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Original Article

The effects of prochloraz on the levels of nuclear receptor genes expressions and global DNA methylation in human prostate carcinoma cells

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ABSTRACT

Background and Aims: Prochloraz (PCZ) is an imidazole fungicide which is used in agriculture and gardening. PCZ, with endocrine disrupting effect, disrupts reproductive and developmental functions. Previously, the effects of PCZ on the estrogen and androgen receptors have been shown *in vitro* and *in vivo*. Because of endocrine disrupting effects, PCZ could influence nuclear receptors which acted as ER and AR antagonists. Besides, PCZ has been thought to have no genotoxic effects. Therefore, we aimed to investigate possible effects of PCZ on nuclear receptor genes and epigenetic mechanisms in human prostate carcinoma (PC-3) cells.
Methods: In the present study, MTT and LDH tests were applied to evaluate the cell viability. Expression levels of nuclear receptor genes such as *AhR, PXR, PPARα, PPARγ* were studied on real-time quantitative PCR. For global DNA methylation analysis, the levels of 5-methylcytosine (5-mC%) were measured by elisa kit.
Results: According to MTT and LDH test results, IC50 value of PCZ has been determined as 144.19 and 116.65 μM, respectively. There were significant changes for the expression levels of *AhR, PPARα* and *PPARγ* genes after 5-50 μM of PCZ treatments. 5 and 50 μM of PCZ decreased the levels of 5-mC% in the rates of 22.6% and 26.9%, respectively.
Conclusion: It has been suggested that PCZ may cause alterations on the expressions of nuclear receptor genes which could be related to endocrine disrupting effects and may have implications on global DNA methylation.

Keywords: Cytotoxicity, DNA methylation, nuclear receptor genes, PC-3 cells, prochloraz

INTRODUCTION

Prochloraz (PCZ) (CAS no. 67747-09-5; N-propyl-N-[2-(2,4,6-trichlorophenoxy)ethyl]-*1H*-imidazole-1-carboxamide) is an imidazole fungicide used in gardening and agriculture. The action of imidazole fungicides is based on the blocking cytochrome P450-dependent enzyme activity, for the conversion of an essential component of fungal cell membranes (Henry & Sisler, 1984). Exposure to PCZ , mainly through consumption of fruits and vegetables such as wheat, barley, rice, cereal, cherries, has been important worldwide (Claeys et al., 2011). PCZ exhibited low acute toxicity and has been shown to display hepatotoxic effects (Goettel et al. 2015; Heise et al., 2015, 2018; Marx-Stoelting et al., 2017), induction of oxidative stress (Lundqvist, Hellman & Oskarsson, 2016; Alpertunga et al., 2014; Sanchez, Piccini & Porcher, 2008), endocrine disrupting effects (Vinggaard, Hnida, Breinholt & Larsen 2000; Vinggaard et al., 2002, 2006) and also tumorigenic effects following chronic exposure (EFSA, 2011). The modes mechanisms of action of PCZ

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were antagonism of the androgen and estrogen receptors and inhibition of steroidogenic enzymes which play a key role in biosynthesis and metabolism of steroid hormones as well as a potent aromatase inhibitor (Laier et al., 2006; Andersen, Vinggaard, Rasmussen, Gjermandsen & Bonefeld-Jørgensen, 2002; Vinggaard et al., 2002). PCZ also showed its effects by activating the aryl hydrocarbon receptor (AhR) through up-regulation of *CYP1A1* expression in different liver cell lines and rat liver (Marx-Stoelting et al., 2017; Halwachs, Wassermann, Lindner, Zizzadoro & Honscha, 2013; Long et al., 2003; Vinggaard et al., 2006).

DNA methylation, one of the most studied epigenetic modifications, plays crucial roles in aging, cell proliferation and various diseases such as cancer, diabetes (Anderson, Sant & Dolinoy, 2012; Kulis & Esteller, 2010; Jones & Baylin, 2007; Moggs, Goodman, Trosko, & Roberts, 2004; Baylin, 1997). Mutagenicity and genotoxicity of PCZ have been summarized by JMPR and EFSA that PCZ exerted in some test systems with negative results except in *in vitro* sister chromatid exchange assay (JMPR, 2001). Based on these results, JMPR (2001) and EFSA (2011) clarified that PCZ is improbable to be potentially genotoxic. In our previous study, PCZ also showed no evidence of mutagenicity and DNA damage in NRK-52E cells (Alpertunga et al., 2014). Controversially, Lundqvist et al. (2016) have indicated that non-toxic concentrations of PCZ triggered DNA damage in HepG2 cells by Comet assay. Therefore, non-gentoxic mechanisms may have a role in PCZ toxicity. Therefore, in present study we aimed to investigate global DNA modifications of PCZ for the first time. We studied potential toxic effects of PCZ on nuclear receptor genes and global DNA methylation as an epigenetic mechanism.

MATERIALS AND METHODS

Chemicals

PCZ, Pestenal, analytical standard (purity 99.2%), was obtained from Sigma-Aldrich Chemicals Co (St. Louis, Missouri, USA). A stock solution of PCZ was prepared by dissolving it in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis, Missouri, USA). Cell culture media and all other supplements were purchased from Wisent Bioproducts (Saint-JeanBaptiste, QC, Canada), and sterile plastic materials were purchased from Nest Biotechnology (Jiangsu, China). Cytotox-LDH-XTT 2 Parameter Cytotoxicity kit was used for cytotoxicity (Xenometrix AG, Allschwil, Switzerland). DNA, RNA isolation kits and cDNA synthesis kits were obtained from Roche Life Sciences (Penzberg, Upper Bavaria, Germany). 5-methylcytosine (5-mC) DNA ELISA kit was purchased from Zymo Research (Irvine, CA, USA). Syber green master mix was obtained from Bioline (London, UK), and primers for gene expressions were obtained from Sentromer DNA Technologies (Istanbul, Turkey).

Cell culture and treatments

The human prostate adenocarcinoma cell line (PC-3 cells) was obtained from American Type Culture Collection (ATCC®CRL-1435™) and cultured in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM-F12) under standard cell culture conditions as described previously (Senyildiz, Karaman, Baş, Pirinççi, Özden, 2016, 2017).

For gene expression and global DNA methylation analysis 1×10^6 cells were incubated in a 25 cm^2 culture flask for 24 h in CO_2 incubator prior to the treatment. The exposure concentrations of PCZ used in the experiments have been determined with our cytotoxicity studies and based on our previous study (Alpertunga et al., 2014).

Cell viability

Effects of PCZ on cell viability were assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, a tetrazole] and lactate dehydrogenase (LDH) cytotoxicity tests. Principle of the MTT test; yellow MTT is reduced to purple formazan in the mitochondria of living cells by the enzyme succinate dehydrogenase, and it measures cell viability and proliferation (Alley et al., 1988; Mossman, 1983). The LDH test is a cell viability test method, based on measuring membrane integrity via % LDH release (lactate dehydrogenase activity) (Decker & Lohmann-Matthes, 1988; Korzeniewski & Callewaert, 1983).

For cytotoxiciy assay, 1×10^4 cells (in 100 µL medium) were seeded in 96-well plates and were treated with PCZ in the concentration range of 7.81-500 µM and DMSO (1%, exposure concentration in culture media) as solvent control for 24 h. Then, cell viability and % LDH release were measured and assessed using the MTT and LDH test as described in our previous study (Karaman & Ozden, 2019; Demirel, Alpertunga & Ozden, 2015).

Gene expression analysis of nuclear receptor genes

After 5, 10 and 50 µM of PCZ treatments for 24 h, total RNA was isolated from PC-3 cells using High Pure RNA Isolation kit (Roche Life Science). Reverse transcription was performed by Transcriptor First Strand cDNA Synthesis kit from 500 ng of total RNA, and the mixture of anchored-oligo(dT) and random hexamer primers. 5 µL of the 1/10 diluted RT-reaction was used as the template in real-time quantitative PCR. Gene expressions of nuclear receptor genes such as *AhR* (aryl hydrocarbon receptor), *PXR* (pregnane-X receptor), *PPARα* (peroxisome proliferator-activated receptor alpha), *PPARγ* (peroxisome proliferator-activated receptor gamma) were measured using BioLine SensiFast™ Syber® No-Rox kit (London, UK) on LightCycler® 480 Instrument II (Roche Life Science). Primer sequences and their annealing temperatures of genes are illustrated in Table 1. Evaluations of results for all genes were performed as described previously (Karaman & Ozden, 2019).

Global DNA methylation

5, 10 and 50 µM of PCZ exposed to PC-3 cells for 24 h, then genomic DNA was isolated from cells using the High Pure PCR Template Preparation kit (Roche Life Sciences, Penzberg, Germany) according to the instructions provided by the manufacturer. To measure global levels of DNA methylation, 100 ng of DNA samples were applied to 5-mC DNA elisa kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions as previously described (Senyildiz, Karaman, Bas, Pirincci & Ozden, 2017). Also, gene expression analysis of DNA methyltransferase genes including *DNMT1* (DNA methyltransferase 1) and *MGMT* (O-6-methylguanine-DNA methyltransferase) were performed by real-time quantitative PCR analysis using BioLine SensiFast™ Syber® No-Rox kit (London, UK).

Table 1. Primers used real-time PCR analysis of nuclear receptor genes, DNA methyltransferase and the corresponding annealing temperatures.

Gene	Primer sequence (5'-3')	Tm (°C)	Reference
AhR	F: TGGACAAGGAATTGAAGAAGC R: AAAGGAGAGTTTTCTGGAGGAA	53	Ayed-Boussema et al., 2011
PPARα	F: CATTACGGAGTCCACGCGT R: ACCAGCTTGAGTCGAATCGTT	58	Rogue et al., 2011
PPARγ	F: CTGAATGTGAAGCCCATTGAA R: GTGGAAGAAGGGAAATGTTGG	54	Harada et al., 2005
DNMT1	F: CCTCCAAAAACCCAGCCAAC R: TCCAGGACCCTGGGGATTTC	60	Ahmadnejad et al., 2017
MGMT	F: TGCACAGCCTGGCTGAATG R: GGTGAACGACTCTTGCTGGAA	58	Lai et al., 2008
β-actin	F: AACTACCTTCAACTCCAT R: TGATCTTGATCTTCATTGTG	48	Rosa et al., 2009

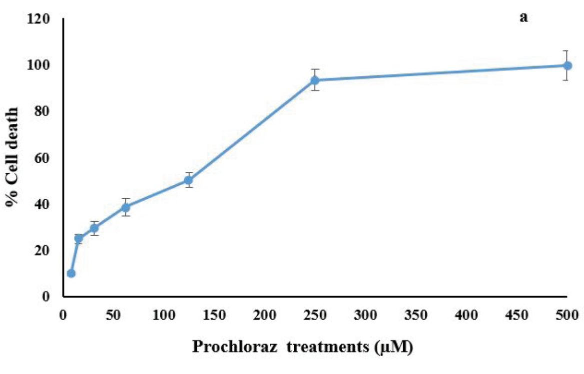
Statistical analysis

Global methylation levels (5-mC%) and cytotoxicity results were represented as mean±standard deviation (SD). Statistical analysis was performed by ANOVA followed by Dunnett's multiple comparison test using "SPSS version 21.0 for Windows", statistical program (IBM Analytics, New York, USA). P values of less than 0.05 and 0.001 were selected as the levels of significance.

RESULTS

Effects of PCZ on the cell viability in PC-3 cells

We performed cytotoxicity of PCZ in the concentration range of 3.906-500 µM in PC-3 cells after 24 h exposure by using MTT and LDH assays. IC_{50} values of PCZ were determined as 144.19 and 116.65 µM, respectively. As shown in Figures 1a and 1b, treatments of PCZ for 24 h significantly decreased the cell viability ≥10.13% and ≥12.74% for MTT and LDH tests, respectively, in comparison to the vehicle control group.



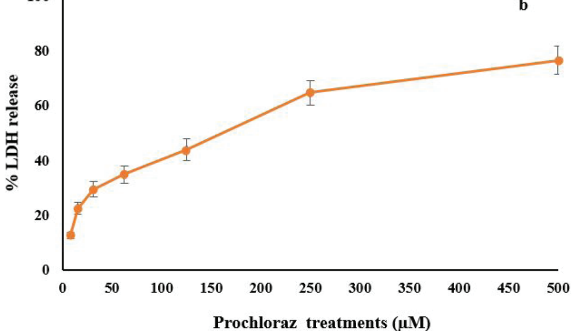


Figure 1. Effects of PCZ (7.81, 15.62, 31.25, 62.5, 125, 250 and 500 µM) on cell viability by MTT (a) and LDH (b) tests in PC-3 cells after 24 h exposure. Data are presented as mean±SD.

Effects of PCZ on global DNA methylation in PC-3 cells

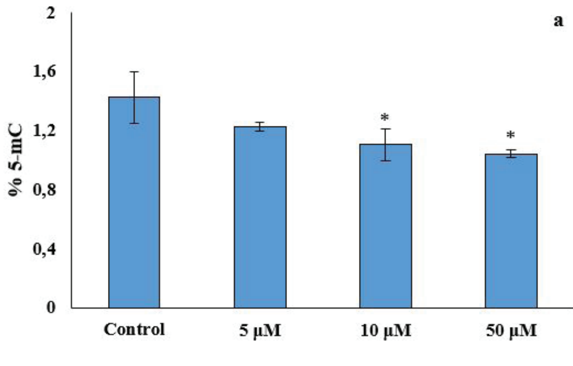
Levels of 5-mC% were observed after 5, 10 and 50 µM treatments of PCZ for 24 h by elisa kit. Exposure to 10 and 50 µM of PCZ for 24 h resulted in a significant decrease in 5-mC% status (22.67%, $p<0.05$ and 26.97%, $p<0.05$, respectively) comparison with the control group (Figure 2a). Consistently, 10 µM (61.9%) and 50 µM (85.21%, $p<0.05$) of PCZ decreased the expression levels of *DNMT1* (Figure 2a) while non-significant decrease was observed for *MGMT* (Figure 2b).

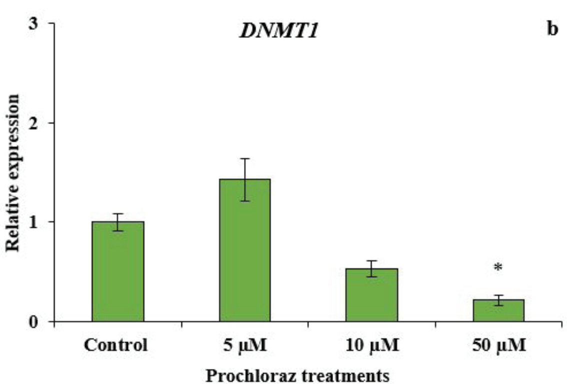
Effects of PCZ on nuclear gene expression levels in PC-3 cells

We analysed the gene expression of selected nuclear receptor genes (*AhR*, *PPARα*, *PPARγ*, *PXR*) to investigate the potential effects of PCZ. In Figure 3, our data showed that 10 µM of PCZ significantly increased expression levels of *PPARα* (2.63 fold, $p<0.05$), 10 and 50 µM of PCZ significantly increased expression levels of *PPARγ* (1.73 fold, $p<0.05$ and 3.09, $p<0.05$, respectively), and 50 µM of PCZ significantly decreased expression levels of *AhR* (53.3%, $p<0.05$). However, no expression of *PXR* gene was observed at all experiment groups, even in control group.

DISCUSSION

PCZ has hepatotoxic properties, exerts tumorigenic effects following chronic exposure and induces gene expression and enzyme activity of CYP1A in animal studies (Goettel et al. 2015; Heise et al., 2015, 2018; Marx-Stoelting et al., 2017; Sturm, Cravedi, Perdu, Baradat & Segner 2001; Vinggaard et al., 2006). It has been reported that PCZ acts as endocrine disruptor through interfering with ER and AR as well as an aromatase inhibition, alterations in the several hormones levels and steroid biosynthesis (Andersen, Vinggaard, Rasmussen, Gjermandsen & Bonefeld-Jørgensen, 2002; Laignelet, Riviere & Lhuguenot, 1992; Ohlsson, Ullerås & Oskarsson, 2009; Robitaille, Rivest & Sanderson, 2015; Vinggaard et al., 2000, 2002, 2006). Moreover, several studies have shown that PCZ caused oxidative damage through induction of lipid peroxidation and alterations in the





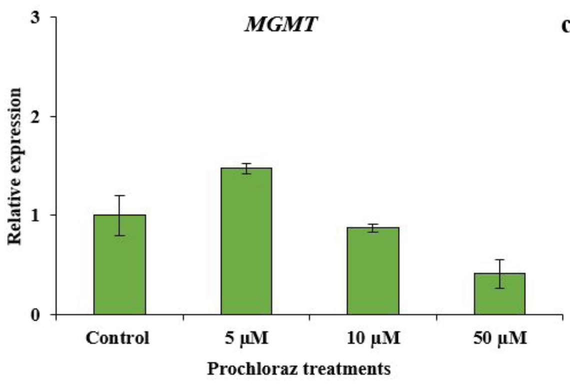


Figure 2. a) Effects of PCZ (0–50 µM) on levels of 5-mC% (a), relative gene expression levels of *DNMT1* (b) and *MGMT* (c) in PC-3 cells after 24 h exposure. Data are presented as mean ± SD. (Statistical analysis was performed by ANOVA + Dunnett post hoc test. Statistically significant changes are indicated by *p<0.05).

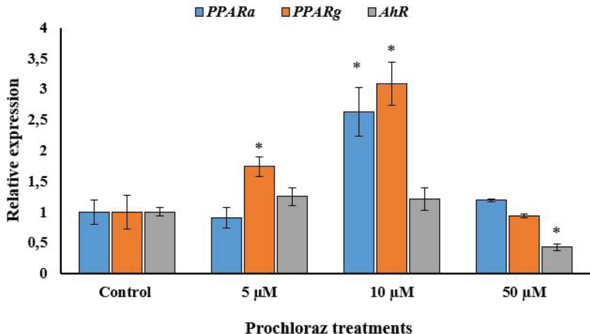


Figure 3. Effects of PCZ (0–50 µM) on relative gene expression levels of *AhR, PPARα, PPARγ* and *PXR* in PC-3 cells after 24 h exposure. Data are presented as mean ± SD. (Statistical analysis was performed by ANOVA + Dunnett post hoc test. Statistically significant changes are indicated by *p<0.05).

levels of antioxidant enzymes and glutathione *in vitro* (Alpertunga et al., 2014; Lundqvist et al., 2016; Sanchez et al., 2008).

In present study, IC_{50} values of PCZ were determined as 144.19 and 116.65 µM in PC-3 cells for 24 h by MTT and LDH tests, respectively. Our cytotoxicty results were consistent with the previous studies (Alpertunga et al., 2014; Rudzok et al., 2011; Strum et al., 2001). It has been reported that IC_{50} values of PCZ were 140 µM in trout hepatocytes by using LDH cytotoxicity test (Strum et al., 2001); 117, 87 and 99 µM in HepG2 cells by using MTT, neutral red uptake and Alamar Blue tests, respectively (Rudzok et al., 2011) and 110.76 µM in NRK-52E rat kidney cells by using MTT test (Alpertunga et al., 2014).

Investigating epigenetic alterations such as DNA methylation could be useful biomarkers for the toxicity assessment of endocrine disrupting chemicals (Casati, Sendra, Sibilia & Celotti, 2015; Greally & Jacobs, 2013; Maqbool, Mostafalou, Bahadar & Abdollahi, 2016). However, there has been any study on epigenetic modifications of PCZ toxicity. In present study, effects of PCZ on global DNA methylation, the most studied epigenetic alteration, have been investigated. PCZ decreased the global DNA methylation levels in a dose-dependent manner. As well as *DNMT* genes regulate DNA methylation in mammals. Especially, *DNMT1* provides regularity and continuity of methyltransferase activity that conserves the methylation state throughout DNA replication (Das & Singal, 2004; Pathania et al., 2015; Pradhan, Bacolla, Wells & Roberts, 1999). Consistent with our 5-mC% levels, expression of *DNMT1* was also decreased. We can suggest that PCZ could cause global DNA methylation which is a common feature in the oncogenesis of many tumor tissues leading to genomic instability (Gama-Sosa et al., 1983).

One of the sensors that enables cells to continually adapt and respond to the molecular changes by the environment, diet and host metabolism is the ligand-activated transcription factor AhR which perceives both endogenous factors and exogenous factors such as environmental toxins (McIntosh, Hogenesch & Bradfield, 2010). It has been suggested that PCZ exerts its effects by activating AhR which has an important role in PCZ-mediated hepatotoxicity (Marx-Stoelting et al., 2017; Rieke et al. 2014; Halwachs et al. 2013), and agonists of AhR could trigger tumor growth in rodent livers (Bock & Kohle 2005). Interestingly, our findings showed that *AhR* gene expression significantly decreased after 50 µM of PCZ in PC-3 cells. Evans et al. (2008) also have observed that higher concentrations of TCDD (2,3,7,8-tetrachlordibenzo-p-dioxin), other AhR agonist, caused a reduction of enzyme activity to 50% of the maximal response value which could reflect a negative feedback through the AhR repressor (AhRR) competition with AhR, similarly to our results.

Members of the PPAR subfamily, one of the nuclear receptors, including PPARα, PPARβ/δ, and PPARγ, are transcription

factors that regulate by activation of fatty acids and have essential roles in several biological processes including cellular differentiation, development, and metabolism (Derosa, Sahebkar & Maffioli, 2018; Michalik & Wahli, 2006). There have been no studies showing the effects of PCZ on *PPARs* gene expression. According to our findings, *PPARα* and *PPARγ* gene expression increased significantly after PCZ exposure. In the meantime, *PPARα* expression has been found among normal prostate and prostate cancer tissues while *PPARγ* expression was observed only in prostate cancer (Segawa et al., 2002). It has been also suggested that PPARγ acts as an oncogenic gene, and its activation promoted the development and progression of prostate cancer (Ahmad et al., 2016; Rogenhofer et al., 2012). Interestingly, it has been also reported that PPARγ signaling was regulated negatively by AR signaling (Olokpa, Bolden & Stewart, 2016). In the present study, while gene expressions of *PPARα* at 10 µM of PCZ and *PPARγ* at 5 and 10 µM of PCZ increased with the treatment, gene expression levels were decreased non-significantly after 50 µM exposure. It has been thought that these effects could be the result of non-monotonic dose responses due to the endocrine disrupting properties of PCZ.

In conclusion, our results, showing that PCZ is able to change nuclear receptor signaling and DNA methylation in cultured human prostate cells, suggests that PCZ might be one of the non-genotoxic promoters. Taken together, these findings are important for future assessment of endocrine disrupting pesticides, and we recommend that further studies should be needed to understand better the toxicity mechanisms of PCZ and especially, its role in epigenetic modifications.

Peer-review: Externally peer-reviewed.

Author contributions: Conception/Design of Study- S.Ö., E.F.K.; Data Acquisition- E.F.K., E.D., D.A.; Data Analysis/Interpretation- S.Ö., E.F.K.; Drafting Manuscript- S.Ö., E.F.K.; Critical Revision of Manuscript- S.Ö., E.F.K.; Final Approval and Accountability- E.F.K., E.D., D.A., S.Ö.

Conflict of Interest: The authors have no conflict of interest to declare.

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