Toxicology Letters 229 (2014) 366–373

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Effects of the mycotoxin patulin at the level of nuclear receptor transcriptional activity and steroidogenesis *in vitro*

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HIGHLIGHTS

- Patulin acts as a potential endocrine disruptor by various modes of action.
- An increase in the glucocorticoid receptor transcriptional activity was observed.
- Patulin was found to be capable of modulating hormone production *in vitro*.

ARTICLE INFO

Article history:
Received 22 April 2014
Received in revised form 27 June 2014
Accepted 30 June 2014
Available online 1 July 2014

Keywords:
Mycotoxin
Patulin
Reporter gene assay
H295R
Cytotoxicity
Endocrine disruptor

ABSTRACT

Patulin (PAT) is a mycotoxin produced by various species of fungi, with *Penicillium expansum* being the most commonly occurring. Apples and apple products are the main sources of PAT contamination. This mycotoxin has been shown to induce toxic effects in animals, a few of which include reproductive toxicity and interference with the endocrine system. Here the endocrine disrupting potential of PAT has been investigated *in vitro* to identify disruption at the level of oestrogen, androgen, progestagen and glucocorticoid nuclear receptor transcriptional activity, and to assess interferences in estradiol, testosterone and progesterone steroid hormone production.

At the receptor level, 0.5–5000 ng/ml (0.0032–32 μM) PAT did not appear to induce any specific (ant) agonistic responses in reporter gene assays (RGAs); however, nuclear transcriptional activity was affected. A >6 fold increase in the glucocorticoid receptor transcriptional activity was observed following treatment with 5000 ng/ml PAT in the presence of cortisol. At the hormone production level, despite cytotoxicity being observed after treatment with 5000 ng/ml PAT, estradiol levels had increased >2 fold. At 500 ng/ml PAT treatment, an increase in progesterone and a decrease in testosterone production were observed. The findings of this study could be considered in assessing the health risks following exposure to PAT.

1. Introduction

The mycotoxin patulin (PAT) (Fig. 1) is a secondary metabolite produced by *Penicillium, Aspergillus, Paecylomtces* and *Byssochlamys* species of fungi, with *Penicillium expansum* considered as the main source (Puel et al., 2010). PAT can be found to occur in many different foods and feeds, including cereals, grapes, oranges, pears and peaches; however, the main sources of contamination are apples and apple products (Beretta et al., 2000; Moake et al., 2005). Apples provide the ideal environment for production of this mycotoxin leading to the formation of blue rot (Moss, 2008). The stability of PAT during processing means that apple-derived products such as juice, compotes and other foods may also be especially susceptible to contamination. The presence of PAT is indicative of the quality of fruit used in the production. Fermentation of apple juice to cider using the yeast *Saccharomyces cerevisiae* does however lead to the degradation of PAT (Moss, 2008).

In a review of studies describing the toxic effects of PAT in various *in vivo* and *in vitro* experiments, PAT has been shown to be capable of inducing a range of toxic effects (Puel et al., 2010). These include mainly gastrointestinal disorders; however, effects such as immunotoxicity, embryotoxicity and genotoxicity have also been noted. PAT is characterised by its strong affinity for sulfhydryl groups, and this can explain at least some of the observed toxic effects. PAT appears to have preference for sulfhydryl-containing compounds such as cysteine or glutathione leading to the

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http://dx.doi.org/10.1016/j.toxlet.2014.06.847
0378-4274/

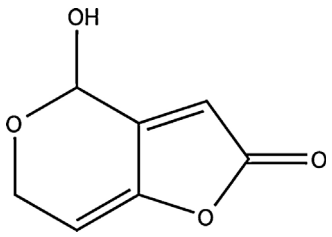

Fig. 1. Chemical structure of patulin (PAT).

formation adducts with reduced toxicity (Fliege and Metzler, 1999, 2000). However, if for example, the cellular glutathione levels are depleted this can in turn lead to PAT induced cytotoxicity (Schumacher et al., 2005; Wu et al., 2008).

Data is scarce regarding the *in vivo* absorption and metabolism of PAT in humans. In the one experiment which has been carried out by Rychlik (2003), apple juice containing PAT at the regulatory limit of 50 μg/kg was consumed. No traces of PAT were detected in blood serum after 1 h when a highly stable isotope dilution assay was employed. This study and another in rats (Rychlik et al., 2004) presumed that PAT firstly binds to intracellular glutathione in the glutathione rich gastrointestinal mucosa cells before reaching the blood system, and if glutathione levels are depleted in the mucosal cells, any amounts entering the blood system are also rapidly degraded by glutathione in blood. For this reason, PAT toxicity in humans is thought to be local and not systemic; this is in agreement with most of the subacute toxicity studies which revealed gastric ulceration and inflammation as main effects (Puel et al., 2010). However, the metabolism and excretion of PAT in humans is still not fully understood; therefore studies on the toxic properties of PAT and any PAT adducts formed *in vivo* are warranted.

A survey conducted by the Scientific Co-operation on Questions relating to Food (SCOOP) to assess human exposure to PAT in the European Union showed that this mycotoxin was present in 57.4% of apple juice samples ($n = 4633$). The mean concentration in these samples was 15.6 μg/kg, while the maximum level was 1150 μg/kg (Majerus and Kapp, 2002). The Joint Expert Committee on Food Additives (JECFA), a scientific advisory body of the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organisation (WHO), has evaluated the hazards of PAT in relation to human and animal health (JECFA, 1990, 1996). JEFCA established a provisional maximum tolerable daily intake (PMTDI) of 0.4 μg/kg bw for PAT, taking into account the no-observed-adverse-effect-level (NOAEL) of 43 μg/kg bw/day and a safety factor of 100. The Codex Alimentarius Commission (CAC), also established by FAO and WHO to develop harmonised international food standards and improve food safety has set a ML of 50 μg/kg for PAT in apple juice (CAC, 1995).

In Europe, the Scientific Committee on Food (SCF) has endorsed the recommended PMTDI set by JECFA (SCF, 2000), while the European Commission has legislated maximum levels (ML) for PAT in certain foods under EC Regulation 1881/2006 (European Commission, 2006). In this legislation, a ML of 50 μg/kg PAT in fruit juices and in fermented drinks made from apples or apple juice has been established, while a ML of 25 μg/kg PAT in solid apple products has also been set. A lower ML of 10 μg/kg PAT in apple juice, solid apple products and baby foods other than processed cereal based foods has been legislated for infants and young children. The European Commission has also introduced measures to prevent and reduce PAT contamination in apple juice and in apple juice ingredients in other beverages (European Commission, 2003).

Exposure assessment studies ($n = 9$) conducted in various countries on apple juice or apple compote consumed by children and infants revealed contamination levels were below the 0.4 μg/kg bw/day PMTDI; however, they did note that they appeared high for this vulnerable group (Marin et al., 2013). As a worst case scenario, a 20 kg child consuming apple juice containing 50 μg/kg PAT would reach the PMTDI by drinking only 160 ml of the juice. As children consume more apples per kilogram of body weight than adults, the potential for exceeding the PMTDI is much greater.

Some evidence does exist indicating that PAT has the ability to act as a potential endocrine disrupting compound (EDC); however, to date PAT has not been tested extensively in this regard. An EDC is defined as an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations (IPCS, 2002). Toxicity of PAT in relation to reproductive development has been noted. In rats, PAT has shown some adverse effects on reproductive parameters (Becci et al., 1981; Choudhary et al., 1992). In another study, Selmanoglu and Koçkaya (2004) measured thyroid and testicular hormones following the treatment of growing male rats with an extremely high dose of 0.1 mg/kg/day PAT for 60 or 90 days. Analysis of plasma levels of the hormones indicated that PAT had the most significant effect on the testes by increasing testosterone levels by 66.6% and 75% after 60 and 90 days exposure, respectively, and luteinizing hormone levels by 146% after 90 days exposure in the 5–6 week old rats. These results suggested possible direct or indirect effects of PAT on the testes or pituitary glands, respectively. Histopathological examination of these testes also revealed Leydig cell hyperplasia which further supported the results. Selmanoglu (2006) showed PAT at the same dose and exposure times to also have effects on sperm count, morphology and reproductive organs in growing male rats.

In this study, we have investigated the endocrine disrupting potential of PAT using various *in vitro* bioassays, namely reporter gene assays (RGAs) and the H295R steroidogenesis assay. The RGA cell lines are human mammary gland cell lines with natural steroid hormone receptors for oestrogens, androgens, progestagens and glucocorticoids (Willemsen et al., 2004), thereby allowing endocrine disruption at the level of nuclear receptor transcriptional activity to be identified. The H295R cell line has the physiological characteristics of zonally undifferentiated human fetal adrenal cells, possessing all of the enzymes in the steroid synthesis pathway and with the ability to produce all of the steroid hormones found in the adult adrenal cortex (Hecker and Giesy, 2008). For this reason, it is useful to identify endocrine disruption of the steroidogenic pathway through inhibition or induction of production of the steroid hormones, indicating potential interference with hormone production *in vivo*. Production of estradiol, testosterone and progesterone steroid hormones following treatment with PAT has been measured in this study. The thiazolyl blue tetrazolium bromide (MTT) and AlamarBlue® assays have also been used to monitor cytotoxicity in the RGA and H295R cell lines, respectively.

2. Materials and methods

2.1. Reagents

Methanol, dimethyl sulfoxide (DMSO), forskolin, patulin (PAT), thiazolyl blue tetrazolium bromide (MTT) and the steroid hormones 17β-estradiol, testosterone, progesterone and cortisol were obtained from Sigma–Aldrich (Poole, Dorset, UK). Cell culture reagents were supplied by Life Technologies (Paisley, UK) unless otherwise stated. All cell lines were grown in tissue culture flasks (Nunc, Roskilde, Denmark) at 37 °C with 5% CO_2 and 95% humidity.

2.2. Reporter gene assays (RGA's)

Four reporter gene cell lines were previously developed (Willemsen et al., 2004). The MMV-Luc cell line is specific for the detection of oestrogens, TARM-Luc for androgens and progestagens, TM-Luc for progestagens and TGRM-Luc for