Toxicology Letters 233 (2015) 84–94

Contents lists available at ScienceDirect

Toxicology Letters

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

An investigation of the endocrine disrupting potential of enniatin B using *in vitro* bioassays

Shewit Kalayou [a,b,*], Doreen Ndossi [c], Caroline Frizzell [d], Per Kristian Groseth [a], Lisa Connolly [d], Morten Sørlie [e], Steven Verhaegen [a], Erik Ropstad [a]

[a] Section of Experimental Biomedicine, Norwegian School of Veterinary Science, Oslo, Norway
[b] Mekelle University College of Veterinary Medicine, Mekelle, Ethiopia
[c] Sokoine University of Agriculture, Morogoro, Tanzania
[d] Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, Northern Ireland, UK
[e] Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Ås, Norway

HIGHLIGHTS

- Endocrine disruption potential of ENN B was investigated using primary and cell lines.
- ENN B modulates cell viability and cell cycle distribution.
- Effect on steroid production in Leydig cells was observed only at higher concentrations used.
- ENN B reduced hormone production and modulated steroidogenic genes in adrenal H295R cells.
- No specific (ant) agonistic responses observed at the receptor level.

ARTICLE INFO

Article history:
Received 17 July 2014
Received in revised form 22 January 2015
Accepted 23 January 2015
Available online 24 January 2015

Keywords:
Leydig cell
H295R
ENN B
Emerging mycotoxins
Steroidogenesis
RGA
Endocrine disruption

ABSTRACT

Evidence that some of the fungal metabolites present in food and feed may act as potential endocrine disruptors is increasing. Enniatin B (ENN B) is among the emerging *Fusarium* mycotoxins known to contaminate cereals. In this study, the H295R and neonatal porcine Leydig cell (LC) models, and reporter gene assays (RGAs) have been used to investigate the endocrine disrupting activity of ENN B. Aspects of cell viability, cell cycle distribution, hormone production as well as the expression of key steroidogenic genes were assessed using the H295R cell model. Cell viability and hormone production levels were determined in the LC model, while cell viability and steroid hormone nuclear receptor transcriptional activity were measured using the RGAs. ENN B (0.01–100 μM) was cytotoxic in the H295R and LC models used; following 48 h incubation with 100 μM. Flow cytometry analysis showed that ENN B exposure (0.1–25 μM) led to an increased proportion of cells in the S phase at higher ENN B doses (>10 μM) while cells at G_0/G_1 phase were reduced. At the receptor level, ENN B (0.00156–15.6 μM) did not appear to induce any specific (ant) agonistic responses in reporter gene assays (RGAs), however cell viability was affected at 15.6 μM. Measurement of hormone levels in H295R cells revealed that the production of progesterone, testosterone and cortisol in exposed cells were reduced, but the level of estradiol was not significantly affected. There was a general reduction of estradiol and testosterone levels in exposed LC. Only the highest dose (100 μM) used had a significant effect, suggesting the observed inhibitory effect is more likely associated with the cytotoxic effect observed at this dose. Gene transcription analysis in H295R cells showed that twelve of the sixteen genes were significantly modulated ($p < 0.05$) by ENN B (10 μM) compared to the control. Genes *HMGR, StAR, CYP11A, 3βHSD2* and *CYP17* were downregulated, whereas the expression of *CYP1A1, NR0B1, MC2R, CYP21, CYP11B1, CYP11B2* and *CYP19* were upregulated. The reduction of hormones and modulation of genes at the lower dose (10 μM) in the H295R cells suggests that adrenal endocrine toxicity is an important potential hazard.

Abbreviations: *HMGR*, hydroxy-methyl-glutaryl CoA reductase; *StAR*, steroidogenic acute regulatory; *CYP11A*, cytochrome P450 11A; *CYP11B1*, cytochrome P450 11B1; *CYP11B2*, aldosterone synthetase; *CYP17*, cytochrome P450 17; *CYP21*, cytochrome P450 21; *3βHSD2*, 3-beta-hydroxysteroid dehydrogenase 2; *17β HSD1*, 17β hydroxysteroid dehydrogenase 1 (17 ketoreductase); *17β HSD4*, 17β hydroxysteroid dehydrogenase 4; *CYP19*, aromatase; *CYP1A*, cytochrome P450 1 A1; *NR5A1*, nuclear receptor 5A1; *NR0B1*, nuclear receptor 0B1; *MC2R*, melanocortin 2 receptor; *EPHX*, epoxide hydrolase (Microsomal).

* Corresponding author at: Section of Experimental Biomedicine, Norwegian School of Veterinary Science, Oslo, Norway. Tel.: +47 22964500; fax: +47 22597309.
E-mail address: shewit.kalayou.teklehaimanot@nmbu.no (S. Kalayou).

http://dx.doi.org/10.1016/j.toxlet.2015.01.014
0378-4274/

1. Introduction

Fusarium fungi frequently infect crops worldwide. They produce a wide array of secondary metabolites, many of which have been associated with adverse health effects both in animals and humans (Placinta et al., 1999). These mycotoxins include the well-known trichothecenes, fumonisins and zearalenone (Jestoi, 2008). The toxicological impact of these well-known *Fusarium* mycotoxins is diverse including reduced growth, immunosuppression, neurological and reproductive disorders (Placinta et al., 1999). In addition to their direct toxic effects, some are linked with the incidence of cancer *e.g.* fumonisins (Gelderblom et al., 1988). Therefore due to these mycotoxins potential health risks to both humans and livestock, much research has been conducted to study their occurrence, toxicity and strategies for their prevention in food chain. However, much less knowledge exists for several other emerging *Fusarium* mycotoxins such as enniatins, beauvericin, moniliformin and fusaproliferin. The physicochemical and biological properties of these emerging mycotoxins have been extensively reviewed by Jestoi (2008). These less-known mycotoxins are co-produced with the well-known *Fusarium* toxins. Unlike many of the frequently occurring *Fusarium* toxins, their maximum levels in foods are not regulated by legislation (EU, 2006). Meanwhile evidence that their incidence is rapidly increasing underlines the need for further investigations and risk assessments.

Among the emerging mycotoxins, enniatins (ENNs) were the most prevalent contaminants in various geographical regions, and were commonly found to contaminate cereals and other feedstuffs (Uhlig et al., 2006; Jestoi, 2008; Meca et al., 2010; Devreese et al., 2013; Vaclavikova et al., 2013). Currently, there are 28 known ENN analogues with the main toxic variants being ENN A, A1, B, B1 and B4 (Ivanova et al., 2006). The relative concentration ratio of ENN mycotoxins reported from Norwegian and Finnish grain samples was as follows: ENN B > ENN B1 > ENN A1 > ENN A (Uhlig et al., 2007). The available occurrence data show that ENN B was found in food with average concentrations of 57.4 mg/kg in Tunisian breakfast cereals (Oueslati et al., 2011), 10.6 mg/kg in Moroccan muesli (Mahnine et al., 2011) and 18.3 mg/kg in Finnish wheat (Jestoi et al., 2004). ENN B was also present in cereals of Spanish commodities with levels ranging between 2.23 and 21.37 mg/kg (Meca et al., 2010). Although ENNs are now recognized as frequent contaminants of grains, data on their potential adverse health effects are sparse.

ENN B and other ENNs possess a wide range of biological properties such as antibacterial, antifungal, antihelmintic, insecticidal and anti-cancer potency (Jestoi, 2008). The available data indicates that ENNs are of low acute toxicity but *in vivo* experiments are scarce. In mice, acute toxicity and death occurred only after intraperitoneal administration of 10–40 mg/kg of body weight per day over 6 days (McKee et al., 1997). Chronic exposure by feeding experiments may induce feed refusal, weight loss and reduced productivity (Jestoi, 2008). However, there are several *in vitro* experiments showing that ENNs are cytotoxic at low micromolar concentrations (Ivanova et al., 2006; Dornetshuber et al., 2007; Behm et al., 2009; Meca et al., 2011; Gammelsrud et al., 2012). The primary toxicity of ENNs results from its ionophoric characteristics that enables transfer of mono-and divalent cations through cell membrane (Jestoi, 2008). Moreover, ENN B also exerts cytotoxic activities by inducing mitochondrial modifications and cell cycle disruption, finally resulting in apoptotic cell death (Dornetshuber et al., 2007; Watjen et al., 2009; Gammelsrud et al., 2012). ENN B in concentrations up to 100 μM has not shown genotoxic activity (Behm et al., 2009).

In recent years, it has been highlighted that there is a risk that some of the fungal metabolites present in the food and feedstuffs may act as potential endocrine disruptors (Yang et al., 2007; Frizzell et al., 2011; Ndossi et al., 2012; Frizzell et al., 2013). Appropriate levels of steroid hormones are pivotal for reproductive success. Exposure at critical periods of development may induce persistent changes in both reproductive and non-reproductive organs, including persistent molecular alterations (Placinta et al., 1999). Despite the overwhelming evidence of global contamination of cereals and food products with ENN B, data on any potential endocrine disrupting effects of ENN B has not been established. ENN B and other ENNs have been previously been suggested to inhibit acyl-CoA: cholesterol acyltransferase (ACAT) activity (Tomoda et al., 1992). ACAT is a regulator of cholesteryl ester formation in the adrenal gland (Liscum and Dahl, 1992). Steroidogenic factor-1 (SF-1, *NR5A1*) dependent up-regulation of ACAT is important for maintaining readily available cholesterol esters, needed at times of active steroidogenesis (Ferraz-de-Souza et al., 2011). Here it is hypothesized that ENN B could have cytotoxic and endocrine disrupting properties. The human adrenocortical carcinoma cell line (H295R), the primary culture of neonatal porcine Leydig cells and reporter gene assays (RGAs) are used to test the potential toxicity of ENN B. The H295R and Leydig cell models may represent a unique *in vitro* system for evaluating chemicals which may affect adrenal and testicular steroidogenesis, respectively. The H295R cell line has the physiological characteristics of zonally undifferentiated human foetal adrenal cells with the ability to produce the steroid hormones found in both the adult adrenal cortex and the gonads (Gracia et al., 2007). Neonatal Leydig cells are testicular cells responsible for steroid production in male mammals. These porcine neonatal stage cells have been shown to be useful in endocrine disruption investigations. Most of the testicular volume at this developmental stage is made up of Leydig cells and this is also a time when dynamic changes in reproductive tract development occurs, thus hormonally active chemicals are known to exhibit greater potency (Van Straaten and Wensing, 1978; Colborn et al., 1993). Meanwhile, the RGA cell lines are human mammary gland cell lines with natural steroid hormone receptors for estrogens, androgens, progestagens and glucocorticoids (Willemsen et al., 2004), which allow endocrine disruption at the level of nuclear receptor transcriptional activity to be identified. This study, therefore, aims at making use of these cell lines to investigate whether ENN B could potentially impact cell viability, cell cycle distribution, hormone production potential and expression of key genes that code steroidogenesis, as well as nuclear receptor transcriptional activity.

2. Materials and methods

2.1. Reagents

ENN B (molecular weight, 639.82 Da; >95% HPLC purity), methanol (MeOH), dimethyl sulfoxide (DMSO), forskolin, 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 Gibco, Invitrogen (Paisley, UK) and the cells cultured at 37 °C and 5% CO_2 atmosphere, unless otherwise stated.

2.2. H295R cell culture

The human adrenocortical carcinoma H295R cells, were obtained from the American Type Culture Collection (ATCC # CRL-2128, ATCC, Manassas, VA, USA) and cultured as previously described (Hilscherova et al., 2004). Briefly the cells were grown in 75 cm^2 flasks with 12.5 mL of 1:1 mixture of Dulbecco's Modified Eagle Medium and Ham's F-12 Nutrient (DMEM/F12) containing 15 mM HEPES buffer, supplemented with 1% ITS™ Premix (BD Biosciences, Bedford, MA, USA) and 2.5% NuSerum (BD Biosciences). The medium was changed 2–3 times a week. Cells were