface of two bladders.
[e]SEM classification significantly different from 0 ppm diuron group, day 7, $p < 0.05$ (one-way nonparametric procedures followed by a chi-square test).
[f]Occasional swollen prenecrotic cells present on the surface of both bladders.
[g]Numerous swollen prenecrotic cells present on the surface of two bladders.
[h]Results for three animals in group excluded due to the presence of bacteria observed on the urothelial surface of the bladder by SEM.
[i]A few swollen prenecrotic cells present on the surface of two bladders.
[j]A few swollen prenecrotic cells present on the surface of one bladder.
[k]A few prenecrotic cells present on the surface of one bladder.

Immunohistochemical determination of BrdU and caspase labeling indices. There was no diuron-induced increase in the BrdU labeling index at any time point (Table 1). There was no increase in the cleaved caspase-3 labeling index on study day 7 (data not shown).

Experiment 2

Clinical observation, body weight, water consumption, food consumption, and organ weight. There were no instances of animal morbidity or mortality, and none of the animals had any visible abnormalities. Similar to experiment 1, from day 7 onward the animals treated with 2500 ppm diuron gained less weight than control ($p < 0.05$) (Supplementary Table 3). Food consumption and water consumption were similar in the control and diuron-treated groups except for a significant decrease in water consumption (g/rat/day) in the diuron-treated group during week 6 (data not shown). Although terminal body weights were decreased and the relative bladder and kidneys weights were increased in diuron-treated animals compared with control group, no statistical differences were detected (Supplementary Table 4).

Histopathology and BrdU labeling index. After 8 weeks of treatment, there was an increased incidence of mild simple hyperplasia (6/10, $p < 0.05$) in the 2500 ppm diuron-treated group compared with the control group (Table 2). In the control and diuron-treated groups, some rats showed minimal chronic progressive nephropathy that is considered to be an age-related change not a treatment-related change. No changes in the BrdU labeling index of the urothelium were detected.

TEM at 7 days. One micrometer sections from bladders from both groups showed the typical structure of the bladder, with urothelium and underlying connective tissue and smooth muscle wall. The urothelium showed the typical distended appearance. The diuron group showed various degrees of epithelial degeneration of all layers of the urothelium up to complete loss of the epithelium, but without penetration through the basement membrane (Fig. 2).

Ultrastructural examination of the urothelium from the control showed a normal urothelium from a distended bladder with three layers of cells (basal, intermediate, and superficial), with normal appearance. The basal cells from this group showed normal ultrastructure: cuboidal cells with normal cytoplasmic organelles. The intermediate layer maintained a somewhat more flattened appearance, with rare apoptotic cells intertwined in the layers.

In the diuron-treated rats, there were profound ultrastructural changes of the urothelium. Minimal changes comprised distention of the cytoplasm, organelles, and nuclei characteristic of cytolysis. This resulted in enlargement of the superficial cells and protrusion of part of the superficial cells into the lumen, corresponding to the swelling of the cells observed by SEM. Occasionally, there was also loss of the luminal cytoplasmic membrane and loss of ultrastructural detail of the cytoplasmic organelles, representing severe degenerative changes and cell death. For most of the

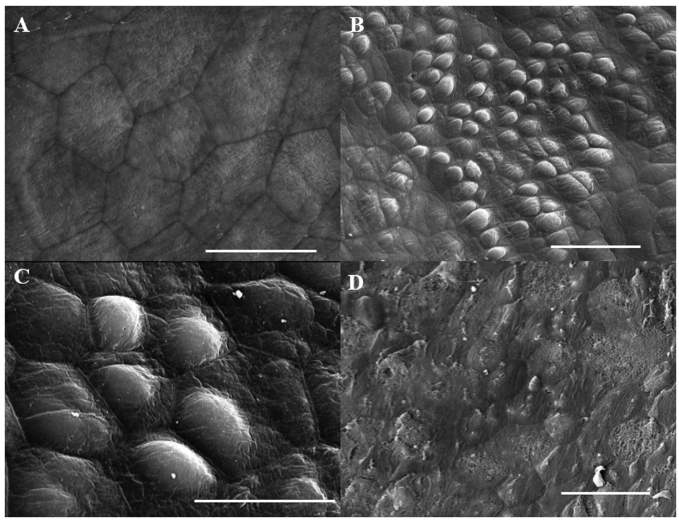

FIG. 1. (A) SEM of normal bladder showing flat, polygonal superficial cells, from control group, day 28 (bar = 100 µm). (B) SEM showing numerous swollen superficial cells protruding into the lumen, from a rat fed diuron for 3 days (bar = 100 µm). (C) Higher magnification of the swollen cells (bar = 50 µm). (D) Bladder surface showing extensive necrosis and exfoliation of superficial cells, from a rat fed diuron for 28 days (bar = 100 µm).

TABLE 2
Effects of Dietary Administration of Diuron for 8 Weeks on the Urinary Bladder of Male Rats

		Histopathology			SEM classification				
Group	Treatment	Normal	Simple hyperplasia	BrdU labeling index (%) Mean ± SE (*n*)	1	2	3	4	5
1	0 ppm diuron	10	0	0.21 ± 0.16 (10)	7	2	1	0	0
2	2500 ppm diuron*	4	6**	0.19 ± 0.14 (10)	1	0	4	4	1

*SEM classification significantly different from 0 ppm diuron group, $p < 0.05$.
**Significantly different from 0 ppm diuron group, $p < 0.05$.

affected area, the typical infolding of the cytoplasmic membrane was maintained, forming deep clefts and stacks of flattened plasmic membrane segments, the fusiform vesicles. However, in more severely affected areas, there was disruption to complete loss of the cell membrane on the luminal side of the superficial cell. In the most severely affected areas, there was loss of the superficial cells and occasionally loss of intermediate and basal cells. Although the basal lamina was not breached, degenerative changes comprised swelling of the underlying fibroblasts with increased intercellular spaces were identified and focally extended to the muscularis. There was no inflammatory cell infiltrate. Numerous cytoplasmic and nuclear fragments were present. For the urothelial cells that remained intact, the junctional complexes were evident although there was increased widening of the intercellular space and loss of cohesion between the cells. The nuclei in many of the surviving cells showed varying degrees of karyolysis and karyorrhexis with irregular contouring and widening of the nuclear envelope. There was loss of margination and clumping of the heterochromatin. The number of primary and secondary lysosomes was increased, particularly in the superficial cells, and some of these were greatly enlarged. Many of the urothelial cells showed varying degrees of cytolysis with loss of organelles (Fig. 2).

SEM examination of the bladder epithelium at 7 days and 8 weeks. On study day 7, swollen cells were present in four bladders of the treated group (two occasional and two numerous); however, none of the bladders showed extensive necrotic or proliferative changes typical of classes 4 and 5 (data not shown). Red blood cells were detected on the surface of bladders in both groups, but were considered to result from artifactual damage during necropsy. It did not affect the urinary bladder classification.

At 8 weeks, there was a significant difference in the SEM classification of bladders in the diuron-treated group compared with the control group. There were four bladders in the diuron group with extensive areas of necrosis and exfoliation (class 4) and one bladder with piling up of round cells (hyperplasia) (class 5) (Table 2).

DISCUSSION

The postulated MOA for diuron-induced urinary bladder tumors in rats is cytotoxicity with prolonged, sustained, consequent regenerative proliferation, leading to urothelial

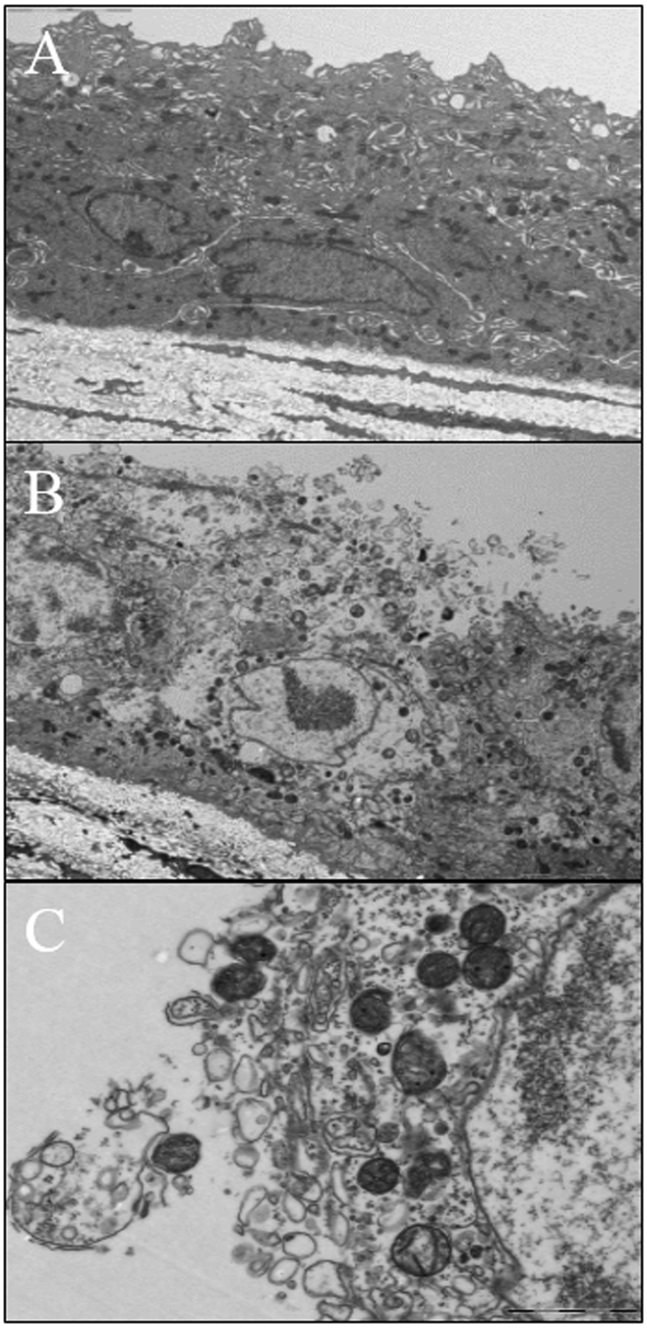

FIG. 2. (A) TEM of normal bladder, from a control rat after 7 days. (B) TEM showing cytolysis, with enlargement of the cytoplasm, swelling of organelles, and disruption of luminal membrane, from a rat fed diuron for 7 days. (C) Higher magnification of (B) showing greater detail of organelle changes.

hyperplasia and ultimately bladder tumors. The present studies support this MOA. The earliest change appears to be the swollen superficial cells detected by SEM already evident 1 day after the start of treatment. The swollen cells increase in number, with clear evidence of necrosis and cell loss (exfoliation) by day 7. The extent of cytotoxicity continues to increase after that. Although there is evidence of increased proliferation by 28 days as detected by SEM and increased BrdU labeling index (not statistically significant), we did not observe significant hyperplasia by light microscopy until 8 weeks of treatment. These observations suggest that the effect of diuron treatment on the rat bladder urothelium is relatively mild and the changes are gradual. Previous studies have shown greater degrees of necrosis and proliferation by 15 and 20 weeks of administration (Da Rocha *et al.*, 2010; Nascimento *et al.*, 2006).

Our results showed the presence of mild diuron-induced changes to the urothelial cells as early as study day 1, as evidenced by the presence of swollen urothelial cells. The presence of these swollen cells increased by study day 3 and persisted through study day 7. TEM analysis on study day 7 of diuron exposure showed that these swollen urothelial cells had cytoplasmic and nuclear swelling typical of degeneration, progressing to actual necrosis and cell loss in some foci. A similar phenomenon occurs in the liver and is called ballooning degeneration or oncosis (Majno and Joris, 1996). It can progress to necrosis or it can reverse if the stimulus is removed.

In previously reported studies with diuron, swollen urothelial cells have not been observed by SEM. This is likely due to a difference in methodology. In previous studies, the urinary bladders were processed for SEM examination using osmium tetroxide fixation (Da Rocha *et al.*, 2010) which causes the urothelial cells to contract. In the present studies, we did not use osmium for tissue processing.

Evidence of cytotoxicity occurred quickly after diuron administration began. Hyperplasia was observed as early as 7 days in the diuron-treated group by light microscopy. Somewhat surprisingly, no significant increase in BrdU labeling index was detected even though light microscopy and SEM evidence of increased cell proliferation was detected on day 28 and was significantly increased by 8 weeks.

The incidence of hyperplasia was 1 of 10 at day 7, 2 of 10 at day 28, and 6 of 10 at 8 weeks, and these were all mild in degree. The labeling index is a rate determined by the number of labeled cells divided by the total number of cells in that population. It reflects the rate of replication, not the actual number. Hyperplasia represents an increase in cell number. However, because bladder shows a low proliferative rate and hyperplasia increases the number of cells, the rate in the labeling index could decrease even with a proliferative response as observed at 28 days and 8 weeks. The main parameter is the number of DNA replications that can either be increased by decreasing the number of cell deaths or, more commonly in epithelial tissues in response to chemical toxicity, increasing the number of cell births, which could be followed by regenerative cell proliferation (Cohen, 1998). Hyperplasia is usually accompanied by an increased proliferation rate, but not always. It could occur without an increase in the BrdU labeling index by inhibiting cell death, either by inhibiting differentiation or inhibiting apoptosis. We did not observe any changes in apoptosis.

Simple hyperplasia was present after treatment with diuron for 20 weeks (Nascimento *et al*., 2006). The simple hyperplasia ranged from mild to severe, with greater severity observed than what was seen in the present studies of up to 8 weeks in duration. Cell proliferation was present in these longer studies as evidenced by an increase in the PCNA labeling index. However, expression of PCNA increases during phase G1, peaks in phase S, and declines during phases G2 and M, whereas BrdU is incorporated into nuclei during the S phase of the cell cycle. Diuron is a relatively weak carcinogen. The mild cytotoxicity and proliferation observed after high doses at early exposure times appeared to increase over time when the high level of exposure was sustained. This continued exposure maintains cytotoxicity and a compensatory proliferative response, which could eventually lead to tumor development. Gene expression changes observed at 7 days (Ihlaseh et al., unpublished data) and 20 weeks (Ihlaseh *et al*., 2011) of diuron dietary exposure showed significant differences, suggesting an initial adaptive response to protect the urothelium from the diuron toxicity that is overcome with long-term exposure (Ihlaseh *et al*., 2011).

Increased proliferation of the urothelium occurs either due to direct mitogenesis, with only one known example (propoxur) (Cohen *et al*., 1994), or more commonly due to increased cell deaths and regeneration (Cohen, 1998). Increased cell death is usually due to necrosis. Regenerative proliferation ensues which is dependent for its continuance on sustained exposure to the inciting stimulus.

Cytotoxicity and necrosis of the urothelium can be caused by urinary solids, cytotoxic chemicals (parent compound and/or a metabolite), or extremes of urinary composition, such as pH > 9 (Cohen, 1998). It has previously been shown that diuron does not induce a change in urinary pH, and urinary solids do not play a role in the rat urinary bladder carcinogenesis induced by high dietary concentrations of diuron. Thus, cytotoxicity appears to be chemically induced by diuron and/or its metabolites (Da Rocha *et al.*, 2010). Urinary bladder gene expression analysis of diuron-treated Wistar rats suggested that persistent exposure to high dietary concentrations of diuron induced oxidative stress, increased cellular metabolism, and enhanced cell death associated with sustained urothelial hyperplasia (Ihlaseh *et al*., 2011).

Although the specific mechanism leading to diuron-induced necrosis is unclear, our results strongly suggest that the MOA of diuron-induced rat bladder carcinogenesis starts with urothelial cell degeneration evidenced by the presence of the swollen urothelial cells. Continued exposure to diuron resulted in necrosis and exfoliation detected by SEM and TEM, with consequent cell proliferation evidenced by hyperplasia at 8 weeks as detected by SEM and light microscopy. These data suggest that cytotoxicity followed by regenerative cell proliferation are the sequential key events of diuron-induced urothelial carcinogenesis in the urinary bladder epithelium that occur after long-term high-diuron exposure in rats.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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MANUSCRIPT 04

ORIGINAL RESEARCH ARTICLE

Diuron Metabolites and Urothelial Cytotoxicity:

In vivo, *In vitro* and Molecular Approaches

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ABSTRACT

Diuron is carcinogenic to the rat urinary bladder at high dietary levels. The proposed mode of action (MOA) for diuron is urothelial cytotoxicity and necrosis followed by regenerative urothelial hyperplasia. Urothelial cytotoxicity can be induced either by urinary solids or by chemical toxicity. However, diuron-induced urothelial cytotoxicity is not due to urinary solids. Diuron is extensively metabolized, and in rats, N-(3,4-dichlorophenyl) urea (DCPU) and 4,5-dichloro-2-hydroxyphenyl urea (2-OH-DCPU) were the predominant urinary metabolites; lesser metabolites included N-(3,4-dichlorophenyl)-3-methylurea (DCPMU) and trace levels of 3,4-dicloroaniline (DCA). In humans, DCPMU and DCPU have been found in the urine after cases of product abuse. To aid in elucidating the MOA of diuron and to evaluate the specific metabolites that are responsible for the diuron toxicity in the bladder epithelium, we investigated the *in vitro* urothelial cytotoxicity of the metabolites and their gene expression profiles. Also, the urinary metabolite concentrations in male Wistar rats treated with 2500 ppm of diuron were determined. For the rat diuron-induced MOA, we suggest that DCPU is the primary metabolite responsible for the urothelial cytotoxicity. DCPU was found in rat urine in concentrations above the IC50. Moreover, DCPU induced more gene expression alterations than the other metabolites tested. Our studies support a MOA for diuron-induced bladder effects in rats consisting of metabolism to DCPU (and 2-OH-DCPU to a lesser extent), concentration and excretion in urine, cytotoxicity, and regenerative proliferation.

Key words:

Bladder, diuron, metabolites, cytotoxicity, DCPU, DCPMU, DCA.

INTRODUCTION

Diuron (3-(3,4-Dichlorophenyl)-1,1-dimethylurea) is a phenylurea herbicide carcinogenic to the urinary bladder of rats at high dietary levels. The United States Environmental Protection Agency (U.S.EPA) in 1996 classified diuron as a "known-likely" human carcinogen based on a two-year bioassay. After continuous dietary exposure at 2500 ppm, Wistar rats of both genders showed an increased incidence of urinary bladder papillomas and carcinomas with a higher incidence in males (35/48) than in females (13/49) (Iyer, 2002).

Male Wistar rats treated with diuron also developed a renal pelvis papilloma and two carcinomas. Diuron-treated female NMRI mice developed mammary gland carcinomas after continuous diuron dietary exposure at 2500 ppm but not at 250 ppm. Although the incidence of mammary gland tumors was significantly higher than the control group (6/50 compared to 2/50) the incidence was within the range of the historical controls from the same test facility and other laboratories indicating that it was not treatment related. The rat urinary bladder is considered to be the target organ for diuron-induced carcinogenesis in rodents. No urothelial carcinogenic effect was observed in mice.

Diuron is used in crop and non-crop areas, such as industrial sites and on rights-of-way, and as an algaecide in commercial fish production and a preservative in paints and stains. It has been used on 33 different crops, including sugar cane, citrus fruit, cotton, cocoa, coffee, alfalfa, grapes, berries, asparagus, pineapple and others. [Australian Pesticides and Veterinary Medicine Authority (APVMA), 2005; U.S.EPA, 2003]. In Brazil, 9245 metric tons of diuron was used in 2011 (*personal communication*). Diuron acts as an herbicide inhibiting photosynthesis by preventing oxygen production in plants, and it is broken down in the environment both by biotic

and abiotic processes. Diuron and its metabolites can be found in the environment, including soil, water, sediments, and groundwater (Giacomazzi and Cochet, 2004).

In rodent studies, male Wistar rats exposed through the diet for 20 weeks to the a carcinogenic dose (2500 ppm) of diuron showed increased urothelial cell proliferation and simple hyperplasia detected by light microscopy (Nascimento et al., 2006). Scanning electron microscopy (SEM) evaluation also showed urinary bladder mucosal necrosis by 15 and 30 weeks after high dose diuron administration (Da Rocha et al., 2010). In a short-term study with the same dose, SEM of the bladder epithelium showed the presence of mild diuron-induced changes evidenced by the presence of swollen urothelial cells within 1-7 days. Transmission electron microscopy (TEM) analysis on study day 7 showed that these swollen urothelial cells had cytoplasmic and nuclear swelling typical of degeneration, progressing to actual necrosis and cell loss in some foci. SEM by day 28 showed extensive necrosis, exfoliation and piling up of cells suggestive of hyperplasia. Continued exposure to diuron resulted in necrosis and exfoliation detected by SEM and TEM with consequent cell proliferation as evidenced by hyperplasia at 8 weeks (Da Rocha et al., 2012).

Based on these studies, the key events for the mode of action for diuron-induced urinary bladder tumors at high doses are cytotoxicity, starting with urothelial cell degeneration, and continued exposure resulting in necrosis and cell exfoliation, followed by sustained regenerative cell proliferation leading to hyperplasia and ultimately bladder tumors. As a non-genotoxic compound (USEPA, 2003; Nascimento et al., 2006; Da Rocha et al., 2010), diuron cytotoxicity could be induced either by formation of urinary solids or by chemical toxicity by the parent compound and/or its metabolites in the urine. However, diuron-induced urothelial cytotoxicity is not due to urinary solids (Da Rocha et al., 2010), and therefore must be chemically induced.

Diuron is absorbed from the gastrointestinal system and excreted in urine and feces. Diuron is extensively metabolized, and in rats, N-(3,4-dichlorophenyl) urea (DCPU) and 4,5-dichloro-2-hydroxyphenyl urea (2-OH-DCPU) were the predominant urinary metabolites; lesser metabolites included N-(3,4-dichlorophenyl)-3-methylurea (DCPMU) and trace levels of 3,4-dicloroaniline (DCA) (APVMA, 2005). In humans, DCPMU and DCPU were found in the urine after cases of product abuse (Boven et al., 1990) with exposures considerably higher than with usual occupational or environmental exposures.

Wistar rats fed different concentrations of diuron – 0, 60, 125, 1250 and 2500 ppm – had urinary bladder mucosal alterations detected by microscopy and transcriptional changes by microarray analysis after 20 weeks of treatment. The dose response was characterized by gradual enhancement of morphological changes and with the number of significantly modulated genes increasing progressively with increasing diuron doses. Microarray analysis showed clear differences in gene expressions at the higher doses (1250 and 2500 ppm) in comparison with the lower doses (60 and 125 ppm). The major categories of altered pathways after exposure to high doses of diuron included amino acid, lipid, phase I, and phase II metabolism, and oxidative stress response. These data suggest that extended exposure to high dietary concentrations of diuron induces oxidative stress, increases cellular metabolism, and enhances cell death which is associated with a sustained regenerative urothelial hyperplasia (Ihlaseh et al., 2011). In a seven-day study, the gene expression profile was different compared with 20 weeks exposure (Ihlaseh et al., unpublished data), suggesting an initial adaptive response to protect the urothelium from the diuron toxicity that is overcome with long term exposure (Ihlaseh et al., 2011).

To aid in elucidating the MOA of diuron and to evaluate the specific metabolites that are responsible for the diuron toxicity in the bladder epithelium, the aims of this study were: 1) Investigate the *in vitro* cytotoxicity of the metabolites DCPU, 2-OH-DCPU, DCPMU and DCA and the gene expression profiles of DCPU, DCPMU and DCA in rat (MYP3) and human (1T1) urothelial cell lines; 2) determine the urinary metabolite concentrations in male Wistar rats treated with 2500 ppm of diuron.

MATERIALS AND METHODS

In vivo

Test material

Diuron was provided by E.I. DuPont de Nemours and Company (Wilmington, DE). The purity of the test material as determined by Advinus Therapeutics Private Ltd. (Bangalore, India) was 98.6%. The diet concentration of 2500 ppm diuron was adjusted based on the purity. The test material was administered in Certified Purina Rodent LabDiet® 5002 (PMI Nutrition International, Richmond, IN) at a concentration of 2500 ppm. The test diet was analyzed by DuPont Haskell Global Centers for Health and Environmental Sciences (Newark, DE) for homogeneity/concentration verification and stability by high performance liquid chromatography (HPLC) with ultraviolet (UV) detection. Analytical results from homogeneity/concentration verification testing of the test diet used in this study showed that diuron was homogeneously mixed into the diet at a concentration of 2500 ppm.

Test animals

Wistar rats 5-weeks old were purchased from Charles River Laboratories, Inc. (Raleigh, NC). This is the same species and strain of animal used in the 2-year carcinogenicity study. On arrival, the animals were placed in an animal facility

accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and quarantined for 1 week before starting treatment. The study protocol was approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee (IACUC), and the level of care provided to the animals met or exceeded the basic requirements outlined in the Guide for Care and Use of Laboratory Animals (NIH Publication #86-23, revised 2011). The animals were housed 2 per cage in polycarbonate cages with dry corn-cob bedding in a room with a targeted temperature of 22^{o}C, humidity of 50% and a light/dark cycle of 12 hours (0600 lights on/1800 lights off) and fed basal chow (Purina 5002). Diet and water were available *ad libitum* throughout the study. These are the same animals used for light and scanning electron microscopic analysis reported previously (Da Rocha et al., 2012).

Study Design

Following quarantine, Group 1 continued to receive the basal chow (Purina 5002) and Group 2 was changed to Purina 5002 supplemented with 2500 ppm diuron. Animals were checked daily for moribundity and mortality. Animals were acclimated to metabolism cages for 24 hours before urine collection. Food and water were available *ad libitum* during acclimation and during urine collection (Cohen et al., 2007). Twenty-four-hour urine samples were collected on ice from 3 animals in the control group and 5 animals in the diuron-treated group during study week 4. The urine volume was determined in our laboratory and creatinine concentration (Beckman Coulter DxC 800, Beckman Coulter Inc., Brea, CA) was determined by The Nebraska Medical Center Clinical Laboratory. The urinary samples were kept frozen for approximately 9 weeks until shipment for the metabolite analysis. The urinary concentrations of diuron and its metabolites DCPU, 2-OH-DCPU, DCPMU and DCA were determined by HPLC/MS (ABC Laboratories, Columbia MO).

In vitro

Test materials

Diuron metabolites DCPU, 2-OH-DCPU, DCPMU and DCA were tested for their relative cytotoxicity to rat and human urothelial cells *in vitro*. Many attempts were made to solubilize diuron (DMSO, acetone, ethanol) for an *in vitro* assay. However, diuron has very low solubility in water (42mg/L), and it precipitated after a few minutes in solution or the solvent concentrations that kept diuron in solution were too high and cytotoxic to the cells. Based on studies in the literature, diuron is largely metabolized and not detected in rat urine (APVMA, 2011); for this reason only the metabolites were tested *in vitro* for cytotoxicity. DCA, DCPU and DCPMU were purchased from Sigma-Aldrich (St. Louis, MO). DCA was purchased with certificate of analysis of 99%. The purity of DCPU and DCPMU were verified by NMR at the University of Nebraska Medical Center. 2-OH-DCPU, 99% purity, was received as a gift from E.I. DuPont de Nemours and Company (Wilmington, DE). All test materials were considered 100% pure for calculations of concentrations, stored at room temperature and protected from light.

Urothelial cell lines

The MYP3 rat urothelial cell line and the 1T1 human urothelial cell line were provided by Dr. Ryoichi Oyasu (Northwestern University, Chicago, IL) and were used to assess the cytotoxic effects of the test substances on urothelial cells. The MYP3 cell line was obtained from a small nodule that developed in a heterotopically transplanted rat urinary bladder after treatment with N-methyl-N-nitrosourea (MNU) (Kawamata et al., 1993). The cell line has retained the characteristics of epithelial cells in culture, expresses keratin 5 mRNA, does not exhibit anchorage-independent growth, and does not cause development of tumors in nude mice. The 1T1 cell line was derived from

normal human ureter epithelium and immortalized by transfection of the HPV-16 E6 and E7 genes (Tamatani et al., 1999).

Cell culture conditions

MYP3 cells were grown in Ham's F-12 medium (Gibco-BRL, Grand Island, NY) supplemented with 10 µM non-essential amino acids, 10 ng/ml EGF, 10 µg/ml insulin, 5 µg/ml transferrin, and 100 U/ml penicillin, and 100 µg/ml streptomycin (supplied as a penicillin and streptomycin mixture) (all from Gibco), 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 250 mg/ml dextrose and 1 mg/ml hydrocortisone from Sigma (St. Louis, MO). 1T1 cells were cultured in Keratinocyte-SFM (1x) supplemented with bovine pituitary extract (25 mg minimum) and human recombinant EGF (2.5 µg minimum) (Gibco-BRL, Grand Island, NY). All cells were grown at 37^{o} C in 5% CO_2.

Evaluation of cytotoxicity

The cytotoxicity of diuron metabolites on MYP3 and 1T1 cells was assessed by viability determination. Diuron metabolites were dissolved in 0.1% DMSO prior to dilution in the appropriate medium for the cell line to the concentrations used for testing. Each concentration was tested in 5 wells. MYP3 cells were seeded at a concentration of 4000 cells/well and 1T1 cells were seeded at a concentration of 6000 cells/well into 96-well plates. Treatment began 24 hours after seeding and continued for 3 days with no change in medium. At the end of treatment, cell viability was determined by the MTT assay. For each plate, the % survivability for each treated well was calculated as the ratio of the mean absorbance of the treated wells to the mean absorbance of the negative control (untreated cells). The means ± SE from all experiments for each chemical were subjected to non-linear regression analysis to determine the IC50. Non-linear regression

analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Gene Expression analyses

RNA extraction

For each cell line, in a six-well plate, three wells per metabolite were treated for 3 days after 24 hours seeding without changing the medium using the IC50 concentration of DCA, DCPU and DCPMU determined in the experiment described previously. One extra plate with one well for each metabolite was used as control to verify the cell number, and to ensure that the IC50 dose for each metabolite was effective for all metabolites. Three wells were used for the control group containing medium in 0.1% DMSO, the same concentration previously used for the metabolite dilution for the cytotoxicity experiment. RNA was isolated from each well using the Qiagen RNeasy Mini Kit (Catalog # 74034) following kit instructions. After isolation, the quantity of isolated RNA was confirmed using optical density measurements (Nanodrop 1000 3.7.1, Thermo Fisher Scientific, Waltham, MA). The quality of isolated RNA was evaluated by optical density measurements at 260 nm and calculation of 260 nm/280 nm and 260 nm/230 nm absorbance ratios. The quality of RNA was analyzed by the Agilent 2100 Bioanalyzer. Only samples having an RNA integrity number (RIN) >7.0 were used for microarray analysis. RNA extracted from the controls and treated cells in each well was analyzed on individual microarray chips. For each cell line and each metabolite, the RNA from the three wells were extracted individually and used for one individual microarray chip totalizing 3 chips per metabolite and control, or 12 chips for the 1T1 cell line and 12 chips for the MYP3 cell line. The RNA was evaluated by the University of Nebraska DNA Microarray Core Facility.

Affymetrix microarray hybridization

Gene expression analyses were conducted using Affymetrix GeneChip Rat Genome 230_2.0 Arrays containing over 31,000 probe sets representing ~28,700 known rat genes and Human Genome U133 Plus_2.0 Arrays representing more than 54,000 probe sets and 1,300,000 distinct oligonucleotide features (Affymetrix, Santa Clara, CA).

Microarray data analysis

Data quality was assessed using R-package affyPLM (Bolstad et al., 2005; R Development Core Team 2004). Low-level analysis, which converts probe level data to a probe set level data on the $\log_2$ scale, was done using robust multiarray average (RMA) in R (Irizarry et al., 2003). The probe set was retained if the percentage of present calls in the MAS 5.0 detection calls exceeded 60% in at least one group. Probe sets labeled as controls were excluded from further analysis. Probe sets with low expression were filtered out as well. These filtering procedures resulted in a subset of 26,317 probe sets for later analyses in the experiment with human cells and 17,083 probe sets for later analyses in rat cells.

The differential expression analysis was conducted with Limma (Linear Models for Microarray Analysis) package in Bioconductor developed by Gordon Smyth (Smyth, 2004). A linear model was fitted to the normalized expression data for each probe set. An empirical Bayes method was used to compute a moderated t-statistic for each comparison on each probe set. By shrinking the probeset-wise sample variances towards a common value and so increasing the degree of freedom for the individual variances, this method borrowed information across probe sets making the analyses stable even for experiments with small numbers of arrays (Smyth, 2004). The associated p-values for moderated t-statistics were adjusted by the false discovery rate (FDR)

method of Storey and Tibshirani (Storey and Tibshirani, 2003) due to multiple hypotheses testing. Probe sets with adjusted p-values below 0.05 and at least 2.0 fold changes were selected and classified subsequently whether they were significantly up or down regulated.

Pathway analysis was perfomed using Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, http://www.ingenuity.com). The most significant biological functions and altered canonical pathways associated with the probe sets for each metabolite were determined using IPA.

Real time PCR

The most differentiated genes considered relevant for this study obtained from the microarray analysis, were confirmed by real time PCR (qPCR). The same RNA samples used for the microarray analysis were used in qPCR. cDNAs were obtained from the samples using the High Capacity RNA-to-cDNA Kit (Life Technologies, Grand Island, NY) following the kit instructions and submitted to qPCR in a StepOnePlus^{tm} Real-time PCR System (Applied Biosystems, Grand Island, NY) for gene expression quantification. A Singleplex reaction was performed using the Inventoried Assays from Taqman® Gene Expression Assays (Life Technologies, Grand Island, NY) targeting MMP1 (Hs00899660_g1) and MMP10 (Hs00233987_m1) in human samples and MMP3 (Rn00591740_m1) and MMP13 (Rn01448199_m1) in rat samples. Actb (Hs99999903_m1) and Actb (Rn00667869_m1) were used to normalize gene expression levels for human samples and rat samples, respectively. Normalization for the results for the gene expression used the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

RESULTS

In Vivo

There were no instances of animal morbidity or mortality, and none of the animals had any visible abnormalities. No differences in the urine volume or creatinine levels were detected (data not shown). The mean of the 5 individual samples submitted for HPLC/MS chromatography analysis of 24-hour urine revealed the metabolite DPU present at the highest concentration followed by 2-OH-DCPU (Table 1). The concentration of DPU was greater than 488 µM in all treated samples. However, the determination of the exact concentration was not performed since the highest standard was 10 ug/ml or 48.8 uM, and a x10 dilution of the sample was the maximum dilution used. OH-DCPU was present at 125 uM, DCA was at 35 uM and DCPMU showed the lowest urinary concentration at 13 uM. Surprisingly, urine samples contained diuron at a concentration of 54 uM, however, since fasting the animals during collection is not appropriate to urine evaluation (Cohen, et al. 2007), our animals received diet during the 24 hour collection period, and it is possible that the presence of diuron in the urine samples was related to diet powder dropping into the collection tubes despite efforts to exclude it. No diuron or metabolites were detected in the urines of the control group (Table 1).

In Vitro

Determination of IC50 for MYP3 cells

The IC50 for DCPU, 2-OH-DCPU, DCPMU and DCA was 185, 230, 104 and 213 uM, respectively (Table 1).

Determination of IC50 for 1T1 cells

The IC50 for DCPU, 2-OH-DCPU, DCPMU and DCA was 157, 329, 224 and 72 uM, respectively (Table 1).

Gene Expression Analyses

MYP3 rat urothelial cells

The gene expression analysis in MYP3 cells showed a total of 17,083 probe-sets available for analysis after filtering. The metabolite with the highest number of altered probe sets compared to the control group was DCPU with 893, followed by DCPMU with 158 and DCA with 108. In the rat cells, DCA and DCPMU showed a higher number of up-regulated probe sets than down-regulated. However, DCPU showed a higher number of down-regulated probe-sets (Table 2). The number of unique probe-sets altered for each metabolite was 740, 31 and 13 for DCPU, DCPMU and DCA, respectively; DCPU and DCPMU shared the highest number of common genes (Figure 1).

It is interesting to note that for all metabolites, the highest fold change differences compared to controls were present in the up-regulated genes. Moreover, all of the metabolites had the same top two up-regulated genes: MMP3 and MMP13 (Supplementary Table 2; Figure1). Especially noteworthy, the metabolite DCPU showed a large number of up-regulated chemokine genes. The list of the top differentiated genes for each metabolite using a cutoff of 0.05 for adjusted p-value and at least 2.0-fold changes in the microarray analyses can be found in the Supplementary data (Table 1).

IPA analysis showed that the networks most affected by metabolite induced gene expression alterations were associated in general with cell-to-cell signaling and interaction, cellular movement, cell morphology, cellular growth and proliferation, cell cycle, cell death, cellular development, tissue morphology, tissue development, lipid

metabolism, small molecule biochemistry, inflammatory response, immune cell trafficking, and antigen presentation.

The top four biological functions most affected by DCPU- and DCPMU-altered genes were: inflammatory response, genetic disorder, cancer and dermatological diseases and conditions, but not in the same order. DCA also induced cancer and genetic disorders gene alterations. In addition to the significant up-regulation of MMP3 and MMP13 genes, canonical pathways affected by DCPU were aryl hydrocarbon receptor (Ahr) signaling and tumor necrosis factor receptor 1 (TNFR1) signaling showed a significant alteration (Figure 2). Ahr expression was also altered by DCA and DCPMU, but with a lower response.

1T1 human urothelial cells

The gene expression analysis in 1T1 cells showed a total of 26,317 probe-sets available for analysis after filtering. Contrary to observations in rat cells, the metabolite with the highest number of altered probe sets compared to the control group was DCA with 2296, followed by DCPMU with 1242 and DCPU with 443. In the human cells, all groups showed more up-regulated probe sets than down-regulated, especially DCPU with 71% of the total up-regulated (Table 2). The number of unique probe-sets altered for each metabolite was 1762, 626 and 108 for DCA, DCPMU and DCPU, respectively; DCA and DCPMU shared the highest number of common genes (Figure 1).

However, like the rat cells, metalloproteinase genes were among the top up-regulated genes with metabolite exposures (Supplementary Table 3; Figure 2). DCPU and DCPMU induced the highest fold change in MMP1 and MMP10 , and DCA exposure resulted in MMP1 as the highest up-regulated gene and MMP10 as the fifth highest.

The top networks affected by DCPU were cellular growth and proliferation, cell death, cellular movement, lipid metabolism, small molecule biochemistry, immune cell trafficking and free radical scavenging. DCPMU induced alterations in cell cycle, cellular assembly and organization, DNA replication, recombination and repair and cell cycle. DCA also induced alterations in cell cycle, cellular assembly and organization, DNA replication, recombination and repair as it top networks. The top biological function affected by all metabolites was cancer. However, the canonical pathways showed some differences.

Some of the pathways altered by DCPU involved p38 MapK signaling, TGF-beta signaling and Ahr receptor signaling. DCPMU affected DNA double strand break repair by homologous recombination, role of BRCA1 in DNA damage response, TGF-beta signaling and mitotic roles of polo-like kinase. DCA also affected genes in the mitotic roles of polo-like kinase pathway, cyclins, cell cycle regulation and NRF-2 oxidative stress response.

The rat (MYP3) cells real time PCR showed an overexpression of the MMP3 and MMP13, confirming the findings in the microarray analysis. Even with an underestimation in gene expression of the human (1T1) cell samples, probably due to the presence of PCR inhibitor enzymes, the real time PCR also showed an overexpression of the MMP1 and MM10, consistent with the microarray data.

DISCUSSION

In the present studies, the metabolite with the highest concentration in the urine of male Wistar rats fed 2500 ppm diuron was DCPU (>488uM), followed by 2-OH-DCPU (125 uM). DCPMU (13.5 uM) and DCA (35.4 uM) were found at considerably lower levels in the urine compared to the levels of DCPU and 2-OH-DCPU. In addition,

we found low levels of diuron (54.4 uM) in the urine of diuron treated animals, however, as explained earlier, this may be due to contamination of the urine samples with treatment diet containing diuron during collection.

In metabolic studies, Sprague-Dawley rats received a single oral dose of [14C]-diuron or multiple doses of unlabeled diuron for 14 days followed by [14C]-diuron at 10 and 400 mg/kg body weight (APVMA, 2011). Diuron was readily absorbed and almost completely metabolized in 24 hours. Metabolites were mainly excreted in the urine (80-91%). As in our study, the predominant urinary metabolites were DCPU and 2-OH-DCPU with lesser amounts of DCPMU and trace levels of DCA. No diuron was detected in the urine, but a small amount was detected in the feces.

Male Wistar rats developed a higher incidence of tumors compared to female rats (U.S.EPA, 2003), and some studies showed that diuron metabolism between male and female rats is different (APVMA , 2011; Kinoshita et al., 1970). In subacute studies via inhalation, male and female Wistar rats were exposed to diuron aerosol at 4.1, 37.4 and 268 mg/m^3 5 days per week for 8 weeks (APVMA, 2011). Twenty-four-hour urine specimens were collected at weeks 4 and 8. The main metabolite excreted in urine was DCPU, and at the two high doses (37.4 and 268 mg/m^3), males excreted more DCPU than females. DCPMU, DCA and small amounts of diuron were also detected. In a study with the Holtzman rat strain, differences in hepatic microsomal enzyme inducing activity were observed between males and females (Kinoshita, 1970).

Similar to the rat, DCPU was the dominant metabolite the urine of dogs fed 125 ppm diuron for one month or 1250 ppm for 2 years, with small amounts of DCPMU, DCA, 3,4-dichlorophenol (DCP) and unchanged diuron also detected. There was a dose responsive increase in the DCPU concentration in the urine at 1250 ppm compared to the concentration at 125 ppm dose. However, no differences were detected in the

urinary concentrations of metabolites between males and females (Hodge et al., 1967) in contrast to rats.

In humans, in a case of product abuse, DCPMU was the metabolite with the highest concentration in the urine followed by DCPU indicating some differences in diuron metabolism between species (Boven et al., 1990). Only trace amounts of DCA (0.2 ppm) were found in the urine, and unchanged diuron was not detected. In addition, in isolated human liver cell homogenate, diuron was a relatively potent inhibitor of CYP1A1/2 (Abass, 2007). When N-demethylation formation was investigated in liver microsomes isolated from various species, the rank order based on intrinsic clearance for different species was dog > monkey > rabbit > mouse > human > minipig > rat (Abass, 2007).

For the rat, cytotoxicity is the initial key event for the mode of action for diuron-induced urinary bladder tumors at high doses (Da Rocha et al., 2012). DCPMU (IC50, 104 uM) was the most cytotoxic to rat MYP3 cells, followed by DCPU (185 uM), however, DCPMU was not present in rat urine (13±5 µM) at cytotoxic concentrations, so it is likely DCPMU made little contribution to the urothelial effects of diuron. Because DCPU was found at concentrations in the urine (>488 uM) that were considerably higher than the *in vitro* cytotoxicity IC50 of 185 uM we suggest that DCPU is the primary metabolite responsible for the urothelial cytotoxicity observed in diuron-treated rats. Moreover, DCPU induced more gene expression alterations (893) in the rat urothelial cell line compared with DCPMU (158) and DCA (108).

As noted above, there are metabolism differences between species, especially between rats and humans (Boven et al., 1990; Abbas, 2007). In humans, the main metabolite appears to be DCPMU, in contrast to the rat where DCPU is the main metabolite. *In vitro* and molecular analysis also showed differences between rat and

human cell lines in response to treatment with the different metabolites. . Contrary to the results found with the rat MYP3 cells , in the human 1T1 cells, DCA was the most cytotoxic metabolite *in vitro* and altered more genes than the other metabolites. However, only trace amounts of DCA were found in human urine in the case of product abuse (Boven et al., 1990), and we and others (APVMA, 2011) found only small amounts of urinary DCA in diuron-treated rats. Thus, DCA does not contribute significantly to the rat urothelial effects of diuron and should not represent a potential risk for humans exposed to diuron.

After male Wistar rats were exposed to diuron for 20 weeks, *in vivo* microarray analysis showed differences between low (60, 125 ppm) and high doses (1250, 2500 ppm) of diuron (Ihlaseh et al., 2011). At high doses of diuron, the most altered pathways included amino acid, lipid, phase I and phase II metabolism, and oxidative stress response. Several signaling pathways that could lead to diuron-induced necrosis included Nrf2-mediated oxidative stress response, glutathione metabolism and AhR signaling as well as cellular intermediate metabolism, including amino acid, lipid, and nitrogen metabolism (Ihlaseh et al., 2011).

Our microarray analysis showed the highest overexpressed genes induced by the diuron metabolites were the metalloproteinases (MMPs), and this was confirmed by real time PCR analyses. MMPs are enzymes involved in extracellular matrix (ECM) degradation (Szarvas et al., 2011). They are involved in tumor invasion, but increased evidence suggests that they are involved in early stages of carcinogenesis, regulating various critical pathways such as cell adhesion (Szarvas et al., 2011). The ECM not only provides structural support, but also generates cell signaling responses and cytoskeletal tension. Cell surface receptors bind the ECM and signal to the nucleus, either indirectly via intracellular signaling pathways or directly via cellular architectural alterations, to

reorganize chromatin, which then modifies gene expression (Aitken and Bagli, 2009). MMPs are often co-regulated in response to stimuli from growth factors such as TNF and other cytokines (Szarvas et al., 2011). Especially in the DCPU rat gene expression analysis, cytokines and TNF genes were increased. In a 7-day study using dietary exposure to diuron, *in vivo* bladder transcriptional results showed altered expression of genes from collagen), matrix metallopeptidase and tissue inhibitor of metalloproteinase families (Ihlaseh et al., unpublished observations). In addition to the upregulation of the metalloproteinase genes, the AhR pathway was also up regulated in the canonical pathways in these *in vitro* microarray studies. The AhR complex induces transcriptional activation of genes encoding xenobiotic metabolizing enzymes (CYP1A1, CYP1A2, CYP1B1), phase II metabolizing enzymes (GST, NADPH-quinone oxidoreductase, UDP-glucuronyl-S-transferase) and other growth factors and proteins involved in cell cycle progression (p27Kip1, p21Cip1) and apoptosis (Bax, Fas, Fasl) (IPA canonical pathways report). AhR is also known to interact with different signaling pathways involved in cell cycle progression, cell proliferation, apoptosis and tumorigenesis (IPA canonical pathways report). In one study, diuron showed an ability to bind and activate the AhR signal transduction pathway with more effective induction of rat cells than cells from the mouse, guinea pig, or human cell lines tested (Zhao et al. 2006). However, the authors of this study suggested that diuron was not a full agonist, but a partial AhR agonist. Furthermore, persistent agonists such as dioxins demonstrated that the AhR mediates liver tumor promotion and carcinogenicity response (Bock and Kohle, 2005). However, no liver carcinogenesis effects were observed in rats treated with diuron (Grassi et al., 2007).

Many studies in Wistar rats showed diuron-induced morphological changes in the urinary bladder at high doses. Scanning electron microscopy showed urothelial cell

swelling at early time points, followed by necrosis and exfoliation of the epithelial cell layer (Da Rocha et al., 2012). There was also a dose responsive difference in the incidence of bladder lesions in male rats fed diuron for 20 weeks. The incidences of simple hyperplasia at low doses were 1/10 at 60 ppm and 0/10 at 125 ppm. At high doses incidences of urothelial simple hyperplasia were 8/10 at 1250 ppm and 7/10 at 2500 ppm (Ihlaseh, et al., 2011). Bladder TEM analysis after 7 days of dietary exposure to diuron showed that the most severely affected areas involved disruption to complete loss of the cell membrane on the luminal side of the superficial cells with loss of superficial cells and occasionally loss of intermediate and basal cells. For the urothelial cells that remained intact, the junctional complexes were evident, although there was increased widening of the intercellular space and loss of cohesion between the cells (Da Rocha et al. 2012). MMPs are the main enzymes involved in extracellular matrix degradation. The up-regulation of urothelial cell MMPs after treatment with diuron metabolites could induce destruction of tissue architecture resulting in the loss of normal cohesion between the cells observed *in vivo* by TEM.

Taken together we suggest that DCPU is the diuron metabolite most responsible for the cytotoxic effects on the urothelium of rats fed diuron at high doses, leading to necrosis and cell exfoliation, regenerative hyperplasia and eventually tumors. Since a sufficiently high dose is required to produce a concentration that will be cytotoxic to the urothelium, exposures below that level will not be expected to produce a cytotoxic effect. Since humans are exposed to levels of diuron that are not expected to produce urinary concentrations of metabolites that would be cytotoxic, we conclude that humans will not have a carcinogenic response at typical occupational or environmental exposures.

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TABLES

Table 1: *In vivo* 24-hour diuron and metabolites urinary concentrations and *in vitro* IC50 for rat (MYP3) and human (1T1) cell lines.

	Diuron	DCPU	2-OH-DCPU	DCPMU	DCA
Rat Urinary Concentration	54±35uM	> 488 uM[a]	125±60uM	13±5uM	35±16uM
MYP3 IC50	_____	185uM	230uM	104uM	213Um
1T1 IC50	_____	157uM	329uM	224uM	72uM

[a] Measure of exact concentration not determined since the highest standard used was 10 ug/ml or 48.8 uM and a x10 dilution of sample was the maximum dilution used.

Table 2. Number of altered probe sets in rat (MYP3) and human (1T1) cell lines.

	Human cells (26317)[a]			Rat cells (17083)[a]		
Treatment	Probe sets[b]	Down-regulated (%)	Up-regulated (%)	Probe sets[b]	Down-regulated (%)	Up-regulated (%)
DCA	2296	1112 (48%)	1184 (52%)	108	44 (41%)	64 (59%)
DCPU	443	129 (29%)	314 (71%)	893	479 (54%)	414 (46%)
DCPMU	1242	594 (48%)	648 (52%)	158	74 (47%)	84 (53%)

[a] Total probe sets available for analysis after filtering

[b] Probe sets with adjusted p-values below 0.05 and at least 2.0 fold change

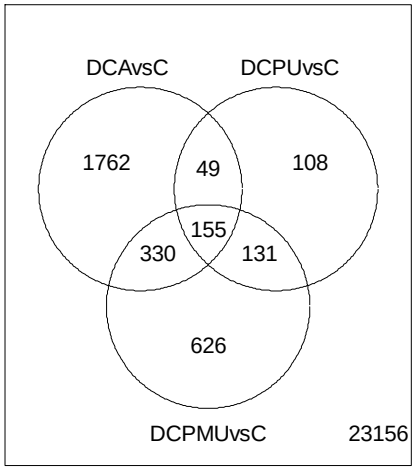
LEGEND FOR FIGURES

Figure 1: Venn Diagram across 3 treatments (DCPU, DCPMU and DCA) in human (1T1) and rat (MYP3) cell lines.

Figure 2: Most expressed DCPU x Control Canonical Pathways – Microarray Analyses

Figure 1.

Humans



Rats

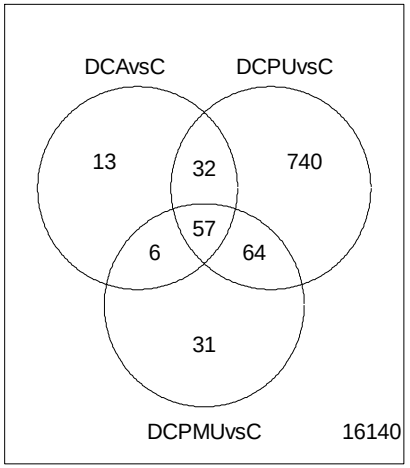
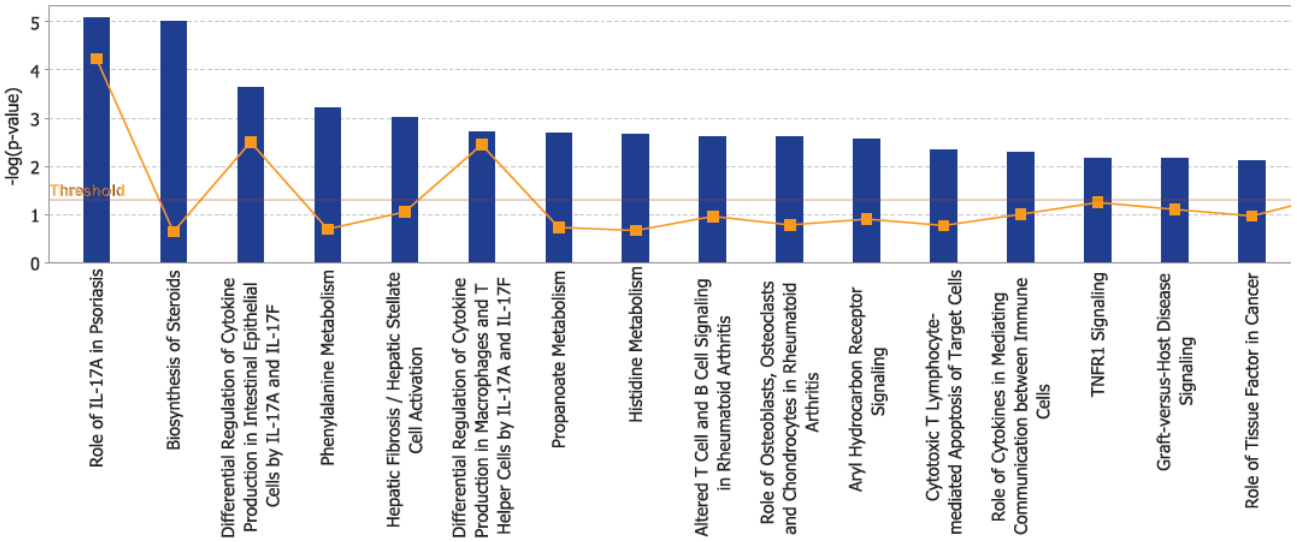


Figure 2.


-log(p-value)
0
1
2
3
4
5
Threshold
Role of IL-17A in Psoriasis
Biosynthesis of Steroids
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F
Phenylalanine Metabolism
Hepatic Fibrosis / Hepatic Stellate Cell Activation
Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F
Propanoate Metabolism
Histidine Metabolism
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis
Aryl Hydrocarbon Receptor Signaling
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells
Role of Cytokines in Mediating Communication between Immune Cells
TNFR1 Signaling
Graft-versus-Host Disease Signaling
Role of Tissue Factor in Cancer

SUPPLEMENTARY DATA

Table 1: Top differentiated expressed genes in MYP3 and 1T1 cells after treatment with DCPU, DCPMU and DCA.

Up-Regulated		**RAT (MYP3) Fold Change**			**HUMAN (1T1) Fold Change**		
Gene	***Name***	***DCPU***	***DCPMU***	***DCA***	***DCPU***	***DCPMU***	***DCA***
Mmp3	matrix metallopeptidase 3	34,94	11,95	6,80	-----	-----	-----
Mmp13	matrix metallopeptidase 13	28,39	19,66	11,18	-----	-----	-----
Cxcl12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	19,17	-----	-----	-----	-----	-----
Cxcl2	chemokine (C-X-C motif) ligand 2	14,36	-----	-----	-----	-----	-----
Cxcl1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	9,50	-----	-----	-----	-----	-----
Cxcl6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	9,37	-----	2,82	-----	-----	-----
Cx3cl1	chemokine (C-X3-C motif) ligand 1	8,85	3,62	-----	-----	-----	-----
Csf3	colony stimulating factor 3 (granulocyte)	8,66	-----	-----	-----	-----	-----
Lcn2	lipocalin 2	7,52	3,33	3,02	-----	-----	35,15
Cyp26b1	cytochrome P450, family 26, subfamily b, polypeptide 1	6,18	-----		-----	-----	-----
C3	complement component 3	-----	7,39	4,77	-----	-----	-----
Cndp1	carnosine dipeptidase 1 (metallopeptidase M20 family)	-----	6,23	5,38	-----	-----	-----
Expi	extracellular proteinase inhibitor	-----	4,91	-----	-----	-----	-----
Upk3a	uroplakin 3[a]	-----	4,57	-----	-----	-----	-----
Slpi	secretory leukocyte peptidase inhibitor	-----	3,53	-----	-----	-----	-----
Ass1	argininosuccinate synthetase 1	-----	3,23	-----	-----	-----	-----
Ctse	cathepsin E	-----	-----	4,07	-----	-----	-----
Prss23	protease, serine, 23	-----	-----	3,41	-----	-----	-----

Rnf128	ring finger protein 128	-----	-----	2,89	-----	-----	-----
Ubd	ubiquitin D	-----	-----	2,81	-----	-----	-----
MMP1	matrix metallopeptidase 1 (interstitial collagenase)	-----	-----	-----	93,07	93,02	77,89
MMP10	matrix metallopeptidase 10 (stromelysin 2)	-----	-----	-----	77,18	83,43	21,83
IL33	interleukin 33	-----	-----	-----	43,94	24,64	-----
AKR1C1 /AKR1C2	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase) / aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	-----	-----	-----	9,53	13,98	-----
SCG5	secretogranin V (7B2 protein)	-----	-----	-----	9,10	-----	-----
CXCR7	chemokine (C-X-C motif) receptor 7	-----	-----	-----	8,12	-----	-----
SPRR1B	small proline-rich protein 1B (cornifin)	-----	-----	-----	7,87	-----	-----
S100A8	S100 calcium binding protein A8	-----	-----	-----	7,33	-----	-----
AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	-----	-----	-----	6,70	10,35	-----
TMEM132 B	transmembrane protein 132B	-----	-----	-----	6,56	-----	-----
AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	-----	-----	-----	6,52	-----	-----
C14orf34	chromosome 14 open reading frame 34	-----	-----	-----	-----	23,40	-----
FYB	FYN binding protein	-----	-----	-----	-----	19,75	-----
ATP6V0D2	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2	-----	-----	-----	-----	15,99	-----
HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	-----	-----	-----	-----	15,18	-----
PAG1	phosphoprotein associated with glycosphingolipid microdomains 1	-----	-----	-----	-----	10,93	-----
IL1RL1	interleukin 1 receptor-like 1	-----	-----	-----	-----	-----	33,13
PI3	peptidase inhibitor 3, skin-derived	-----	-----	-----	-----	-----	27,44
CLGN	Calmegin	-----	-----	-----	-----	-----	16,98
RALBP1	ralA binding protein 1	-----	-----	-----	-----	-----	15,96
HSPA6	heat shock 70kDa protein 6 (HSP70B')	-----	-----	-----	-----	-----	15,82
SNHG12	small nucleolar RNA host gene 12 (non-protein coding)	-----	-----	-----	-----	-----	15,06
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	-----	-----	-----	-----	-----	14,74

Down-Regulated		RAT (MYP3) Fold Change			HUMAN (1T1) Fold Change		
Gene	***Name***	***DCPU***	***DCPMU***	***DCA***	***DCPU***	***DCPMU***	***DCA***
Lgals4	lectin, galactoside-binding, soluble, 4	-10,01	-----	-----	-----	-----	-----
Calml4	calmodulin-like 4	-8,67	-----	-----	-----	-----	-----
Hist2h2be	histone cluster 2, H2be	-6,96	-----	-----	-----	-----	-----
Hist1h2bc / Hist1h2bcl1 /Hist1h2bm	histone cluster 1, H2bc /// histone cluster 1, H2bc-like 1 /// histone cluster 1, H2bm	-6,76	-5,46	-3,64	-----	-----	-----
Fxyd4	FXYD domain-containing ion transport regulator 4	-6,59	-----	-----	-----	-----	-----
Aldh3a1	aldehyde dehydrogenase 3 family, member A1	-6,34	-----	-----	-----	-----	-----
Serpinb1a	serine (or cysteine) proteinase inhibitor, clade B, member 1a	-5,52	-----	-----	-----	-----	-----
Serpinb2	serine (or cysteine) peptidase inhibitor, clade B, member 2	-5,49	-----	-2,55	-----	-----	-----
Tchh	Trichohyalin	-5,34	-----	-----	-----	-----	-----
Ttc39a	tetratricopeptide repeat domain 39[a]	-5,32	-----	-----	-----	-----	-----
Trib3	tribbles homolog 3 (Drosophila)	-----	-3,30	-4,04	-----	-----	-----
Dusp14	dual specificity phosphatase 14	-----	-3,07	-----	-----	-----	-----
Scrn1	secernin 1	-----	-2,94	-----	-----	-----	-----
Igfbp6	insulin-like growth factor binding protein 6	-----	-2,83	-----	-----	-----	-----
Acss2	acyl-CoA synthetase short-chain family member 2	-----	-2,68	-2,81	-----	-----	-----
Dem1	defects in morphology 1 homolog (S. cerevisiae)	-----	-2,67	-----	-----	-----	-----
Lce1d	late cornified envelope 1D	-----	-2,60	-----	-----	-----	-----
Plcd4	phospholipase C, delta 4	-----	-2,48	-----	-----	-----	-----
Fgfbp1	fibroblast growth factor binding protein 1	-----	-----	-4,23	-----	-----	-----
Egr1	early growth response 1	-----	-----	-3,03	-----	-----	-----
Elovl6	ELOVL family member 6, elongation of long chain fatty acids (yeast)	-----	-----	-2,92	-----	-----	-----
Zc3h13	zinc finger CCCH type containing 13	-----	-----	-2,52	-----	-----	-----
Idi1	isopentenyl-diphosphate delta isomerase 1	-----	-----	-2,52	-----	-----	-----

Hsd17b7	hydroxysteroid (17-beta) dehydrogenase 7	-----	-----	-2,50	-----	-----	-----
VGLL1	vestigial like 1 (Drosophila)	-----	-----	-----	-5,79	-7,25	-----
SCEL	Sciellin	-----	-----	-----	-5,22	-----	-----
RRAD	Ras-related associated with diabetes	-----	-----	-----	-4,93	-9,98	-----
CTGF	connective tissue growth factor	-----	-----	-----	-3,89	-----	-----
FAM46B	family with sequence similarity 46, member B	-----	-----	-----	-3,87	-----	-----
PER2	period homolog 2 (Drosophila)	-----	-----	-----	-3,83	-----	-----
PDK4	pyruvate dehydrogenase kinase, isozyme 4	-----	-----	-----	-3,74	-----	-----
TMEM40	transmembrane protein 40	-----	-----	-----	-3,71	-----	-----
TAF9B	TAF9B RNA polymerase II, TATA box binding protein (TBP)-associated factor, 31kDa	-----	-----	-----	-3,67	-5,60	-----
PLK2	polo-like kinase 2 (Drosophila)	-----	-----	-----	-3,62	-----	-----
CLDN11	claudin 11	-----	-----	-----	-----	-8,28	-22,96
JUB	jub, ajuba homolog (Xenopus laevis)	-----	-----	-----	-----	-7,56	-----
TRIM6	tripartite motif-containing 6	-----	-----	-----	-----	-5,55	-----
IL6	interleukin 6 (interferon, beta 2)	-----	-----	-----	-----	-5,54	-----
PRNP	prion protein	-----	-----	-----	-----	-5,42	-16,63
OXCT1	3-oxoacid CoA transferase 1	-----	-----	-----	-----	-5,41	-----
NNMT	nicotinamide N-methyltransferase	-----	-----	-----	-----	-5,40	-----
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	-----	-----	-----	-----	-----	-18,27
MXRA5	matrix-remodelling associated 5	-----	-----	-----	-----	-----	-15,96
KLRC3	killer cell lectin-like receptor subfamily C, member 3	-----	-----	-----	-----	-----	-15,23
TPRG1	tumor protein p63 regulated 1	-----	-----	-----	-----	-----	-13,09
GPR109B	G protein-coupled receptor 109B	-----	-----	-----	-----	-----	-12,51
DST	Dystonin	-----	-----	-----	-----	-----	-12,34
COL8A1	collagen, type VIII, alpha 1	-----	-----	-----	-----	-----	-10,65
KLRC1 /KLRC2	killer cell lectin-like receptor subfamily C, member 1 /// killer cell lectin-like receptor subfamily C, member 2	-----	-----	-----	-----	-----	-10,18

Table 2: Common differentiated genes between DCA/DCPMU/DCPU metabolites – Rat (MYP3)

Gene	DCA	DCPMU	DCPU
Mmp13	**11,18**	**19,66**	**28,39**
Mmp3	**6,8**	**11,95**	**34,94**
Cndp1	**5,38**	**6,23**	**5,69**
C3	**4,77**	**7,39**	**5,86**
Ctse	**4,07**	**3,19**	**2,73**
Lcn2	**3,02**	**3,33**	**7,52**
Cxcl6	**2,82**	**2,98**	**9,37**
Rnf128	**2,73**	**2,73**	**3,99**
Cx3cl1	**2,61**	**3,62**	**8,85**
Gdf15	**2,61**	**2,17**	**5,74**
Mex3b	**2,55**	**2,27**	**5,65**
Klhl24	**2,55**	**2,09**	**3,38**
Ass1	**2,36**	**3,23**	**6,18**
St6gal1	**2,32**	**2,18**	**3,49**
Nedd9	**2,28**	**2,17**	**4,26**
Smpd3	**2,2**	**2,29**	**2,69**
Itga1	**2,16**	**2,1**	**4,09**
Aldh1a3	**2,15**	**3,02**	**4,03**
Sult1b1	**2,11**	**2,22**	**2,02**
Ifitm3	**2,02**	**2,03**	**4,46**
Scrn1	**-2,03**	**-2,94**	**-5,28**
Tgfbi	**-2,07**	**-2,39**	**-3,92**
Tp63	**-2,11**	**-2,12**	**-3,12**
Igfbp6	**-2,3**	**-2,83**	**-3,93**
Serpinb2	**-2,55**	**-2,31**	**-5,49**
Acss2	**-2,81**	**-2,68**	**-5,05**
Hist1h2bc / Hist1h2bcl1 / Hist1h2bm	**-3,64**	**-5,46**	**-6,76**
Trib3	**-4,04**	**-2,94**	**-2,17**
Fgfbp1	**-4,23**	**-2,32**	**-3,26**

Figure 1: Common differentiated genes between DCA/DCPMU/DCPU metabolites – Rat (MYP3)

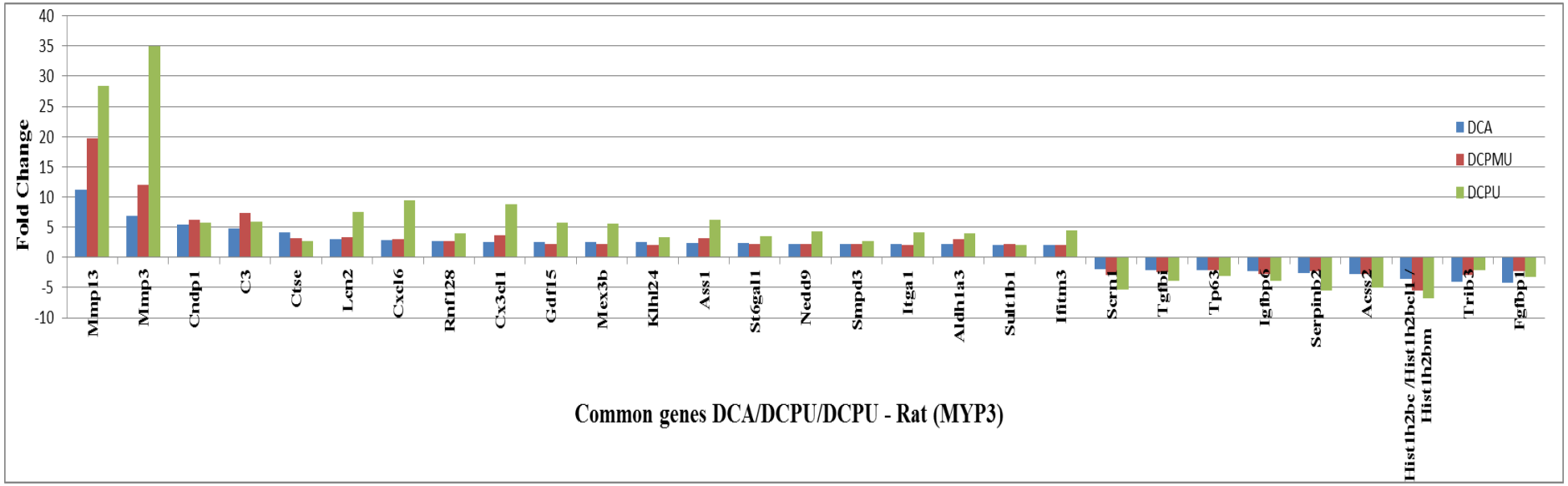


Table 3: Common differentiated genes between DCA/DCPMU/DCPU metabolites – Human (1T1)

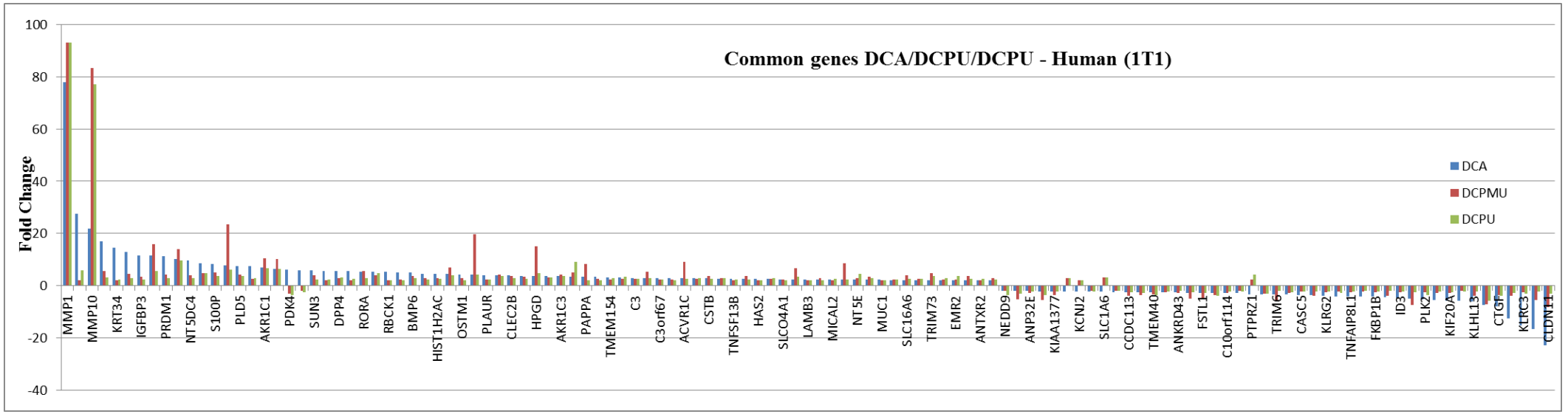
Gene	DCA	DCPMU	DCPU
MMP1	**77,89**	**93,02**	**93,07**
PI3	**27,44**	**2,11**	**5,84**
MMP10	**21,83**	**83,43**	**77,18**
CLGN	**16,98**	**5,56**	**3,22**
KRT34	**14,41**	**2,06**	**2,26**
IL1RN	**13,03**	**4,4**	**2,99**
IGFBP3	**11,51**	**3,39**	**2,29**
ATP6V0D2	**11,41**	**15,99**	**5,67**
PRDM1	**11,28**	**4,23**	**2,85**
AKR1C1 /// AKR1C2	**10,16**	**13,98**	**9,53**
NT5DC4	**9,54**	**3,82**	**2,82**
HSD17B2	**8,46**	**4,69**	**4,9**
S100P	**8,32**	**5,09**	**3,8**
C14orf34	**7,67**	**23,4**	**6,15**
PLD5	**7,54**	**4,26**	**3,65**
CYP1A1	**7,4**	**2,5**	**2,83**
AKR1C1	**7,03**	**10,35**	**6,7**
AKR1C2	**6,42**	**10,1**	**6,52**
PDK4	**6,09**	**-3,15**	**-3,74**
ATF3	**5,93**	**-2**	**-2,47**
SUN3	**5,86**	**3,84**	**2,22**
ZBTB1	**5,68**	**2,1**	**2,41**
DPP4	**5,55**	**2,94**	**3,01**
DNER	**5,54**	**2,17**	**2,65**
RORA	**5,34**	**5,64**	**2,94**
SERPINB2	**5,25**	**4**	**4,71**

RBCK1	**5,19**	**2,14**	**2,12**
C1orf210	**5,17**	**2,35**	**2,05**
BMP6	**5,16**	**3,7**	**2,87**
TMCC3	**4,63**	**2,95**	**2,32**
HIST1H2AC	**4,58**	**2,93**	**2,6**
RARRES1	**4,53**	**7**	**3,98**
OSTM1	**4,14**	**2,99**	**2,02**
FYB	**4,14**	**19,75**	**4,34**
PLAUR	**3,97**	**2,28**	**2,27**
ADAMTS9	**3,94**	**4,23**	**3,72**
CLEC2B	**3,9**	**3,79**	**2,99**
CYP4F3	**3,74**	**3,32**	**2,71**
HPGD	**3,7**	**15,18**	**4,77**
KRT6B	**3,66**	**3,24**	**3,19**
AKR1C3	**3,58**	**4,14**	**3,56**
SCG5	**3,4**	**5,17**	**9,1**
PAPPA	**3,35**	**8,24**	**2,13**
CDCP1	**3,3**	**2,63**	**2,1**
TMEM154	**3,26**	**2,34**	**2,75**
ENTPD3	**3,22**	**2,69**	**3,43**
C3	**2,95**	**2,58**	**2,48**
MUC16	**2,84**	**5,36**	**2,78**
C3orf67	**2,84**	**2,42**	**2,22**
PNLIPRP3	**2,83**	**2,23**	**2,03**
ACVR1C	**2,79**	**9,07**	**2,72**
CYP1B1	**2,75**	**2,51**	**2,84**
CSTB	**2,74**	**3,57**	**2,64**
TIMP1	**2,67**	**2,86**	**3**
TNFSF13B	**2,56**	**2,01**	**2,23**
ENO2	**2,51**	**3,79**	**2,23**
HAS2	**2,5**	**2,14**	**2,14**
TMEM117	**2,49**	**2,58**	**2,97**

SLCO4A1	2,46	2,4	2,18
CEACAM1	2,44	6,67	3,44
LAMB3	2,4	2,1	2,18
PGBD5	2,34	2,94	2,04
MICAL2	2,34	2,12	2,64
MCTP1	2,33	8,68	2,23
NT5E	2,32	2,74	4,56
PTGS2	2,31	3,36	2,85
MUC1	2,25	2,01	2,09
SCD	2,13	2,31	2,4
SLC16A6	2,07	3,89	2,48
TNFRSF10D	2,06	2,56	2,47
TRIM73	2,05	4,81	3,73
MYO10	2,03	2,22	2,93
EMR2	2,02	2,27	3,7
NDRG1	2,02	3,77	2,52
ANTXR2	2,02	2,11	2,59
MAF	2	2,99	2,28
NEDD9	-2,09	-2,03	-3,54
AOX1	-2,09	-5,17	-2,99
ANP32E	-2,13	-2,91	-2,15
TAF9B	-2,15	-5,6	-3,67
KIAA1377	-2,22	-3,7	-2,27
GJB6	-2,29	2,82	2,78
KCNJ2	-2,29	2,1	2,05
SOHLH2	-2,34	-2,14	-2,35
SLC1A6	-2,35	3,02	3,13
TRIM59	-2,42	-2,01	-2,07
CCDC113	-2,47	-3,84	-2,42
MSRB3	-2,5	-3,69	-2,91
TMEM40	-2,51	-3,41	-3,71
ACAD11 /// NPHP3	-2,52	-2,67	-2,15

ANKRD43	**-2,64**	**-2,94**	**-2,06**
C11orf70	**-2,73**	**-4,89**	**-2,62**
FSTL1	**-2,73**	**-5,35**	**-2,79**
FAM46B	**-2,84**	**-3,7**	**-3,87**
C10orf114	**-2,86**	**-2,82**	**-2,29**
AXL	**-2,91**	**-2,05**	**-2,17**
PTPRZ1	**-3,24**	**2,33**	**4,09**
CYR61	**-3,26**	**-3,2**	**-3,02**
TRIM6	**-3,26**	**-5,55**	**-2,05**
MATN3	**-3,33**	**-2,87**	**-2,43**
CASC5	**-3,58**	**-2,24**	**-2,16**
ERP27	**-3,76**	**-3,9**	**-2,4**
KLRG2	**-3,96**	**-2,42**	**-2,38**
PTGER4	**-4,19**	**-2,36**	**-2,91**
TNFAIP8L1	**-4,19**	**-2,57**	**-2,35**
NUSAP1	**-4,25**	**-2,31**	**-2,03**
FKBP1B	**-4,25**	**-2,63**	**-2,19**
SPC24	**-4,45**	**-3,81**	**-2,07**
ID3	**-4,68**	**-2,56**	**-2,4**
JUB	**-5,02**	**-7,56**	**-2,47**
PLK2	**-5,38**	**-2,68**	**-3,62**
ANLN	**-5,65**	**-2,71**	**-2,19**
KIF20A	**-5,85**	**-2,69**	**-2,48**
FAM198B	**-5,86**	**-2,09**	**-2,18**
KLHL13	**-5,97**	**-3,96**	**-2,2**
VGLL1	**-7,47**	**-7,25**	**-5,79**
CTGF	**-7,89**	**-3,76**	**-3,89**
GPR109B	**-12,51**	**-3,92**	**-2,89**
KLRC3	**-15,23**	**-2,63**	**-3,1**
PRNP	**-16,63**	**-5,42**	**-2,15**
CLDN11	**-22,96**	**-8,28**	**-3,11**

Figure 2: Common differentiated genes between DCA/DCPMU/DCPU metabolites – Human (1T1)



CONCLUSIONS

1) The hypothesized diuron rat urothelial MOA consists of cytotoxicity with consequent regenerative cell proliferation. However, the urothelial cytotoxicity is not due to urinary solids; rather, it is chemically induced.
2) The temporal association supports the hypothesis that urothelial cytotoxicity followed by regenerative cell proliferation are the sequential key events that occur with high-dose diuron exposure in rats.
3) The urothelial carcinogenic MOA of diuron in rats exposed at high doses starts with formation of cytotoxic metabolites; the diuron metabolite most responsible for urothelial cytotoxicity is DCPU.

In summary, the carcinogenic urothelial MOA of diuron in rats consists of metabolite-induced urothelial cytotoxicity with necrosis and cell exfoliation, consequent regenerative hyperplasia and eventually tumors.

This MOA is plausible to occur in humans. However, the starting key event is formation of cytotoxic metabolites and high doses are required to produce the urothelial effects. Since humans are expected to be exposed to levels of diuron that will not produce urinary concentrations of metabolites that would be cytotoxic, it is plausible to assume that humans will not have a carcinogenic response at usual occupational or environmental exposures.

ATTACHMENTS