**QUEEN'S
UNIVERSITY
BELFAST**

An in vitro investigation on the cytotoxic and nuclear receptor transcriptional activity of the mycotoxins fumonisin B1 and beauvericin

Fernández-Blanco, C., Frizzell, C., Shannon, M., Ruiz, M-J., & Connolly, L. (2016). An in vitro investigation on the cytotoxic and nuclear receptor transcriptional activity of the mycotoxins fumonisin B1 and beauvericin. *Toxicology Letters*, 257, 1-10. <https://doi.org/10.1016/j.toxlet.2016.05.021>

Published in:
Toxicology Letters

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
[Link to publication record in Queen's University Belfast Research Portal](#)

Publisher rights

© 2016 Elsevier

This is an open access article published under a Creative Commons Attribution-NonCommercial-NoDerivs License (<https://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits distribution and reproduction for non-commercial purposes, provided the author and source are cited.

General rights

Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

An *in vitro* investigation on the cytotoxic and nuclear receptor transcriptional activity of the mycotoxins fumonisin B1 and beauvericin.

Celia Fernández-Blanco¹, Caroline Frizzell², Maeve Shannon², Maria-Jose Ruiz¹, Lisa Connolly^{2*}.

Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain.

Institute for Global Food Security, School of Biological Sciences, Queen's University, Belfast, Northern Ireland, United Kingdom.

Abstract

Fumonisin B1 (FB1) and beauvericin (BEA) are secondary metabolites of filamentous fungi, which under appropriate temperature and humidity conditions may develop on various foods and feeds. To date few studies have been performed to evaluate the toxicological and endocrine disrupting effects of FB1 and BEA. The present study makes use of various *in vitro* bioassays including; oestrogen, androgen, progestagen and glucocorticoid reporter gene assays (RGAs) for the study of nuclear receptor transcriptional activity, the thiazolyl blue tetrazolium bromide (MTT) assay to monitor cytotoxicity and high content analysis (HCA) for the detection of pre-lethal toxicity in the RGA and Caco-2 human colon adenocarcinoma cells.

At the receptor level, 0.001-10 µM BEA or FB1 did not induce any agonist responses in the RGAs. However at non-cytotoxic concentrations, an antagonistic effect was exhibited by FB1 on the androgen nuclear receptor transcriptional activity at 10 µM and BEA on the progestagen and glucocorticoid receptors at 1 µM. MTT analysis showed no decrease in cell viability at any concentration of FB1, whereas BEA showed a significant decrease in viability at 10 µM. HCA analysis confirmed that the reduction in the progestagen receptor transcriptional activity at 1 µM BEA was not due to pre-lethal toxicity. In addition, BEA (10 µM) induced significant toxicity in both the TM-Luc (progestagen responsive) and Caco-2 cells.

Keywords: Mycotoxin, Beauvericin, Fumonisin B1, Reporter gene assay, High Content Analysis.

* **Corresponding author:** Tel.: +44 28 90976668; fax: +44 28 90976513. E-mail address: l.connolly@qub.ac.uk (L. Connolly).

1. Introduction

Mycotoxins are secondary metabolites of filamentous fungi, which under appropriate temperature and humidity conditions may develop on various foods and feeds. They are mainly produced by fungi belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps* (Fung et al., 2004). *Fusarium* species are contaminants of wheat, maize, and other grains worldwide, capable of producing high levels of fumonisin mycotoxins. Fumonisin B1 (FB1) is the most prevalent of the fumonisins, accounting for approximately 70% of total fumonisins (Martins et al., 2012). Studies have also highlighted that *Fusarium* species can co-produce other mycotoxins such as Beauvericin (BEA) simultaneously (Dombrink-Kurtzman, 2003).

Total fumonisin concentrations in feed materials have been reported to vary from a few µg/kg to tens of mg/kg (EFSA, 2005). Dietary fumonisin estimates, by the Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO, 2001), indicate exposure levels ranging from 0.02-0.2 µg/kg in body weight (b.w.)/day, thus remaining below the Tolerable Daily Intake (TDI) of 2 µg/kg b.w./day as set in Europe by the Scientific Committee on Food (SCF, 2003). Nevertheless, a wide range of animal diseases and pathophysiological effects such as leukoencephalomalacia, porcine pulmonary oedema, liver and kidney toxicity and liver cancer, as well as human oesophageal carcinoma are associated with FB1 ingestion (Harrison et al., 1990; Kellerman et al., 1990; Gelderblom et al., 1997; Hussein et al., 2001). While the molecular mechanism of FB1 toxicity is poorly understood, it appears to be related to the deregulation of sphingolipid metabolism (Merrill et al., 2001).

BEA is predominantly found in cereal grains such as wheat, maize and rice (Serrano et al., 2012) as well as other matrices such as nuts and dried fruits (Tolosa et al., 2013). The mean dietary exposure to BEA varies from a minimum of 0.003 µg/kg b.w./day to a maximum of 0.050 µg/kg b.w./day (EFSA, 2014). However, the Panel on Contaminants in the Food Chain (CONTAM) concluded that there was insufficient data to establish a TDI or/and an acute reference dose (ARfD) for BEA in humans (EFSA, 2014). BEA possesses a wide range of biological activities. These substances are known as ionophores, forming a complex with essential cations (Ca²⁺, Na⁺, K⁺), which increases ion permeability of biological membranes, therefore potentially affecting ionic homeostasis (Chen et al., 2006). Many mycotoxins such as ochratoxin A, patulin, alternariol and zearalenone have been found to possess endocrine disrupting capabilities (Frizzell et al., 2011, 2013a, 2013b and 2014).

Endocrine disruptors (EDs) include both natural and man-made substances that may interfere with the body's endocrine system by acting like endogenous hormones and inducing adverse developmental, reproductive, neurological and immune effects (IPCS, 2002). A few studies suggest that FB1 may act as a potential ED (Collins et al., 1998; Gbore et al., 2009). While there is not enough data to confirm that FB1 is a developmental or reproductive toxicant in animals or humans, Collins et al., (1998) reported that FB1 was toxic to maternal rats and the foetus at 15 mg/kg of feed consumption. In addition, Gbore (2009) reported that FB1 affected fertility in pigs by causing a delay in sexual maturity and poor sperm production and quality. There are no *in vivo* toxicological studies available on reproduction and developmental toxicity, neurotoxicity or carcinogenicity for BEA. However, it has been shown to be absorbed and rapidly metabolised to a range of uncharacterised metabolites as detected in the eggs of laying hens and several tissues of turkeys and broilers (Jestoi, 2008).

In vitro bioassays may be used to investigate the toxicity and endocrine disrupting potential of compounds (Connolly et al., 2011). The emerging technology, High Content Analysis (HCA) is a highly powerful multi-parameter bio-analytical based tool incorporating fluorescent microscopy with automated *in vitro* cell analysis software. HCA provides assays with high sensitivity and specificity for pre-lethal cytotoxicity and multiple biological endpoints for use as a high throughput-screening tool to monitor the cytotoxicity, endocrine disruption and biological effects of compounds on exposed cells (Clarke et al., 2015).

In this study, we have investigated the endocrine disrupting and cytotoxic potential of FB1 and BEA using various *in vitro* bioassays. Reporter gene assays (RGAs) utilising human mammary gland cells with natural steroid hormone receptors for oestrogens, androgens, progestagens and glucocorticoids (Willemsen et al., 2004) are employed for the identification of endocrine disruption at the level of nuclear receptor transcriptional activity. HCA is used to detect early cytotoxicity, via multiple markers in the progestagen responsive (TM-Luc) cell line exposed to 0.001-10 μ M BEA, to ensure that a reduction in transcriptional activation of endocrine receptors is not correlated with pre-lethal toxicity. HCA is also used to assess cytotoxicity in colon adenocarcinoma (Caco-2) cells because the ingestion of food contaminated with FB1 and BEA is the main exposure route for animals and humans.

2. Materials and methods

2.1 Reagents

Methanol, thiazolyl blue tetrazolium bromide (MTT), FB1, BEA and the steroid hormones 17 β -estradiol, testosterone, progesterone and hydrocortisone were obtained from Sigma–Aldrich (Poole, Dorset, UK). Cell culture reagents were obtained from Life Technologies (Paisley, UK). Multiparameter cytotoxicity 2 multiplex kit (8400202) containing mitochondrial probe and cell membrane permeability dye was supplied by Thermo Scientific, UK. Stock solutions of FB1 and BEA were prepared in methanol and stored at -20°C. FB1 and BEA were dissolved in methanol at a final concentration of 0.5% (v/v) in media for the RGAs, MTT assays and HCA.

2.2 Cell culture

All cells were routinely cultured in 75 cm² tissue culture flasks (Nunc, Roskilde, Denmark) at 37 ° with 5% CO₂ and 95% humidity.

Four RGA cell lines were previously developed by the transformation of human mammary gland cells with the luciferase gene under the control of a steroid hormone inducible promoter (Willemssen et al., 2004). The MMV-Luc cell is specific for the detection of oestrogens, TARM-Luc for androgens and progestagens, TM-Luc for progestagens and TGRM-Luc for glucocorticoids and progestagens. The RGA cells were routinely grown in cell culture medium containing Dulbecco's Modified Eagle Medium (DMEM), 10% foetal bovine serum (FBS) and 1% penicillin streptomycin. As phenol red is a weak oestrogen, DMEM without phenol red was used when culturing the MMV-Luc cells. Cells were transferred prior to RGA analysis into assay media, which was composed of DMEM and 10% hormone depleted serum.

The Caco-2 cell line (ATCC HTB-37) was routinely grown in DMEM medium, 10% FBS and 1% penicillin streptomycin.

2.3 Reporter gene assay (RGA).

RGAs were carried out as previously described by Frizzell et al. (2011). Briefly, cells were seeded at a concentration of 4×10^5 cells/ml, 100 μ l/well, into white walled 96 well plates with clear flat bottoms (Greiner Bio-One, Germany). The cells were incubated for 24 h and then exposed to BEA and FB1 (0.001, 0.01, 0.1, 1, 10 μ M) for the agonist test. The positive control used with each cell line was as follows: 1.35 ng/ml 17 β -estradiol (MMV-Luc cells), 14.5 ng/ml testosterone (TARM-Luc cells), 157 ng/ml

progesterone (TM-Luc cells) and 181 ng/ml hydrocortisone (TGRM-Luc cells). A solvent control 0.5% (v/v) methanol in media was also added to each plate. Antagonist tests were carried out by incubating BEA and FB1 (0.001, 0.01, 0.1, 1, 10 μ M) with the relevant positive control for each cell line. The cells were incubated for 48 h, after which, the media was discarded and the cells washed once with phosphate buffered saline (PBS). The cells were lysed with 30 μ l cell culture lysis buffer (Promega, Southampton, UK) and then 100 μ l luciferase (Promega, Southampton, UK) injected into each well and the response measured using the Mithras Multimode Reader (Berthold, Other, Germany). The response of the cells to the various compounds was measured and compared with the solvent control.

2.4 Cell viability assay

The MTT assay, based on the ability of viable cells to metabolize the yellow tetrazolium salt to a blue formazan product by the mitochondria, was performed in parallel to the RGA assays to monitor for cytotoxic effects of the mycotoxins and their concentrations tested.

Briefly, the cells were exposed exactly as for the RGAs but in clear flat bottomed 96 well plates (Nunc, Roskilde, Denmark). Following removal of the media, 50 μ L of MTT solution (2 mg/ml stock in PBS diluted 1:2.5 in assay media) was added to each well and incubated for 4 h. The supernatant was removed and 200 μ L/well of DMSO added to dissolve the formazan crystals. The absorbance was measured at 570nm and a reference absorbance of 630nm using an automatic plate reader (Tecan, Safire, USA). Cell viability was calculated as a percentage absorbance of the sample when compared to the absorbance of the solvent control (0.5% (v/v) methanol in media).

2.5 HCA multi-parameter assay

HCA is a rapid and robust technology which can determine multiple cytotoxic effects, including early (pre-lethal) as well as late-stage occurrences of cytotoxicity simultaneously. The cytotoxicity of BEA and FB1 was assessed on Caco-2 cells as an effective indicator of toxicity to the human gut. The TM-Luc cell line was also investigated by HCA to confirm whether pre-lethal toxicity was inducing the antagonist response observed at 1 μ M.

Briefly, cells were seeded at a concentration of 2×10^4 cells/ml, 100 µl/well, into 96 well plates (Nunc, Roskilde, Denmark). The cells were incubated for 24 h and then exposed to (0.001, 0.01, 0.1, 1, 10 µM) of BEA (TM-Luc cells for 48 h) and BEA or FB1 (Caco-2 cells for 24 and 48 h).

Cellomics® HCA reagent series multi-parameter cytotoxicity dyes were utilised. Mitochondrial membrane potential dye was prepared by adding 117 µl of anhydrous DMSO to make a 1 mM stock. Permeability dye was used as provided in the multiparameter cytotoxicity 2 multiplex kit (8400202). The live cell staining solution was prepared by adding 2.1 µl permeability dye to 6 ml of complete media that had been preheated to 37°C, and then 21 µl of mitochondrial membrane potential (final concentration 3.5 mM). Nuclear stain solution was prepared by adding 5.5 µl Hoechst 33342 dye to 11 ml 1X Wash Buffer.

After incubation, 50 µl of live cell staining solution was added to each well. Cells were incubated in the dark at 37°C and 5% CO₂ for 30 min. The staining solution was aspirated and 100 µl of 10% formalin solution (fixation solution) added. The cells were incubated for 20 min at room temperature before discarding the fixation solution and washing the cells with 100 µl of PBS. Nuclear staining solution (100 µl) was then added, and the cells incubated for 10 min at room temperature protected from light. The cells were then washed twice and the wells filled with 100 µl of PBS. Cell number (CN), nuclear area (NA), nuclear intensity (NI), plasma membrane permeability (PMP), mitochondrial membrane potential (MMP) and mitochondrial mass (MM) were measured using the CellInsight™ NXT High Content Screening platform (Thermo Fisher Scientific, UK).

2.6 Statistical analysis

Assay exposures were carried out in triplicate wells and in three independent experiments. Results were expressed as the mean $\pm$ standard error of the mean (SEM) of the triplicate exposures. For the RGAs, data was analysed using Microsoft Excel and Graphpad PRISM software (San Diego, CA). A one way analysis of variance (ANOVA) and Dunnett's multiple comparison test was used to determine significant differences between the treatments and the corresponding controls in the RGAs, MTT assays and HCA. The mean concentrations were tested for significant difference at the 95% confidence level. A *p* value of < 0.05 was considered statistically significant, $p = \leq 0.05$ (*), ≤ 0.01 (**) and ≤ 0.001 (***).

3. Results

3.1. Cell viability

The MTT assay was used to determine the viability of the RGA cells following exposure to FB1 or BEA (0.001-10 μ M). No cytotoxicity was observed in any of the RGA cell lines exposed to 0.001-10 μ M FB1 (Fig.1) or 0.001-1 μ M BEA. However, at 10 μ M BEA, a decrease in cell viability for all RGA cell lines was observed ($p \leq 0.001$) (Fig. 1).

3.2. Reporter gene assays

Neither FB1 nor BEA (0.001-10 μ M) exhibited an agonist response in any of the four RGA cell lines (data not shown). However FB1, at the highest concentration tested (10 μ M), exhibited an antagonistic effect ($p \leq 0.05$) on the androgen nuclear receptor transcriptional activity (Fig. 2b). No antagonist effects were observed in the progestagen, glucocorticoid or oestrogen RGAs (Fig. 2a, c and d). BEA, at the highest concentration tested (10 μ M), exhibited a strong antagonistic response ($p \leq 0.001$) in the oestrogen, androgen, progestagen and glucocorticoid RGAs (Fig. 3a-d). However, the MTT assay results indicate that this response is due to the cytotoxicity of BEA at 10 μ M on all of the RGA cell lines. Antagonistic effects on nuclear receptor transcriptional activity in the progestagen ($p \leq 0.05$) and glucocorticoid ($p \leq 0.01$) RGAs were also observed at non-toxic concentrations of 1 μ M BEA (Fig. 3c and d). Considering that BEA is cytotoxic to all of the RGA cell lines at 10 μ M, it is possible that the antagonism observed at 1 μ M BEA is not a true response and instead may be due to pre-lethal toxicity being initiated within the cells. The validity of this response was further explored by HCA in the progestagen responsive, TM-Luc cell line.

3.3 High Content Analysis (HCA).

In the TM-Luc (progestagen responsive) cell line, BEA (10 μ M) was not possible to analyse due to lethal cytotoxic effects. BEA (1 μ M) did not show any significant differences when compared to the control. Therefore, no pre-lethal toxicity was observed at 1 μ M BEA, confirming that the antagonism observed in the progestagen RGA was a true response (Fig. 4).

Exposure of Caco-2 cells to 0.001-10 μ M FB1 or BEA revealed that 1 μ M BEA caused a significant ($p \leq 0.01$) decrease in the CN (Fig. 5). Nevertheless, 10 μ M BEA was not possible to analyse due to lethal cytotoxic effects on the Caco-2 cells.

4. Discussion

The MTT assay confirmed that FB1 (0.1 -10 μ M) was not cytotoxic to any of the four RGA cell lines. This value is consistent with other publications, Meca et al., (2010) showed that exposure of Vero cells (monkey kidney) to 0-100 μ M FB1 for 24 h decreased cellular viability to 60 % at 100 μ M when compared to the control. In addition, Wan et al., (2013) did not observed a reduction of viability from 0 to 20 μ M FB1 in IPEC-J2 (porcine jejunal epithelial) cell line after 48 h of exposure.

BEA reduced cell viability at a concentration of 10 μ M in all of the RGA and Caco-2 cell lines. BEA (1 μ M) also decreased viability in the Caco-2 cell line upon 48 h exposure. This data is consistent with previous studies whereby 24 and 48 h 0-30 μ M BEA exposure of Caco-2 cells decreased viability to 80% and 87% respectively and HT-29 (human colon adenocarcinoma) cells presented a decrease of 85% at 24 h and 90% at 48 h (Prosperini et al., 2012). Similar results were obtained by Calo et al. (2004) with two human cell lines of myeloid origin (U-937 and HL-60 cells) and Ferrer et al. (2009) who investigated 0-100 μ M BEA exposure on Chinese hamster ovary cells (CHO-K1). They observed a decline in viability at a concentration of 10 μ M or higher after 24 h.

The application of HCA in toxicity studies is based on the parallel analysis of multiple markers for cytotoxicity, which allows early reversible and late irreversible effects to be distinguished, and thus provides a more detailed analysis of compound-induced toxicity (Ramirez et al. 2010; Tolosa et al., 2015). In this context, HCA can identify gross toxicity and pre-lethal toxicity, whereby exposed cells are not dead but are becoming unhealthy. While traditional end-point toxicity assays such as MTT can identify gross toxicity, they cannot do so for pre-lethal toxicity.

In the current study, an antagonist response was observed in the progesterone responsive TM-Luc cell line after exposure to 1 μ M BEA. While the MTT assay was able to confirm cytotoxicity via BEA exposure at 10 μ M but not at 1 μ M, the potential for pre-lethal toxicity being responsible for the perceived antagonist response was considered. Consequently, HCA analysis was utilised to confirm the absence of pre-

lethal toxicity and thus confirm the validity of the progesterone receptor antagonist response.

The Caco-2 cell line is a well-recognised human gut cell model (Sambuy et al., 2004) and as such is suited to investigating the toxic effects of food contaminants. HCA analysis confirmed that FB1 was not cytotoxic at any of the concentrations tested on the Caco-2 cell line. However, BEA exhibited cytotoxicity at 1 μ M on the Caco-2 cell line. Furthermore, in this study was observed a slight decrease in MMP at 1 μ M BEA. According to Jow et al. (2004), Ca^{2+} -dependent pathway by BEA involves cell death, in which it induced an increase in intracellular $[\text{Ca}^{2+}]$ that leads to a combination of cellular apoptosis and necrosis responses. Moreover, Tonshin et al., (2010) in isolated mitochondria BEA induced a loss of MMP where K^{+} inflow into the mitochondrial matrix and uncoupling of oxidative phosphorylation, followed by induction of apoptosis. In addition, Prosperini et al., (2013) investigated that Caco-2 cells exhibit mitochondrial dysfunction leading a stable depolarized state of MMP and cell death after exposure of 1.5 and 3 μ M BEA. Low BEA concentrations might be reached due to food consumption and based on tissue accumulation (Jestoi et al., 2007). Moreover, with regard to food intake, BEA might increase the absorption of commonly co-occurring mycotoxins probably leading to higher toxicity. Thus, exposure to low BEA concentrations activates diverse cellular stress response and protection systems (Mallebrera et al., 2014). This indicates that continuous exposure to BEA might lead to alter the intestinal epithelial barrier (Dornetshuber et al., 2009).

Antagonism of the androgen receptor in the TARM-Luc cell line was observed following exposure to 10 μ M FB1. A reduction in the transcriptional activity of the androgen, glucocorticoid, oestrogen and progestagen receptor was correlated to the cytotoxic effects of BEA at 10 μ M rather than true antagonism. An antagonistic response was also observed in the TGRM-Luc (glucocorticoid) and TM-Luc (progesterone) cell lines following exposure to 1 μ M BEA. HCA established that no pre-lethal toxicity was evident in the TM-Luc cell line at 1 μ M BEA and thus the reduction in progesterone receptor transcriptional activity was confirmed as a true antagonist response. To the authors' knowledge, this is the first study investigating the endocrine disrupting effects of FB1 and BEA at the level of nuclear receptor activity. The actions of progesterone, glucocorticoid and androgen are mediated by its receptor. In the target cell, progesterone, glucocorticoid and androgen produce a change in

conformation of its receptors that is associated with transforming receptors from a non-DNA binding form to one that will bind to DNA (Spitz et al., 2003). This transformation is go with a loss of associated heat shock proteins and dimerization. The activated receptors dimers then binds to specific DNA sequences within the promotor region of progesterone, glucocorticoid and androgen responsive genes. Antagonist impair the ability of receptors to interact with coactivators allowing the recruitment of corepressors (Liu et al., 2002). The antagonist activity of an antihormone may depend on the cell or tissue type. In addition, these transformations in the structure and function of the receptor results in numerous endocrine disorders. Many antagonists of progesterone receptor display antiproliferative effects in the endometrium by suppressing follicular development and blocking the LH flood. Moreover, progesterone antagonists are potent antiglucocorticoid agents (Neulen et al., 1996). GR signalling is required for homeostatic control of pyramidal neurons. Thus, GR hormone influence memory, mood, and neuronal survival (Savory et al., 2001) Therefore, inhibition of the GR may affect the peripheral glucose metabolism, the stress response, and the regulation of the hypothalamic pituitary axis (Honer et al., 2003; Deroche-Gamonet et al., 2003). The regulatory steroidal sex hormones role in developmental processes such as sex determination and differentiation is of particular interest with regard to endocrine disruption (Kelce et al., 1995; 1997). Androgens, through interaction with the androgen receptor, play decisive roles in sexual differentiation of the male reproductive tract, accessory reproductive organs, and other tissues during fetal development. They also influence male pubertal maturation and the maintenance of secondary sex characteristics in adults. (Wilson et al., 2001)

This *in vitro* investigation has demonstrated the potential for FB1 and BEA to modulate the endocrine system by antagonism of nuclear transcriptional activity as observed for BEA (1 μ M) on the glucocorticoid and progesterone receptor and FB1 (10 μ M) on the androgen receptor. HCA has also proven to be an added value cytotoxic assessment tool in establishing pre-lethal toxicity in exposed cells and confirming antagonistic responses. In addition, while FB1 did not show any significant cytotoxic effects on mammalian gut cells, BEA did at a concentration of 1 μ M. Further investigation is needed to investigate the risk of BEA and FB1 exposure in humans and animals.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgement

The authors wish to thank Rachel Clarke for her help and training the High Content Analysis and to the Economy and Competitiveness Spanish Ministry (AGL2013-43194-P)

338

339 **References**

340

341 Calò, L., Fornelli, F., Ramires, R., Nenna, S., Tursi, A., Caiaffa, M.F., Macchia, L.,
342 2004. Cytotoxic effects of the mycotoxin beauvericin to humans cell lines of myeloid
343 origin. *Pharmacol. Res.* 49, 73-77

344 Chen, BF, Tsai, MC and Jow, GM., 2006. Induction of calcium influx from
345 extracellular fluid by beauvericin in human leukemia cells. *Biochem. Biophys. Res.*
346 *Com.* 340, 134–139.

347 Clarke, R., Connolly, L., Frizzell, C., Elliott, C.T., 2015. High content analysis: A
348 sensitive tool to detect and quantify the cytotoxic, synergistic and antagonistic effects of
349 chemical contaminants in food. *Tox.Lett.* 18, 278-86.

350 Collins, T.F., Shackelford, M.E., Sprando, R.L., Black, T.N., Láborde, J.B., Hansen,
351 D.K., Eppley, M., Trucksess, M.W., Howard, P.C., Bryant, M.A., Ruggles, D.I.,
352 Olejnik, N and Rorie, J.I., 1998. Effects of Fumonisin B1 in Pregnant Rats. *Food Chem.*
353 *Toxicol.* 36, 397-408.

354 Connolly, L., Ropstad, E., Verhaegen, S., 2011. In vitro bioassays for the study of
355 endocrine-disrupting food additives and contaminants. *Trends Anal. Chem.* 30, 227–
356 238.

357 Deroche-Gamonet, V., Sillaber, I., Aouizerate, B., Izawa, R., Jaber, M., Ghozland, S.,
358 Kellendonk, C., Le Moal, M., Spanagel, R., Schütz, G., Tronche, F., Piazza, P.V., 2003.
359 The glucocorticoid receptor as potential target to reduce cocaine abuse. *J. Neurosci.* 11,
360 4785-4790.

361 Dombrink-Kurtzman, M.A., 2003. Fumonisin and beauvericin induce apoptosis in
362 turkey peripheral blood lymphocytes. *Mycopathologia* 4, 357-364

363 Dornetshuber, R., Heffeter, P., Lemmens-Gruber, R., Elbling, L., Marko, D., Micksche,
364 M., Berger, W., 2009. Oxidative stress and DNA interactions are not involved in
365 enniatin- and beauvericin-mediated apoptosis induction. *Molecular Nutrition and Food*
366 *Research* 53, 1112–1122.

367 EFSA (European Food Safety Authority)., 2005 Opinion of the Scientific Panel on
368 Contaminants in Food Chain on a request from the Commission related to fumonisins as
369 undesirable substances in animal feed. EFSA, 10, 2005, p. 1004

370 EFSA (European Food Safety Authority). 2014 Scientific Opinion on the risks to human
371 and animal health related to the presence of beauvericin and enniatins in food and feed.

372 EFSA, 12, 2014, p. 3802FAO/WHO (Food and Agriculture Organization/World Health
373 Organization), 2001. Safety evaluation of certain mycotoxins in food. Prepared by the

374 Fifty-sixth meeting of the Joint FAO/WHO Expert Committee on Food Additives.
375 WHO Food Additives Series, No. 47; FAO Food and Nutrition Paper 74, 2001.

376 Ferrer, E., Juan-García, A., Font, G., Ruiz, M.J., 2009. Reactive oxygen species induced
377 by beauvericin, patulin and zearalenone in CHO-K1 cells. *Toxicol. In Vitro* 1504–1509.

378 Frizzell, C., Ndossi, D., Verhaegen, S., Dahl, E., Eriksen, G., Sørli, M., Ropstad,
379 E., Muller, M., Elliott, C.T., Connolly, L., 2011. Endocrine disrupting effects of
380 zearalenone, alpha- and beta-zearalenol at the level of nuclear receptor binding and
381 steroidogenesis. *Toxicol. Lett.* 206, 210–217.

382 Frizzell, C., Ndossi, D., Kalayou, S., Eriksen, G.S., Verhaegen, S., Sørli, M.,
383 Elliott, C.T., Ropstad, E., Connolly, L., 2013a. An in vitro investigation of endocrine
384 disrupting effects of the mycotoxin alternariol. *Toxicol. Appl. Pharmacol.* 271, 64–71.

385 Frizzell, C., Verhaegen, S., Ropstad, E., Elliott, C.T., Connolly, L., 2013b. Endocrine
386 disrupting effects of ochratoxin A at the level of nuclear receptor activation and
387 steroidogenesis. *Toxicol. Lett.* 217, 243–250.

388 Frizzell, C., Elliott, C. T., Connolly, L., 2014 Effects of the mycotoxin patulin at the
389 level of nuclear receptor transcriptional activity and steroidogenesis in vitro. *Tox. Lett.*
390 229, 366–373

391 Fung Frederick and Clark Richard., 2004. Health effects of mycotoxins: A toxicological
392 overview. *J. Toxicol.* 42, 217–234.

393 Gbore FA. 2009 Growth performance and puberty attainment in growing pigs fed
394 dietary fumonisin B1. *J Anim. Physiol. Anim. Nutr.* 93, 761–7.

395 Gelderblom, W.C.A., Smuts, C.M., Abel, S., Snyman, S.D., Van Der Westhuizen, L.,
396 Huber, W.W., Swanevelder, S., 1997. Effect of Fumonisin B1 on the levels and fatty
397 acid composition of selected lipids in rat liver in vivo. *Food Chem. Toxicol.* 35, 647–
398 656.

399 Harrison, L.R., Colvin, B.M., Green, J.T., Newman, L.E., Cole, J.R., 1990. Pulmonary
400 oedema and hydrothorax in swine produced by fumonisin B1, a toxic metabolite of
401 *Fusarium moniliforme*. *J. Vet. Diagn. Invest.* 2, 217–221

402 Honer, C., Nam, k., Fink, C., Marshall, P., Ksander, G., Chatelain, R.C., Cornell, W.,
403 Steele, R., Schweitzer, R., Schumacher, C., 2003. Glucocorticoid receptor antagonism
404 by cyproterone acetate and RU486. *Molecular Pharmacology.* 63, 1012–1020.

405 Hussein, H. S.; Brasel, J. M., 2001. Toxicity, metabolism, and impact of mycotoxins on
406 humans and animals. *Toxicology* 167, 101–134

407 International Programme on Chemical Safety, 2002. Global Assessment of the State-of-
408 the-Science of Endocrine Disruptors. World Health Organization, Geneva.
409 http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/.

410 Jestoi M., 2008. Emerging Fusarium–mycotoxins fusaproliferin, beauvericin, enniatins,
411 and moniliformin a review. *Crit. Rev. Food. Sci. Nutr.* 48, 21–49

412 Jestoi, M., Rokka, M., Peltonen, K., 2007. An integrated sample preparation to
413 determine coccidiostats and emerging Fusarium- mycotoxins in various poultry tissues
414 with LC MS/MS, *Mol. Nutr. Food Res.* 51, 625–637.

415 Jow, G.-M., Chou, C.-J., Chen, B.-F., Tsai, J.-H., 2004. Beauvericin induces cytotoxic
416 effects in human acute lymphoblastic leukemia cells through cytochrome c release,
417 caspase 3 activation: the causative role of calcium. *Cancer Letters* 216, 165–173

418 Kelce, W.R., Lambright, C.R., Gray, L.E., Jr., Roberts, K.P., 1997. Vinclozolin and p,
419 p'-DDE after androgen-dependent gene expression: In vivo confirmation of an androgen
420 receptor-mediated mechanism. *Toxicol. Appl. Pharmacol.* 142, 192–200.

421 Kelce, W.R., Stone, C.R., Laws, S.C., Gray, L.E., Jr, Kemppainen, J.A., Wilson, E.M.,
422 1995. Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist.
423 *Nature.* 375, 581–585.

424 Kellerman, T.S., Mararas, W.I.-O., Thiel, G., Gelderblom, W.C.A., Cawood, M.,
425 Coetzer, J.A.W., 1990. Leukoencephalomalacia in two horses induced by oral dosing of
426 fumonisin B1. *J. Vet. Res.* 57, 269–275

427 Liu, Z., Auboeuf, D., Wong, J., Chen, J.D., Tsai, S.Y., Tsai, M.J., O'Malley, B.W., 2002.
428 Coactivator/corepressor ratios modulate PR-mediated transcription by the selective
429 receptor modulator RU486. *Proc. Natl. Acad. Sci.* 99, 7940–4

430 Mallebrera B, Font G and Ruiz M J., 2014. Disturbance of antioxidant capacity
431 produced by beauvericin in CHO-K1 cells. *Toxicol. Lett.* 226, 337–342.

432 Martins, F.A., Dias Ferreira, F.M., Dias Ferreira, F., Bando, É. Nerilo, S.B., Hirooka,
433 E.Y., Machinski Jr. M., 2012. Daily intake estimates of fumonisins in corn-based food
434 products in the population of Parana, Brazil. *Food Control.* 23, 614–618.

435 Meca, G., Fernández-Franzón, M., Ritieni, A., Font, G., Ruiz, M.J., Mañes, J., 2010.
436 Formation of Fumonisin B1-Glucose reaction product, in vitro cytotoxicity, and lipid
437 peroxidation on kidney cells. *J. Agric. Food Chem.* 58, 1359–1365.

438 Merrill, A.H., Sullards, M.C., Wang, E., Voss, K.A., Riley, R.T., 2001. Sphingolipid
439 metabolism: roles in signal transduction and disruption by fumonisins. *Environ. Health*
440 *Perspect.* 109, 283–289.

441 Neulen J , Williams RF, Breckwoldt M, Chwalisz K, Baulieu EE, Hodgen GD., 1996.
442 Non-competitive anti-oestrogenic actions of progesterone antagonists in primate
443 endometrium: enhancement of oestrogen and progesterone receptors with blockade of
444 postreceptor proliferative mechanisms. *Hum Reprod.* 11, 1533–7.

445 Prosperini A, Meca, G, Font G and Ruiz M J., 2012. Study of the cytotoxic activity of
 446 beauvericin and fusaproliferin and bioavailability in vitro on Caco-2 cells. Food. Chem.
 447 Toxicol. 50, 2356–2361

448 Prosperini A, Juan-García A, Font G and Ruiz M J., 2013. Beauvericin-induced
 449 cytotoxicity via ROS production and mitochondrial damage in Caco-2 cells. Toxicol.
 450 Lett. 222, 204–211.

451 Ramirez CN, Antczak C, Djaballah H., 2010. Cell viability assessment: toward content-
 452 rich platforms. Expert Opin. Drug Discov. 5, 223–233.

453 Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M.L., Stammati, A., Zucco, F., 2005.
 454 The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-
 455 related factor son Caco-2 cell functional characteristics. Cell Biol. Toxicol. 21, 1-26.

456 Savory, J.G.A., Préfontaine, G.G., Lamprecgt, C., Liao, M., Walther, R.F., Lefebvre,
 457 Y.A., Haché, R.J.G., 2001. Glucocorticoid receptor homodimers and Glucocorticoid-
 458 Mineralcorticoid receptor heterodimers form in the cytoplasm through alternative
 459 dimerization interfaces. Mol. Cell. Biol. 21, 781-793

460 Serrano AG, Font G, Ruiz MJ and Ferrer E., 2012. Co-occurrence and risk assessment
 461 of mycotoxins in food and diet from Mediterranean area. Food Chem. 135, 423–429.

462 Spitz, I.V., 2003. Progesterone antagonists and progesterone receptor modulator: an
 463 overview. Steroids, 68, 981-993

464 Tolosa J, Font G, Mañes J and Ferrer E., 2013. Nuts and dried fruits: natural occurrence
 465 of emerging Fusarium mycotoxins. Food Control, 33, 215–220.

466 Tolosa, L., Gómez-Lechón, M.J., Donato, M.T., 2015. High- content screening
 467 technology for studying drug-induced hepatotoxicity in cell models. Arch.Toxicol. In
 468 press.DOI 10.1007/s00204-015-1503-z

469 Tonshin, A.A., Teplova, V.V., Andersson, M.A., Salkinoja-Salonen, M.S., 2010. The
 470 Fusarium mycotoxins enniatins and beauvericin cause mitochondrial dysfunction by
 471 affecting the mitochondrial volume regulation, oxidative phosphorylation and ion
 472 homeostasis. Toxicology 276, 49–57.

473 Wan, L.Y.M., Turner, P.C., El-Nezami, H., 2013. Individual and combined cytotoxic
 474 effects of Fusarium toxins (deoxynivalenol, nivalenol, zearalenone and fumonisins B1)
 475 on swine jejunal epithelial cells. Food and Chem. Tox. 57, 276-283.

476 Willemsen, P., Scippo, M., Kausel, G., Figueroa, J., Maghuin-Rogister, G., Martial, J.,
 477 2004. Use of reporter cell lines for detection of endocrine-disrupter activity. Anal.
 478 Bioanal. Chem. 378, 655–663.

479 Wilson, V.S., Bobseine, K., Lambright, C.R., L.E.Gray, Jr. 2002. A novel Cell line,
480 MDA-kb2 that stably express an androgen and glucocorticoid responsive reporter for
481 the detection of hormone receptor agonists and antagonists. *Toxicol.Sci.* 60, 69-81

482

Legends of Figures:

Fig.1 Viability of the RGA cell lines a) MMV-Luc b) TARM-Luc c) TM-Luc and d) TGRM-Luc following exposure to 0.001-10 μ M of FB1 and BEA for 48 h and compared to the solvent control, as determined in the MTT assay. Values are means $\pm$ SEM for the three separate experiments (n=3), $p \leq 0.001$ (***).

Fig.2 Results of RGA antagonistic test following co-exposure of the positive control with FB1 (0.001-10 μ M) in the a) MMV-Luc (oestrogen responsive), b) TARM-Luc (androgen responsive), c) TM-Luc (progestagen responsive) and d) TGRM-Luc (glucocorticoid responsive) RGA cells. Responses measured are compared to the solvent and the positive control (1.36 ng/ml 17 β -estradiol, 14.5 ng/ml testosterone, 157 ng/ml progesterone and 181 ng/ml cortisol, respectively). Results are expressed as the mean percentage response $\pm$ SEM for the three separate experiments (n=3), $p \leq 0.05$ (*).

Fig.3 Results of RGA antagonistic test following co-exposure of the positive control with BEA (0.001-10 μ M) in the a) MMV-Luc (estrogen responsive), b) TARM-Luc (androgen responsive), c) TM-Luc (progestagen responsive) and d) TGRM-Luc (glucocorticoid responsive) RGA cells. Responses measured are compared to the solvent and relevant positive controls (1.36 ng/ml 17 β -estradiol, 14.5 ng/ml testosterone, 157 ng/ml progesterone and 181 ng/ml cortisol, respectively). Responses are expressed as the mean percentage response $\pm$ SEM for the three separate experiments (n=3), $p \leq 0.05$ (*), ≤ 0.01 (**), ≤ 0.001 (***).

Fig.4 Quantification of the cytotoxic effects of 0.001-1 μ M BEA in the progestagen responsive TM-Luc cells as measured by HCA. a) cell number (CN) b) nuclear area (NA), c) nuclear intensity (NI), d) plasma membrane permeability (PMP), e) mitochondrial membrane potential (MMP) and f) mitochondrial mass (MM). Data are expressed as mean values $\pm$ SEM for the three separate experiments (n=3). $p \leq 0.05$ (*) and $p \leq 0.01$ (**) indicate significant differences from the solvent control.

Fig.5 Quantification of the cytotoxic effects of 0.001-10 μ M FB1 and BEA in the gut

derived Caco-2 cells after 48 h exposure as measured by HCA. a) cell number (CN) b)
nuclear area (NA), c) nuclear intensity (NI), d) plasma membrane permeability (PMP),
e) mitochondrial membrane potential (MMP) and f) mitochondrial mass (MM). Data are
expressed as mean values $\pm$ SEM for the three separate experiments (n=3). $p \leq 0.001$
(***) indicate significant differences from the solvent control.

Fig.1

Figure
[Click here to download Figure: Fig. 2.doc](#)

Figure
Click here to download Figure: Fig. 3.doc

Fig. 4

Fig. 5.