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Fusarium mycotoxin enniatin B: Cytotoxic effects and changes in gene expression profile

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ABSTRACT

The mycotoxin enniatin B, a cyclic hexadepsipeptide produced by the plant pathogen *Fusarium*, is prevalent in grains and grain-based products in different geographical areas. Although enniatins have not been associated with toxic outbreaks, they have caused toxicity *in vitro* in several cell lines. In this study, the cytotoxic effects of enniatin B were assessed in relation to cellular energy metabolism, cell proliferation, and the induction of apoptosis in Balb 3T3 and HepG2 cells. The mechanism of toxicity was examined by means of whole genome expression profiling of exposed rat primary hepatocytes. Enniatin B altered cellular energy metabolism and reduced cell proliferation in Balb 3T3 and HepG2 cell lines. Furthermore, the proportion of apoptotic cell populations of Balb 3T3 cells slightly increased. On the other hand, enniatin B caused necrotic cell death in primary hepatocytes. Gene expression studies revealed the alteration of energy metabolism due to effects on mitochondrial organization and function and the assembly of complex I of the electron transport chain.

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1. Introduction

Enniatins (Enns), produced by the plant pathogenic fungi *Fusarium*, are common contaminants of grain in Northern Europe. In a 2001 Finnish survey on the mycotoxin content of grains, low levels of Enns were recurrently detected in 95–100% of the grain samples, with 18.3 mg/kg wheat being the highest concentration (Jestoi et al., 2004). Similar findings were reported in a Norwegian survey, with Enn contamination of up to 100% of grain samples and the highest enniatin B (EnnB) concentration being 790 µg/kg in wheat (Uhlir et al., 2006).

Enniatins are N-methylated cyclic hexadepsipeptides, with 3 residues of D-2-hydroxyisovaleric acid alternating with 3 amino acid residues of valine, leucine, or isoleucine. The molecule has a disk form and is highly lipophilic (Jestoi, 2005; Desjardins, 2006). More than 20 isoforms of Enns exists and are synthesized by enniatin synthetase

enzymes by several *Fusarium* species, including *F. avenaceum*, *F. poae*, *F. tricinctum*, *F. oxysporum*, and *F. sporotrichoides* (Desjardins, 2006).

The clinically most important fusariotoxins, trichothecenes, zearalenone, and fumonisin, induce immunotoxicity, reproductive and developmental toxicity, and carcinogenicity in several animal species and humans (Desjardins, 2006; Jestoi, 2005). Enns, however, have not been associated with clinical outbreaks. A few *in vivo* studies have indicated a low level of or no toxicity. The LD₅₀ of a commercial mixture of Enns (fusafungine) in mice was as high as 350 mg/kg b.w. (Berdy, 1980; EFSA Panel on Contaminants in the Food Chain (CONTAM), 2014). Furthermore, no adverse effects were detected in a 28-day subchronic feeding study on Wistar rats with a daily oral dose of 20.91 mg/kg b.w. EnnA (Manyes et al., 2014). As highly lipophilic compounds, Enns have been shown to bioaccumulate in eggs (Jestoi et al., 2009), and low levels were recovered from broiler meat, liver, and skin in a poultry feeding trial (CODA-CERVA, 2011–2012). Two metabolites have also been recovered from liver and serum samples of broilers, indicating a carry over to food and feed (Ivanova et al., 2014).

Although not very toxic *in vivo*, Enns have caused toxicity *in vitro* in several cell lines. The mode of action of Enns is thought to be related to the formation of cation-selective channels, which transport mono- and divalent cations across biological membranes, resulting in non-physiological concentrations in the cell (Ivanov et al., 1973; Kamyar

Abbreviations: Bea, beauvericin; ELISA, enzyme-linked immunosorbent assay; Enn, enniatin; ED₅₀, effective dose at which 50% of cells are affected; ETC, electron transport chain, a central part of cellular respiration.; IC₅₀, inhibitory concentration at which 50% of enzyme activity is inhibited.; FDR, false discovery rate; GO, gene ontology, describes gene products in associated biological processes, cellular components and molecular functions.; o/n, over night; RT, room temperature.

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et al., 2004, EFSA Panel on Contaminants in the Food Chain (CONTAM), 2014). ATP-driven efflux pumps in intestinal epithelial cells form a barrier against xenobiotics by pumping them back to the gut lumen. Enns have been found to alter the efflux properties of two ATP-binding cassette (ABC) transporters, ABCB1 and ABCG2, involved in multidrug resistance. Moreover, Enn exposure inhibited the efflux of pheophorbide (PhA) and rhodamin 123 in lung carcinoma A549 cells and leukemia HL60/Vinc cells, and led to the accumulation of these substrates in cells (Dornetshuber et al., 2009b). Enns may thereby influence the absorbance of other xenobiotics from the intestine.

Enns have been reported to cause a wide range of cytotoxic effects, including immunotoxicity (Gammelsrud et al., 2012), lysosomal alteration (Ivanova et al., 2012) and inhibition of enzyme activity. For example, Enns inhibited CoA:cholesterol acyltransferase (ACAT), involved in bile acid production (Tomoda et al., 1992) and calmodulin dependent 3',5'-cyclic nucleotide phosphodiesterase (PDE) hydrolyzing the secondary messengers cyclic-AMP and cyclic-GMP (Mereish et al., 1990). Gammelsrud et al., reported alteration of the immune response due to reduced mitochondrial metabolic activity and lysosomal damage in RAW 267.4 macrophages (Gammelsrud et al., 2012). Furthermore, cell proliferation was altered due to cell cycle arrest, appearing in the G0/G1 phase, and a concomitant decrease in cyclin D1 levels in exposed cells. EnnB also reduced the endocytosis capacity of macrophages exposed during the differentiation process of monocytes into macrophages (Ficheux et al., 2013). In a study by Ivanova et al., a dose-dependent disruption of lysosomal membranes post exposure to EnnB in human colon adenocarcinoma Caco-2 cells was discovered using neutral red staining. A loss of mitochondrial membrane potential was also detected. Furthermore, EnnB caused cell death by increasing the level of necrotic cell populations of Caco-2 cells, rather than by induced apoptosis (Ivanova et al., 2012).

Dornetshuber et al., demonstrated that Enn cytotoxicity did not involve oxidative stress in HL-60 leukemia cells or cervix carcinoma KB-3-1 cells, or cause DNA damage in the form of DNA intercalation (Dornetshuber et al., 2009a).

Despite several toxicological studies on enniatins, the toxic impacts in different cell types and the exact mode of action remain unclear. In particular, the relevance of *in vitro* findings to toxic effects *in vivo* are difficult to define. Our study aimed to shed light on the toxic effects of EnnB on cellular energy metabolism, cell proliferation, and cell death in mouse embryo fibroblast Balb 3T3 cells and human hepatocellular carcinoma HepG2 cells. We also investigated the toxic mechanism of EnnB in rat primary hepatocytes, monitored at the gene expression level.

2. Materials and methods

2.1. Chemicals

2.1.1. Mycotoxins

The *Fusarium* mycotoxin enniatin B used in the toxicity assays was purchased from A.G. Scientific, Inc. (San Diego, California) and was isolated from *Fusarium orthoceras* var. *enniatum*. The purity was demonstrated to be ≥ 90 –99% (HPLC analysis).

In gene expression studies, enniatin B isolated from *Gnomonia errabunda* was used (Sigma Aldrich, Saint Louis, USA), with a purity of 95% (HPLC analysis).

2.2. Bioassays

2.2.1. Cell maintenance and subcultivation

The mouse embryo fibroblast cell line Balb 3T3, clone A31 (CCL-163, American Type Culture Collection, ATCC, USA) was used in all bioassays. The cells were cultured in DMEM (#30-2002, ATCC) supplemented with 10% calf serum (#30-2031, ATCC, USA) and 2 mM L-glutamine (Sigma Chemical Co.), the antibiotics penicillin (100 IU/ml, Orion, Finland)

and streptomycin (200 µg/ml, Sigma-Aldrich, Finland). Human hepatocellular carcinoma HepG2 cells (HB-8065, American Type Culture Collection, ATCC, USA), also used in the bioassays, were cultured in MEM (#30-2003, ATCC) and supplemented as above, however, using 10% fetal bovine serum (FBS, #30-2020, ATCC). The cells were maintained in 25 cm² filter cap flasks (Nunclon™, Thermo Scientific) at 37 °C in a humidified incubator with 5% CO₂. Subcultivation for bioassays was performed twice a week at a ratio 1:10 in supplemented culture medium for 24 h (confluent growth) and dispersed on white, flat, clear-bottom 96-well plates (ViewPlate-96TC, Perkin-Elmer) at a density of 10,000 cells per well for the ATP viability assay and 3000 cells per well on black 96-well plates (CellBIND surface, Corning, USA) for cell proliferation assay, in standard conditions o/n. Antibiotics were excluded to avoid synergistic effects with enniatin.

2.2.2. Cell viability tests and cellular energy metabolism

The lowest EnnB level tested (1 µM/0.64 µg/ml), equals to a level of 0.66 µg/ml in plasma, if water content is assumed to be 93% in plasma and 90% in growth media, respectively (Rhoades and Bell, 2009). These levels are approximately 10 times (9.04 times) higher than the EnnB plasma levels in a feeding trial study of pigs (plasma levels 0.073 µg/ml) after an oral dose of 50 µg/kg b.w., equal to an EnnB level of 1 mg/kg feed (Devreese et al., 2013). Furthermore these values are about 10 times higher than the EFSA highest (mean) acute exposure estimate in Europe (4.67 µg/kg b.w./day, toddlers)(EFSA Scientific Opinion, 2014). The lowest tested level (1 µM EnnB) in our study refers to nearly a 100 times higher exposure level. However, as the levels of Enns in cereals show a high annual variation (from traces to 18.3 mg/kg wheat, Finland, Jestoi et al., 2004) this lowest exposure level may be relevant in the worst case *in vivo* situations.

Cell membrane integrity was tested using the Toxilight bioluminescent assay (LT07-217, Lonza, Rockland, USA), which measures the release of the enzyme Adenylate kinase (AK) through damaged plasma membranes of cells. The effect of EnnB on cellular energy metabolism (ATP production) was assessed with Vialight Plus Bioassay (LT07-221, Lonza, Rockland, USA). The cells were exposed to different toxin concentrations in growth medium (100 µl/well) ranging from 1.5 µM to 100 µM EnnB for 24 h. Both experiments were performed according to manufacturer's protocols, using the same cell samples. AK release was measured from the growth media post EnnB exposure and ATP levels from the disrupted cell layers. The luminescence of the samples was measured using a Victor³ Multilabel Plate Reader 1420 (Perkin Elmer, Belgium). The emitted light intensity is linearly related to the released AK concentration and to ATP concentration in cells.

2.2.3. Cell proliferation assay (BrdU ELISA)

The effects of enniatin B exposure on the proliferation (DNA synthesis) of Balb 3T3 and HepG2 cells was examined using a chemiluminescent cell proliferation ELISA immunoassay (Ref 1166915001, Roche Diagnostics GmbH, Mannheim, Germany). The cells were exposed to EnnB concentrations ranging from 3 to 12 µM in growth medium and incubated as above for 24 h. The experiment was performed according to manufacturer's instructions. Luminescence was measured with a Victor³ Multilabel Plate Reader 1420 (Perkin Elmer, Belgium). The chemiluminescence (rlu/s), produced by luminol, correlates with the level of DNA synthesis in the cell.

2.3. Apoptosis

To study changes in the quantity of pro-apoptotic cell populations by flow cytometry, the Annexin V-FITC/PI double-staining kit (Cat # K101-25, Biovision) was used. Balb 3T3 cells were cultured in standard conditions for 24 h on 6 well-plates (Cat. No. 140685, Nunc surface, Nunc®) at 450,000 cells/well. The growth medium was removed and the cells were exposed to three different toxin concentrations: 11 µM, 22.5 µM, and 45 µM EnnB for 24 h and 48 h. Camptothecin (10 µM and

20 μM) was used as a positive control. Unexposed cells were used as negative controls and for flow cytometer calibration: unstained cells, cells stained with only PI or Annexin V-FITC, and one control with cells stained with both dyes. The cells were detached by adding 2 ml or 1 ml of 0.25% trypsin – 0.53 mM EDTA in HBSS and collected by centrifugation (Eppendorf 5810R) at 1700 rpm for 5 min at +4 °C. The cells were resuspended in 500 μl $1 \times$ binding buffer following the addition of 5 μl of both Annexin V-FITC and propidium iodide (PI) stains. The cells were incubated 5 min in RT in the dark and immediately analyzed by flow cytometry (BD FACSAria II, Becton, Dickinson and Company). For each experiment, 10,000 events were acquired, single cells were gated, and FITC and PI fluorescence were measured using 530 nm and 617 nm band-pass filters. The data were analyzed using BD FACSDiva software for early apoptotic/necrotic populations.

2.4. Gene expression studies

2.4.1. Rat primary hepatocyte cell cultivation

Primary hepatocytes were chosen for this study because they are considered to better correspond to *in vivo* studies than immortalized cell lines. However, the lifespan of cryopreserved primary hepatocytes is rather short, being only 12–24 h, including a 4–8 h period of attachment to the culture plate surface. This limited the duration of the experiments.

Cryopreserved rat primary hepatocytes (RTCP 10, Gibco, Life Technologies, USA), used in the gene expression studies, were maintained and cultured according to the manufacturer's instructions, using ready-to-use cryopreserved hepatocyte recovery medium (CHRM, APSciences Inc., USA) and cryopreserved hepatocyte plating medium (CHPM, APSciences Inc., USA). Only cell suspensions with more than $\geq 79\%$ viability were used. Cells were diluted and plated at a density of 2000 viable cells per well on collagen-coated 6-well plates (Thermo Scientific, NY, USA) and incubated at 37 °C in a humidified incubator with 5% CO₂ o/n to allow the cells to attach to the collagen surface of the plates.

2.4.2. Microarray method

The cells were exposed for 1 and 4 h in standard conditions to 0 μM , 1 μM , 10 μM , and 20 μM EnnB in maintenance medium (William's E medium, without phenol red (WEM)), supplemented with 4% cocktail B (Gibco, Life Technologies, UK) respectively, representing a negative control, a low toxin level, the EC₅₀, and a high level of toxin. RNA was isolated using the RNeasy Plus Mini Kit (Cat. No. 74134, Qiagen). The growth medium was removed and 300 μl of lysis buffer RLT, containing β -mercaptoethanol, was added to each well. The cells were detached by scraping and the cell suspensions were homogenized using QIAshredder columns (Cat. No. 79654, Qiagen). RNA isolation was performed according to manufacturer's instructions. RNA samples were immediately placed on ice and stored at –80 °C.

Genome-wide expression analysis of the exposed rat primary hepatocyte samples was carried out using rat total genome RaGene 2.0 ST expression arrays (Ref 520784, Affymetrix, USA) and the Affymetrix standard protocol. The expression analysis was performed by the Functional Genomics Unit at the University of Helsinki in April 2013. The labeling and hybridization were carried out according to the manufacturer's instructions with minor modifications, using the Ambion WT Expression Kit for Affymetrix GeneChip Whole Transcript Expression Arrays (Part Number 4425209 Rev. C 09/2009, Applied Biosystems). The probe-group intensity data were collected using a GeneChip® Scanner (Affymetrix, USA). The data were collected from 4 independent experiments.

2.4.3. qPCR method

Seven genes were chosen on the basis of the microarray results for further expression analysis by quantitative real-time PCR (RT-qPCR). Rat primary hepatocytes were exposed to 0 μM and 10 μM EnnB in growth medium (representing a negative control and the EC₅₀) for

1 h and 4 h in standard conditions. RNA was isolated as in the microarray experiments using the RNeasy Plus Mini Kit (Cat. No. 74134, Qiagen). The cDNA synthesis was performed with the ReadyScript™ cDNA Synthesis Mix (Sigma-Aldrich) according to manufacturer's instructions. Predesigned KiCqStart SYBR green primers (Sigma-Aldrich) specific for rat genes were used in the analysis (Table 5).

Quantitative real-time PCR was conducted using 100 ng of cDNA as a template, KiCqStart® SYBR Green qPCR Ready MIX for BioRad CFX (Sigma-Aldrich, KCQS00), and 450 nM of each primer per reaction. The PCR cycles for BioRad CFX96 were as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 15 s. The rat SDHA gene (Table 5) served as reference gene. Three technical repeats were used for each gene. The efficiency of each reaction was analyzed, and as it was slightly different between the genes, relative quantification was analyzed by the Pfaffl method (Bio-rad, 2006). The experiment was performed twice.

2.5. Statistics

2.5.1. Statistical analysis in cell viability and cell proliferation studies

A three-parameter Weibull function with parameterization 2 was used in the modeling of the toxic effects on ATP production and cell proliferation of Balb 3T3 cells. For HepG2 cells, a three-parameter Weibull function with parameterization 1 was used in the modeling of toxic responses on ATP production and a four-parameter Weibull function with parameterization 1 was used for the cell proliferation model. The best model function was selected based on the minimum values of residual variance and by comparing the alternative models using ANOVA tests (ANOVA function in R). The models were fitted using the R package 'drc' (Ritz and Streibig, 2005).

2.5.2. Statistical analysis in cell death studies (apoptosis)

The statistical analyses were conducted using GraphPad Prism 5.0 software. A two-tailed nonparametric Mann-Whitney *U* test or Kruskal-Wallis test together with Dunn's Multiple Comparison Test was used to assess the significance of the differences between untreated and treated samples in the apoptotic cell death data.

2.5.3. Statistical analysis of gene expression

2.5.3.1. Statistical analysis in microarray experiments. The gene expression data were pre-processed and log₂ transformed using the R library RMA (Irizarry et al., 2003) and Ensembl gene custom probe mappings (version 17.0.0 based on Ensembl rat genome build 70) (Dai et al., 2005). To list differentially expressed probe sets between samples, a moderated paired *t*-test (pairing carried over individual rat samples) was conducted using the R library "limma" (Smyth, 2004). Differential expression *p*-values were false discovery rates (FDR) adjusted using the *q*-value Bioconductor package (Storey and Tibshirani, 2003). Probes with a *q*-value ≤ 0.05 were accepted as significantly different between two conditions.

2.5.3.1.1. Enrichment analysis of microarray data. Gene ontology (GO) enrichment analysis of the transcriptome was performed using Blast2GO software (Conesa et al., 2005). Lists of differentially expressed genes (with absolute log₂-fold change $\geq \pm 1$, *q*-value ≤ 0.05) were analyzed with Fisher's exact test together with false discovery rate (FDR) correction (Benjamini–Hochberg correction) to find enriched gene ontology categories in up- and downregulated genes. The enrichment analysis was run with default values. A two-tailed test was used to discover over- and under-representation of probe sets in functional categories and a false discovery rate (FDR) value of 0.01 was used as a cut-off value for significance.

2.5.3.1.2. Cluster Analysis of Microarray Data. Cluster analysis was conducted on all probe sets with statistically significant changes in expression compared to the negative control sample of the series (i.e. 1 h, 0 μM EnnB and 4 h, 0 μM EnnB samples), as well as on probe sets

that were part of the gene ontology term mitochondrial organization (GO: 0007005) present on the chip, including 37 probe sets that were enriched. The cluster analysis was performed using the TMeV Multiple Experiment Viewer 4.9.0. program (Saeed et al., 2003). In all analyses, hierarchical clustering was performed using the Euclidean distance.

3. Results

Mouse fibroblast Balb 3T3 cells were chosen for testing the different cytotoxic effects of EnnB, as this cell line is considered to be very sensitive to the adverse effects of xenobiotics. HepG2 cells were used to study possible hepatotoxic effects and enable the comparison of toxic effects between different cell lines. EnnB clearly reduced the viability of both Balb 3T3 cells and HepG2 cells, altering cell proliferation and ATP production at relatively low concentrations. However, light microscopy studies did not reveal changes in typical cell morphology or cell death in the studied concentration ranges in Balb 3T3 or HepG2 cells (Fig. 1A and B). This observation is in line with the results of Toxilight cytotoxicity assay (Lonza), measuring adenylate kinase (AK) leakage through the plasma membrane in damaged cells, as no leakage of AK from EnnB exposed Balb 3T3 cells to the growth media could be detected (Fig. 2). Furthermore, flowcytometric studies performed to study apoptotic effects of EnnB (Section 3.3 Apoptosis) revealed the majority of the cells to be intact post a 24 h exposure to EnnB (11 μ M, 22 μ M, 45 μ M). The ratio of intact cells vs. damaged cells was comparable to the negative control cells, as the proportion of intact cells in the negative control samples were $86 \pm 15\%$ and in EnnB exposed cells $96.6 \pm 0.25\%$ (11 μ M), $91.0 \pm 4.9\%$ (22 μ M) and $84.3 \pm 12\%$ (45 μ M), respectively. In contrast to the cell lines, the effect of EnnB on rat primary hepatocyte morphology was prominent with an exposure of only 4–5 h to 10 μ M EnnB,

Fig. 2. Relative leakage of adenylate kinase (AK) enzyme through the plasma membrane of Balb 3T3 cells was not detected in any tested EnnB levels (3 μ M–60 μ M), post a 24 h exposure (black line). Ethanol was used as a positive control for cell damage (grey line). Each data point represents the result of three independent experiments.

causing the majority of the primary hepatocytes to round up and detach from the plate surface and release cell contents in a necrotic manner (Fig. 1C and D).

3.1. Cell energy metabolism (ATP production)

The effect of EnnB on cellular energy metabolism was determined by measuring ATP levels in cells using the ViaLight Plus Bioassay (Lonza). A 24-h EnnB exposure of Balb 3T3 cells reduced intracellular ATP levels in a steeply declining and dose-dependent manner, with an ED_{50} value of 8.4 ± 0.76 μ M EnnB (Fig. 3). EnnB concentrations below 3 μ M had no influence on Balb 3T3 cells. The effect on HepG2 cells elicited a similar pattern, and the estimated ED_{50} value (2.9 ± 0.7 μ M) was somewhat lower than for Balb 3T3 cells, suggesting that HepG2 cells might be more sensitive to EnnB.

3.2. Cell proliferation

Exposure to EnnB altered DNA synthesis, measured by BrdU incorporation into the DNA of dividing cells (BrdU Elisa, Roche). DNA synthesis markedly declined in both cell lines (Balb 3T3 and HepG2) after EnnB exposure. The toxic effect could be detected within a narrow concentration range, with an estimated ED_{50} value of 4.24 ± 0.06 μ M in Balb 3T3 cells and 0.50 ± 0.09 μ M in HepG2 cells. Concentrations under 3.5 μ M showed no inhibition in Balb 3T3 cells (Fig. 4).

3.3. Apoptosis

Induction of apoptosis was studied by flow cytometric measurements of early apoptotic and late apoptotic/necrotic cell populations and intact cells using Annexin V-FITC/PI staining. Mouse Balb 3T3 cells were exposed to three EnnB concentrations (11 μ M, 22 μ M, and 45 μ M) for 24 h and 48 h. Camptothecin concentrations of 10 μ M and 20 μ M were used as positive controls and cells receiving fresh growth media as a negative control. Treatment for 24 h slightly increased the number of early apoptotic cells in a dose-dependent manner, from 0.3% of negative control cells to 1.25% at a dose of 11 μ M ($\approx ED_{50}$) and to 2.5% and 4.4% at high doses of 22 μ M and 45 μ M EnnB, respectively. The effect was comparable to the positive control, camptothecin, with an increase in the proportion of early apoptotic cells to 1.85% and 3.0% at doses of 10 μ M and 20 μ M, respectively (Fig. 5). The proportion of late apoptotic/necrotic cells also displayed a rising trend with an increase in EnnB levels, although the trend was not statistically significant. The 48-h EnnB exposure gave rise to similar findings, with a statistically significant increase in the early apoptotic cell population from 0.0% to 3.6% at the highest EnnB dose of 45 μ M (data not shown).

3.4. Gene expression studies

The overall gene expression of rat primary hepatocytes treated with a low dose (1 μ M) of EnnB for 4 h did not significantly differ from the

Fig. 1. (A) Light microscopy images of mouse Balb 3T3 cells (negative control) and cells exposed to 22 μ M EnnB for 24 h (B). Exposed cells remain intact, however stop proliferating, giving rise to a less dense cell culture compared to the negative control. (C) Images of untreated rat primary hepatocytes (negative control) and primary hepatocytes exposed to 10 μ M EnnB for 4 h (D). Exposed cells round up, detach from culture plate surface and die. (20 $\times$ objective, EVOS[®] XL Imaging System, Thermo Fisher Scientific).

Fig. 3. The effect of a 24 h ENN B exposure on ATP production in Balb3T3 cells (A) and HepG2 cells (B), resulting in a drastic drop in ATP production in both cell types with ED_{50} values of $8.41 \mu\text{M} \pm 0.76 \mu\text{M}$ ENN B in Balb 3 T3 cells and $2.87 \pm 0.77 \mu\text{M}$ ENN B in HepG2 cells.

expression of untreated cells. However, a 4-h exposure to 10 μM and 20 μM EnnB resulted in an altered gene expression profile of the rat genome. The 10- μM dose compromised the normal expression levels most, with 4.8% (985 genes) of the genes in the rat total genome array significantly downregulated and only 0.89% (179 genes) significantly upregulated (Fig. 6). A limit of the $\log_2$ fold change (a 100% change in expression) and a q value ≤ 0.05 was considered significant.

Enrichment analysis (Fisher's exact test) of downregulated genes suggested the most enriched gene ontology (GO) terms to include mainly catabolic and metabolic processes and the GO term mitochondrial organization (Table 1).

The 37 downregulated genes forming part of the GO term mitochondrial organization are listed in Table 2. Interestingly, downregulated genes were also modestly enriched in genes associated with apoptotic processes (apoptotic mitochondrial changes, $FDR \leq 3.3E-01$), cell signal transduction (TOR signaling, $FDR \leq 1.78E-01$, ERBB signaling pathway, $FDR \leq 1.85E-01$, MAPK activated stress cascade, $FDR \leq 3.9E-01$), and the iron-sulfur cluster assembly ($FDR \leq 3.0E-01$).

Enrichment analysis revealed no functional categories with significant FDR values among upregulated genes. Nonetheless, the most over-represented GO terms among upregulated genes were protein kinase C-activating G-protein coupled receptor signaling pathway ($FDR \leq 2.3E-01$), negative regulation of hexokinase activity ($FDR \leq 9.3E-01$), negative regulation of glucokinase activity ($FDR \leq 9.3E-01$), and chromatin organization ($FDR \leq 9.3E-01$, Table 1). G-protein-coupled receptor signaling increased cytoplasmic Ca^{2+} levels in the cytosol. Cytosolic Ca^{2+} is involved in several signaling pathways in the cell, such as the calmodulin signal transduction pathway, which is important in carbohydrate metabolism and apoptosis

(Mereish et al., 1990, Kamyar et al., 2004). Hexokinases phosphorylate hexoses and are involved in glycolysis and glycogen production, and negative regulation could cause energy deprivation in the cell (Wilson, 2003).

Since our study revealed a drastic decrease in ATP production in Balb 3T3 and HepG2 cells post-exposure to EnnB and there was enrichment among the downregulated genes of the GO term mitochondrial organization (GO: 0007005, $FDR \leq 8.4E-03$) in rat primary cells, this group of genes was further analyzed. The GO term included more than 300 genes involved in mitochondrial assembly, the arrangement of constituent parts, or disassembly (The European Bioinformatics Institute, the EBI, 2015). Hierarchical clustering analysis of these genes was conducted and revealed 3 distinctly clustered gene sets, 2 of which consisted of downregulated genes and 1 of upregulated genes (Fig. 7).

Among the downregulated clusters, three were members of the *Ndufs* family of genes (*Ndufs1*, *Ndufs4* and *Ndufs8*). The *Ndufs* genes are subunits of complex I the first enzyme in the electron transport chain in the mitochondrial inner membrane, leading to ATP production. The microarray data revealed an expression trend of downregulation among several of the *Ndufs* family of genes, although *Ndufs4* is the only gene that reached the (self-set) threshold for statistical significance (Fig. 8). *Ndufs* proteins are NADH dehydrogenase Fe-S proteins, and form parts of the iron-sulfur fragment of complex I (Genecards, 2015). In addition to the clustering of the *Ndufs* family, three *Timm* family genes, involved in the transport of proteins across the mitochondrial intermembrane space, were present in the downregulated clustered genes in array data were downregulated, but only *Timm21* and *8b* with statistical significance (Fig. 8).

Fig. 4. The effect of a 24 h ENN B exposure on cell proliferation in Balb3T3 cells (A) and HepG2 cells (B), resulting in a drastic drop in DNA synthesis in both cell types with ED_{50} values of $4.2 \pm 0.06 \mu\text{M}$ ENN B in Balb 3 T3 cells and $0.50 \pm 0.09 \mu\text{M}$ ENN B in HepG2 cells.

Fig. 5. Flow cytometer studies on Annexin V-FITC/PI labeled Balb 3T3 cells, after 24-h EnnB treatment. Positive control cells were treated with camptothecin and negative control cells with fresh growth media. An 11 μM dose of EnnB ($\approx$ ED50) increased the proportion of early apoptotic cells from 0.3% (negative control cells) to 1.25%. The proportion of early apoptotic cells increased in a dose-dependent manner to 2.5% in cells treated with 22 μM EnnB and 4.4% with 45 μM EnnB. The statistical significance of the difference between treated cells and negative control is denoted as: * ($p \leq 0.05$), ** ($p \leq 0.005$).

RT-qPCR was used to confirm the expression results of the microarray data for a few selected genes, *Ndufs4* and *Timm21* (the genes with most significant fold change among the studied gene families mentioned above), resulting in downregulated relative expression due to EnnB exposure, thus supporting array data results (Fig. 9).

The microarray gene expression study did not reveal any drastic changes in the gene expression of the key proteins involved in cell proliferation compared to the negative control (Table 3). Cyclin-dependent kinases, formed from a catalytic kinase together with regulatory cyclins, play a major role in cell cycle control (Lewin, 2000). The expression of most of these kinases and cyclins remained unchanged compared to the unexposed control following exposure to EnnB for 4 h. No significant enrichment in GO terms was discovered among up- or downregulated genes related to cell proliferation. This may be because primary cells proliferate poorly under in vitro conditions, unless chemically stimulated by growth factors such as platelet-derived growth factor (PDGF), and did not provide a means to efficiently study this endpoint in our experimental settings. However, in array data, the expression of three cyclins (B2, G1, and L2) was significantly changed compared to the negative control following 4-h EnnB (10 μM) treatment. Cyclins B2 and G2 were

Fig. 6. A 4-h exposure of rat primary hepatocytes to 10 μM EnnB caused a 4.85% downregulation and only a 0.89% upregulation of the genes of the rat total genome compared to the negative control. The smaller dose (1 μM) changed (upregulated) the expression of only 2 genes: Sip1/2 (signal-induced proliferation-associated 1-like protein 2) involved in signal transduction (GTPase mediated) and an unknown gene.

Table 1

The most and p-values enriched GO terms regarding biological function among downregulated and upregulated genes (Fisher's exact test). Enrichment among upregulated genes was not statistically significant, as FDR values were too high. FDR values ≤ 0.01 and p-values ≤ 0.05 were considered significant.

The most enriched GO terms among downregulated genes		FDR value	p-Value
GO:0008152	Metabolic process	2.33E-09	4.53E-12
GO:0019941	Modification-dependent protein catabolic process	1.01E-08	2.02E-11
GO:0043632	Modification-dependent macromolecule catabolic process	1.34E-08	2.82E-11
GO:0006511	Ubiquitin-dependent protein catabolic process	3.60E-08	7.94E-11
GO:0044237	Cellular metabolic process	5.08E-08	1.15E-10
GO:0070647	Protein modification by small protein conjugation or removal	5.70E-08	1.34E-10
GO:0044257	Cellular protein catabolic process	1.27E-07	3.05E-10
GO:0030163	Protein catabolic process	5.64E-07	1.42E-09
GO:0051603	Proteolysis involved in cellular protein catabolic process	6.50E-07	1.70E-09
GO:0044260	Cellular macromolecule metabolic process	1.69E-06	4.63E-09
GO:0032446	Protein modification by small protein conjugation	3.21E-06	9.04E-09
GO:0071704	Organic substance metabolic process	4.78E-06	1.40E-08
GO:0044238	Primary metabolic process	4.82E-06	1.43E-08
GO:0044267	Cellular protein metabolic process	1.29E-05	4.50E-08
GO:0016567	Protein ubiquitination	1.44E-05	5.09E-08
GO:0043412	Macromolecule modification	3.02E-05	1.25E-07
GO:0043170	Macromolecule metabolic process	5.49E-05	2.36E-07
GO:0006464	Cellular protein modification process	6.38E-05	2.81E-07
GO:0036211	Protein modification process	6.38E-05	2.81E-07
GO:0010498	Proteasomal protein catabolic process	1.06E-04	4.89E-07
GO:0006184	Obsolete GTP catabolic process	1.55E-04	7.23E-07
GO:0043161	Proteasome-mediated ubiquitin-dependent protein catabolic process	2.68E-04	1.30E-06
GO:0031329	Regulation of cellular catabolic process	4.83E-04	2.52E-06
GO:0000209	Protein polyubiquitination	6.89E-04	3.74E-06
GO:0019538	Protein metabolic process	7.50E-04	4.11E-06
GO:0034660	ncRNA metabolic process	9.15E-04	5.11E-06
GO:0044265	Cellular macromolecule catabolic process	1.15E-03	6.54E-06
GO:0044248	Cellular catabolic process	2.38E-03	1.43E-05
GO:0009057	Macromolecule catabolic process	2.42E-03	1.46E-05
GO:0009894	Regulation of catabolic process	7.62E-03	5.35E-05
GO:2000641	Regulation of early endosome to late endosome transport	8.18E-03	5.87E-05
GO:0007005	Mitochondrion organization	8.43E-03	6.09E-05
The most enriched GO terms among Upregulated genes			
GO:0007205	Protein kinase C-activating G-protein coupled receptor signaling pathway	2.33E-01	7.84E-05
GO:1903300	Negative regulation of hexokinase activity	9.33E-01	8.24E-04
GO:0033132	Negative regulation of glucokinase activity	9.33E-01	8.24E-04
GO:0006325	Chromatin organization	9.33E-01	9.58E-04

downregulated and cyclin L2 upregulated. Cyclin B2 is involved in growth factor beta-mediated cell control and cyclin G2 is involved in DNA damage-induced G2/M-phase arrest. The M-phase kinase inactivating protein Wee1 was also downregulated. The upregulated L2 cyclin is involved in RNA polymerase II regulation and pre-RNA splicing (Table 3).

Enrichment analysis did not reveal significant enrichment among GO terms linked to the third endpoint of our study, apoptosis. However, borderline enrichment among downregulated genes was observable (GO terms apoptotic mitochondrial changes, $\text{FDR} \leq 3.3\text{E}-01$, and regulation of apoptotic DNA fragmentation, $\text{FDR} \leq 6.4\text{E}-01$). Enrichment among upregulated genes was not detected even at this modest borderline significance level, indicating that upregulation of genes is not associated with apoptotic processes. Furthermore, the expression of central genes involved in apoptosis (e.g. caspase-3, -8, -9, *Tradd*, *Bid*, *Apaf-1*, *Aifm*) were downregulated or unchanged compared to the unexposed control in microarray studies (Table 4). Notably, both caspase-3 and *Aifm*, which are executing molecules in the death receptor pathway

Table 2

Significantly downregulated target genes belonging to the enriched GO term mitochondrion organization, following a 4-h, 10- μ M EnnB exposure of rat primary hepatocytes. RT-qPCR results are reported as the change in expression compared to untreated cells, the expression of which is referred to as 1.

Target genes of the enriched GO term: GO:0007005				Mitochondrion organization
Gene	Array data: log2 fold change	Array data: q-value	RT-qPCR	Gene function (Reference: www.genecards.org)
NDUFS4	−1.086106118	1.65E−07	0.65	NADH dehydrogenase (ubiquinone) Fe–S protein 4. A subunit of complex I.
Timm21	−1.880576292	2.97E−09	0.65	Translocase of inner mitochondrial membrane 21 homolog. Involved in the translocation of proteins across the mitochondrial inner membrane. Required for assembly of complex I and complex IV.
Timm8b	−1.114771134	2.57E−07		Translocase of inner mitochondrial membrane 8 homolog B. Guide proteins across the mitochondrial intermembrane space before they are added into the mitochondrial inner membrane.
Tomm5	−1.849071608	2.90E−07		TOMM5 (translocase of outer mitochondrial membrane 5 homolog (yeast))
Mpv17l	−1.038709587	7.78E−05		Mitochondrial membrane protein 17-like protein. Regulates the genes of antioxidant enzymes.
Malsu1	−1.016988016	9.61E−06		Mitochondrial assembly ribosomal large subunit protein. Preventing the formation of functional ribosomes, thus repressing translation.
Nrf1	−1.228319951	1.91E−05		Nuclear respiratory factor 1a. Transcription factor activating some key genes regulating cellular growth and respiration, heme biosynthesis and mitochondrial DNA transcription and replication (ref Wikipedia)
Tfb2m	−1.003915687	8.77E−05		Transcription factor B2, mitochondrial. Dimethylates mitochondrial 12S RNA and is required for transcription of mitochondrial DNA.
Trmt10c	−2.047727163	2.91E−08		Mitochondrial ribonuclease P protein 1. A subunit of the mitochondrial ribonuclease P, involved in 5' processing of mitochondrial tRNAs.
Dctn6	−1.010134783	1.60E−07		Dynactin subunit 6. Dynactins are involved with intracellular and mitochondria biogenesis.
Dnm1	−1.149074708	5.85E−08		Dynamin 1 is involved in clathrin-mediated endocytosis and vesicular trafficking processes.
Smcr7l	−1.218873937	4.48E−07		Mitochondrial dynamic protein MID51. Regulates mitochondrial fission.
Atg3	−1.400830673	2.64E−07		Autophagy related protein 3. Enzyme required for the cytoplasm to vacuole transport (Cvt), autophagy and mitochondrial homeostasis.
Tubb5	−1.264937216	3.13E−07		Tubulin, beta polypeptide. Microtubules act as a scaffold to determine cell shape, and provide a backbone for cell organelles and vesicles to move on.
Golph3	−1.07172804	9.47E−07		Golgi phosphoprotein 3. Links golgi membranes to the cytoskeleton
magmas	−1.242760796	5.04E−07		Mitochondria-associated granulocyte macrophage CSF signaling molecule [Source: RefSeq peptide; Acc:NP_001093606]
Sh3glb1	−1.062385706	1.90E−07		SH3-domain GRB2-like endophilin B1. The encoded protein interacts with the proapoptotic member of the Bcl-2 family Bcl-2-associated X protein (Bax) and may be involved in regulating apoptotic signaling and in maintaining mitochondrial morphology
Bcl2l11	−1.015601743	0.0004518		Bcl-2-like protein 11. Induces apoptosis and anoikis.
Tnfsf10	−1.041252965	8.84E−05		Tumor necrosis factor (ligand) superfamily, member 10. A cytokine. Associated with the activation of MAPK8/JNK, caspase 8, and caspase 3.
Aifm1	−1.03404913	1.51E−07		Apoptosis-inducing factor 1, mitochondrial [Source: UniProtKB/Swiss-Prot; Acc:Q9JLM53] Functions as an antiapoptotic factor in normal mitochondria and as a proapoptotic factor in a caspase-independent pathway.
Casp3	−1.604286218	8.19E−07		Caspase-3 executioner caspase.
Atg12	−1.61538308	5.19E−08		ATG12 is involved in autophagy. Interaction with anti-apoptotic members of the Bcl-2 family
Cisd2	−1.217212828	3.6E−07		CDGSH iron sulfur domain-containing protein 2. A zinc finger protein that is required for BCL2-mediated depression of endoplasmic reticulum Ca(2+) stores during autophagy.
ppp2cb	−1.48270962	3.06E−07		Phosphatase 2A catalytic subunit. Protein phosphatase 2A is one of the four major Ser/Thr phosphatases, involved in the negative control of cell growth and division.
Tmem11	−1.549475024	3.03E−06		Transmembrane protein 11, mitochondrial.
Ywhag	−1.367469554	1.61E−06		14-3-3 protein gamma, N-terminally processed [Source: UniProtKB/Swiss-Prot; Acc:P61983]
Gabrarpl1	−1.300833291	4.37E−08		Gamma-aminobutyric acid receptor-associated protein-like 1 [Source: UniProtKB/Swiss-Prot; Acc:Q0VGGK0]
Mef2a	−1.129488547	3.04E−07		Myocyte enhancer factor 2. A transcription factor that activates many muscle-specific, growth factor-induced, stress induced genes. Control of cell growth, survival and apoptosis via p38 MAPK signaling.
Map3k1	−1.091264569	0.0006314		Mitogen-activated protein kinase kinase kinase 1. Involved in the kinase signal transduction cascade. Activates the ERK and JNK kinase pathways
Rrm2b	−1.64098889	2.17E−08		Ribonucleotide reductase M2 B. Has an important role in cell survival by repairing damaged DNA.
Mterfd2	−1.120340882	1.39E−07		Supplies deoxyribonucleotides for DNA repair in cells arrested at G1 or G2
Bnip3l	−1.684610402	1.21E−07		mTERF domain-containing protein 2 [Source: UniProtKB/Swiss-Prot; Acc:Q4G078]
Cln8	−1.46642584	1.13E−07		BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like [Source: RefSeq peptide; Acc:NP_543164]
Cxadr	−1.107408415	1.33E−05		Ceroid-lipofuscinosis, neuronal 8. Involved in cell proliferation at neuronal differentiation and in protection against cell death.
Mpv17l	−1.038709587	7.78E−05		Coxsackievirus and adenovirus receptor homolog [Source: UniProtKB/Swiss-Prot; Acc:Q9R066]
				Mpv17 transgene, kidney disease mutant-like (Uncharacterized protein [Source: UniProtKB/TrEMBL; Acc:D4A908])

and mitochondrial pathway, respectively, were significantly downregulated in array data (fold change ≤ -1). Furthermore, in RT-qPCR studies, the change in expression compared to the unexposed control was not significant among any caspase gene tested (Fig. 10).

In array data, genes involved in apoptosis inhibition, *c-Jun* (fold change 0.8), *Birc3* (fold change 0.7), and *Faim3* (fold change 0.8), were somewhat upregulated, suggesting the inhibition of apoptotic cascades.

Together, these results indicate that apoptotic cell death is not involved in the cytotoxicity of EnnB in rat primary hepatocytes.

4. Discussion

Although enniatins (Enns) have not been reported to be involved in any human outbreaks, harmful effects on humans and livestock cannot

Fig. 7. Cluster analysis of genes involved with the GO term mitochondrion organization, following a 4-h EnnB exposure of rat primary hepatocytes. Hierarchical cluster analysis using the Euclidean distance was performed with the program TMEV 4.9.0. Only the higher 10 μ M and 20 μ M doses changed the expression pattern in rat primary hepatocytes, compared to the negative control, as the low dose (1 μ M) did not show a marked effect. Three distinguishable areas could be seen. 1 area with upregulated gene expression (red) and 2 with downregulated expression (blue). Several of genes belonging to the Ndufs gene family (Ndufs4, 1, 8) and Timm gene family (Timm8b, 21, 9) are represented in downregulated cluster areas.

be ruled out, especially on a long-term and low-dose basis, as only a very limited amount of subacute or chronic *in vivo* data is available. Additionally, numerous *in vitro* studies point towards cytotoxic effects of Enns on several different cell types at relatively low concentrations (Gammelsrud et al., 2012, Ivanova et al., 2012, Dornetshuber et al., 2009b). The cytotoxic effects of Enns have long been associated with the ionophoric properties of biological membranes, as they are thought to form sandwich-structured dimers transporting cations (especially K^+) across biological membranes (Ovchinnikov et al., Kamyar et al., 2004), but the exact mechanism of toxicity is still unclear.

Fig. 8. Gene expression data of microarray analysis post a 4-h, 10- μ M EnnB exposure of the Ndufs-family of genes (subunits of complex I), required in the electron flow chain in mitochondria leading to ATP production. Although only Ndufs4 is significantly downregulated (fold change ≥ 1), the overall expression trend among the Ndufs family of genes is repressed. Ndufs are NADH dehydrogenase Fe-S proteins and parts of the iron-sulfur fragment of complex I. Ndufs1 is the largest subunit of the enzyme. (Ref: www.Genecards.org) The Timm family of genes is involved in the transport of proteins in the mitochondrial intermembrane space and incorporation of proteins in the inner mitochondrial membrane.

The aim of this investigation was to determine the cytotoxic effects of enniatin B on the very sensitive mouse embryo fibroblast cell line Balb 3T3 and the hepatocyte HEPG2 cell line in relation to cellular energy metabolism, cell proliferation, and apoptotic cell death. Additionally, the mechanism of toxicity was examined on the basis of gene expression using rat primary hepatocytes and Affymetrix gene chip analysis. Primary hepatocytes were chosen for this study because they are considered to better correspond to *in vivo* studies than immortalized cell lines.

According to our results, Enn B was already cytotoxic to both cell lines (Balb 3T3 and HepG2) and rat primary hepatocytes at low (μ M) concentrations. In both cell lines, the levels of ATP and degree of cell proliferation were reduced in a very steeply declining manner, post a

Fig. 9. Real-time quantitative RT-PCR analysis of mitochondrial genes Ndufs4 and Timm21 in rat primary hepatocytes in response to 1 h and 4 h exposure to 10 μ M EnnB. Relative transcript abundance of non-treated cells (1 h/4 h contr) at corresponding time points.

Table 3

Impact of EnnB exposure on the expression in rat primary hepatocytes of genes involved in cell proliferation and its regulation. Rat primary hepatocytes were exposed for 4 h to 10 μ M EnnB and analyzed using the Affymetrix rat whole genome gene chip.

Gene	Fold change array data	Function (Reference: www.genecards.org)
Cyclin A1	0.56	A cyclins bind both CDK2 and CDC2 kinases, the other active in G1/S phase, the other in G2/M-phase.
Cyclin A2	−0.19	
Cyclin B1	–	Associate with Cdc2 kinase, the “M-phase kinase”,
Cyclin B2	−1.53	
Cyclin C	−0.87	Involved in phosphorylation of RNA polymerase II
Cyclin D1	−0.2	
Cyclin D2	0.5	D cyclins form a complex with and functions as a regulatory subunit of CDK4 or CDK6, required for cell cycle G1/S transition
Cyclin D3	−0.37	
Cyclin E1	0.51	E cyclins are regulatory subunits of CDK2 and their activity is required for cell cycle G1/S transition.
Cyclin E2	−0.6	
Cyclin F	−0.8	Cyclin F belong to the F-box protein family, subunits of the ubiquitin protein ligase complex
Cyclin G1	−2.23	
Cyclin G2	−0.58	Involved in p53 mediated G2/M phase arrest if DNA is damaged.
Cyclin H	−0.4	
Cyclin I	−0.9	Regulates CDK7, the catalytic subunit of the CDK-activating kinase (CAK).
Cyclin K	−0.76	
Cyclin L1	0.83	The function has not been determined.
Cyclin L2	1.7	
Cyclin T2	−0.21	May have a role in regulating CDK and RNA polymerase II activities.
Cyclin Y	−0.33	
Kinases		Pre RNA-splicing, RNA polymerase II regulation
Cdc2 or Cdk1	−0.1	
Cdk2	−0.57	Cyclin dependent kinase 2
Cdk4	−0.04	
Cdk5	0.16	Cyclin dependent kinase4
Cdk5r1	0.48	
Cdk5r2	0.72	Cyclin dependent kinase5 activator1
Cdk6	−0.1	
Regulators		Cyclin dependent kinase 6
Rb1 or pRB	−0.6	
E2F1	0.64	A negative regulator of the cell cycle. Acts as a transcription repressor of E2F1 target genes.
E2F4	−0.5	
E2F5	−0.9	A member of the E2F family of transcription factors and mediates both cell proliferation and apoptosis
E2F6	−1.4	
Wee1	−1.33	Transcription activator that controls cell-cycle progression from G1 to S phase
Trp53		

24-h exposure to EnnB, indicating a severe cytotoxic effect (Figs 3 and 4), with ED₅₀ values below 8.4 μ M. Nevertheless, the cell lines remained alive and showed a rather normal morphology as seen in Fig. 1A and B. Furthermore, the majority of the Balb 3T3 cells remained intact in the cell viability assay, as no leakage of AK through the cell membrane was measured (Fig 2). The cell density, however, was much lower than in culture plates of negative control cells supporting the finding of a decrease in DNA synthesis and cell division.

Interestingly, the effect of EnnB on rat primary hepatocyte morphology was very different from that of the two cell lines. An exposure time of only 4–5 h to 10 μ M EnnB caused the majority of the cells to round up and detach from the plate surface and die in a necrotic manner (Fig 1C and D). One reason could be stress due to cryopreservation and the new artificial environment and medium. The cells were, however, acclimatized for a long period (o/n) before exposure, and an optimized medium was used in all steps. Moreover, the morphology of negative control cells remained normal.

As our data indicate reduced ATP levels post EnnB exposure in the cell lines, down regulation of the genes involved with energy metabolism were expected in the primary hepatocytes. Enrichment analysis of microarray data on exposed primary hepatocytes revealed that genes involved in metabolic and catabolic processes were generally downregulated, which could point towards cellular energy deprivation, as for Balb 3T3 and HEPG2 cell lines. The enrichment results could, furthermore, refer to mitochondrial dysfunction given that GO terms linked with “mitochondrion organization” were significantly enriched among the downregulated genes. No enrichment among upregulated genes was discovered. Further cluster analysis of the gene expression patterns of the enriched genes, associated with mitochondrion

organization, revealed the presence of three gene clusters, two consisting of downregulated genes and one of upregulated genes (Fig. 7). Three genes of the *Ndufs* gene family, *Ndufs1*, 4, and 8, were found within these downregulated gene clusters (Fig. 7), indicating that EnnB exposure may alter the function of the mitochondrial electron transport chain (ETC) and hence, ATP production. *Ndufs* gene family members are involved in cellular respiration, as they are subunits of the mitochondrial ETC protein complex I (Breuer et al., 2013). This complex consists of 45 subunits, and the gene products of the *Ndufs* genes are NADH dehydrogenase iron-sulfur protein subunits (Breuer et al., 2013, GeneCards, 2015). The iron-sulfur protein cluster of complex I transfers the electrons to ubiquinone, the next carrier of the ETC (Garrett and Grisham, 1999). *Ndufs1* is the largest subunit of complex I and the *Ndufs4* gene encodes an 18 kDa subunit, phosphorylated by intracellular cAMP, leading to the stimulation of complex I (Petrizzella et al., 2001). Another clustered family of downregulated genes includes the *Timm* gene family. The gene product of the *Timm 21* gene is a translocase of the mitochondrial inner membrane and is required for the assembly of complex I and complex IV (GeneCards, 2015), and the gene *Timm 8b*, the product of which guides membrane proteins through the intermembrane space as they are imbedded in the inner membrane of the mitochondria (The NCBI, 2015).

Downregulation of these genes, affecting complex I function and assembly, could provide an explanation for the low cellular ATP levels after exposure to EnnB, with a different outcome depending on the cell type: a loss of cell proliferation in Balb 3T3 and HepG2 cell lines and necrotic cell death in primary hepatocytes. Mitochondrial dysfunction has been reported to be induced by a number of chemicals (drugs). Drugs can directly affect the DNA transcription of ETC complexes or

Table 4
Changes in gene expression of some central apoptotic proteins of EnnB-exposed (10 μ M, 4 h) rat primary hepatocytes compared to negative control cells. RT-qPCR results are reported as the change in expression compared to untreated cells, the expression of which is referred to as 1.

Changes in gene expression of central apoptotic proteins			
Gene name	Array data: log2 fold change	RT-qPCR	Gene function (Reference: www.genecards.org)
Caspase 1	+0.5	0.61	Activates inflammatory cytokines interleukine-1 β and –18 and induce necrosis
Caspase 2	0.001		Involved in the activation of caspases responsible for apoptosis. May be involved in Alzheimer's disease.
Caspase 3	–1.6	0.94	Executioner caspase, responsible for apoptosis execution.
Caspase4	–0.8		Involved in ER stress-induced apoptosis. Cleaves caspase-1
Caspase7	–0.6	0.96	Executioner caspase, responsible for apoptosis execution.
Caspase8	–0.14		Initiator caspase, the first caspase to be activated in the death receptor pathway.
Caspase9	–0.15	1.20	Initiator caspase, activates caspase-3
Caspase12	–1.4		Initiator caspase, mediates apoptosis in response to ER stress in rodents.
Caspase14	+0.5		May be involved in keratinocyte differentiation, and skin barrier formation.
Casp8ap2	–0.85		Caspase-8 associated protein 2
TRADD	–0.67		TNF (TNFRSF1A)-associated death domain
Bid	–0.3		BH3-interacting domain death agonist. Required for the release of cytochrome C from mitochondria. (Ref Genes VII)
Apaf-1	–0.5		Apoptotic peptidase activating factor 1
Faslg	0.01		Interaction of FAS with this ligand is critical in triggering apoptosis of some types of cells
Daxx	–0.36		Death domain-associated protein 6. Mediates activation of the JNK pathway.
Tp53	–0.6		TP53-dependent transcriptional repression leads to apoptosis. Act as a tumor suppressor.
Aifm	–1.03	0.71	Apoptosis-inducing factor 1, mitochondrial. Aifs are involved in caspase independent apoptosis and affects chromosome condensation and fragmentation.
Apoptosis inhibitors			
Bcl2	+0.2		Prevents the release of cytochrome C from mitochondria, inhibiting apoptosis.
Birc3	+0.73		Inhibits apoptosis by binding to tumor necrosis factor receptor-associated factors TRAF1 and TRAF2
Birc6	+0.68		This protein inhibits apoptosis by facilitating the degradation of apoptotic proteins by ubiquitination.
Faim3	+0.8		Fas apoptotic inhibitory molecule 3

inhibit enzymes of the ETC and glycolysis, or reduce antioxidant levels via free radical production (Neustadt and Pieczenik, 2008). Barbiturates and the pesticide rotenone inhibit oxidative phosphorylation (ATP production) via effects on complex I, with a rapid fall in ATP levels in cells (Neustadt and Pieczenik, 2008). Based on our gene expression results, the rapid necrotic death of the rat primary hepatocytes and the drastic drop in ATP production in the cell lines (Balb 3T3, HepG2) might be due to starvation caused by the effects of enniatin B on complex I function and assembly, leading to dysfunction of the mitochondrial electron transfer chain. This is in line with studies by Tonshin et al., who reported similar findings, as Enns caused mitochondrial alteration and dysfunction in isolated rat liver mitochondria and human neural (Paju) cells, murine insulinoma (Min-6) cells, as well as in boar sperm cells. Enniatins caused a drop in the inner membrane potential and swelling

of the isolated rat mitochondria in the presence of potassium (K⁺) (Tonshin et al., 2010). The mitochondrial membrane potential of intact cells (Paju, Min-6) was also altered and caused necrotic cell death. In sperm cells, enniatins caused a partial depolarization of the mitochondria as a result of K⁺ influx into the mitochondrial matrix (causing swelling), and hyperpolarization of the sperm plasma membrane due to the efflux of potassium ions from the cell cytoplasm to the medium (Tonshin et al., 2010).

Moreover, by reducing ATP production, EnnB exposure resulted in decline in DNA synthesis (proliferation) in Balb 3T3 and HepG2 cell lines, measured by BrdU incorporation into DNA during the S-phase. Low levels of EnnB already had a drastic impact on cell proliferation (ED₅₀ = 4 μ M and 0.5 μ M, respectively, Fig. 4). The effect is even direr than for ATP production, but still lies in the same concentration range. The differences might be due to the different sensitivity of the two test methods.

Fig. 10. Real-time quantitative RT-PCR analysis of the genes Aifm, caspase-3, -7, and -9, involved in apoptosis, and caspase-1 (inducing necrosis and inflammatory cytokines IL-18 and 1 β), in rat primary hepatocytes in response to 10 μ M EnnB exposure. Relative transcript abundance of non-treated cells (1 h/4 h contr) at corresponding time points.

Table 5
Primer sequences used in RT-qPCR analysis.

Gene name	Sequence (5'-3')
FR3_Casp1	GGAGGACATTTTCAGAAAGG
RR3_Casp1	CTTGGATCTTTAATGCTCTGG
FR3_Casp3	GTGTGATTCTAAGTCATGGAG
RR3_Casp3	CATATCATCGTCAGTTCCAC
FR3_Casp7	CGTCTATAATGACTGCTCTTG
RR3_Casp7	TGAGCTGTCAGATCCTTTATC
FR3_Casp9	TTGTGAACATCTCAATGGG
RR3_Casp9	TGTCTTGAGAGGAAGTGAAG
FR3_Ndufs4	CTTCAGTGCCAAAGAAGATG
RR3_Ndufs4	CAGTCAAGCAGAAATGTAGC
FR_Timm21	GAGACAACATGTCAGCTTTAC
RR_Timm21	CAATAGATCTTTTAGGAGCCAC
FR3_Aifm1	GGGAAGTCAAGTCAATTACAG
RR3_Aifm1	GAAGGATCTTCCCCATATTTC
FR3_SDHA	CCAGGACTTGAATTTGTTTCAG
RR3_SDHA	CATATCTCTCCATGAACCTTTC

The mitotic cell cycle is a complex and highly regulated process ending up in mitotic division of a cell. Cyclin-dependent kinases, together with regulators, play a major role in cell cycle control (Lewin, 2000). Our results of decreased cell proliferation are in line with previous studies (Gammelsrud et al., 2012), in which enniatin exposure caused cell cycle arrest in the G0/G1 phase and low cyclin D levels in murine macrophages and human carcinoma cells (HeLa), as well as, arrest in the G2 phase in Caco-2 cells (Ivanova et al., 2012). Low cyclin D levels might be the signal for cells to enter the G0 phase if growth conditions are altered (Lewin, 2000). Hence, ATP depression due to effects of EnnB on ETC complex I, might provide an explanation for our results of decreased cell proliferation (DNA synthesis) in the cell lines Balb 3T3 and HepG2.

The gene expression study on primary hepatocytes, did however, not reveal any drastic changes in the gene expression of the key proteins involved in cell proliferation compared to the negative control (Table 3), except for cyclin B, which was downregulated. Cyclin B is only active during mitosis. There was also no significant enrichment (GO terms) among up- or downregulated genes regarding cell proliferation. This indicates that the toxicity of high doses of EnnB (greater than 10 μ M) is not due to cell cycle arrest in rat primary hepatocytes, but rather an event of necrotic cell death, manifested by swelling of the cells and detaching from the cell culture plate, possibly due to energy deprivation caused by mitochondrial dysfunction (effects on complex I).

The last of the studied endpoints was programmed cell death. Apoptosis can be induced by numerous factors. Unlike necrosis, apoptosis does not cause inflammation in tissues; instead, apoptotic cells undergo typical morphological changes. Apoptotic cells might, however, switch to necrosis if caspase or ATP levels become too low. In some occasions, low levels of chemicals/toxins induce apoptosis but higher doses might cause the cells to die via necrotic cell death (Elmore, 2007, Lewin, 2000).

In our study, exposure of mouse fibroblast Balb 3T3 cells to EnnB for 24 h and 48 h caused a slight increase in the early apoptotic cell population, studied by flow cytometric measurements using Annexin V-FITC/PI staining. The effect was comparable to the positive control, camptothecin. The increase in the pro-apoptotic cell population after 24 h ranged from 0.3% (negative control cells) to 4.4% (45 μ M EnnB exposure). Exposure for 48 h had similar effects. Furthermore, the proportion of late apoptotic or necrotic cells appeared to increase with the EnnB dose; however, the change was not statistically significant. EnnB induction of apoptosis has also been reported in studies by Wätjen et al. and Dornetshuber et al. (Dornetshuber-Fleiss et al., 2014, Wätjen et al., 2009). In these surveys, enniatins caused caspase 3/7 activation in hepatoma H4IIE cells and caspase-7 activation in the KB-3-1 cell line, respectively, as well as nuclear fragmentation. Although EnnB slightly induced apoptosis in the Balb 3T3 cell line in our study, the outcome in rat primary hepatocytes was very different. In contradiction to the results of the cell lines, the gene expression results of rat primary hepatocytes did not support apoptotic cell death. The GO terms linked to apoptosis (apoptotic mitochondrial changes and regulation of apoptotic DNA fragmentation) were slightly, but not significantly, enriched among downregulated genes, while no enrichment was detected among the upregulated genes. Furthermore, the expression of central genes involved in apoptosis (e.g. caspase-3, -8, -9, *TRADD*, *Bid*, *Apaf-1*, *Aifm*) were only slightly downregulated or unchanged compared to the negative control in the microarray experiment. Notably, both caspase-3 and *Aifm*, which are executing molecules in both the death receptor pathway and mitochondrial pathway, were significantly downregulated based on microarray and RT-qPCR studies (Table 4, Fig. 10). Furthermore, the *c-JUN* gene, involved in apoptosis inhibition via the TNF/NF-KB/IAP pathway, was marginally upregulated (log₂ fold change 0.8). *c-JUN* interacts with NF-KB, which results in the transcription of IAPs (inhibitors of apoptosis proteins, e.g. Birc3) (log₂ fold change 0.73), which can bind and inhibit caspases (Dubrez-Daloz et al., 2008, Liu and Lin, 2005). Together with the morphological findings (swelling of cells, rupture of plasma membrane), the gene

expression results indicate that primary hepatocytes died via necrosis. The significance of apoptotic cell death in the overall toxic response of cells to EnnB could be considered minor, as the increase in the early apoptotic cell population in the studied cell lines was less than 5% compared to the negative control and cell death in primary hepatocytes was necrotic rather than apoptotic.

We report clear toxic outcomes of EnnB in *in vitro* assays and the data facilitates the understanding of the mode of action of EnnB at cellular level. However, if the toxic exposure levels are mirrored to the reported EnnB levels in foodstuffs, the levels in our experiments are by far higher (over 1000 times) than is possible in food intake.

5. Conclusions

EnnB exposure of cell lines Balb 3T3 and HepG2 altered cellular energy metabolism by reducing ATP levels, which already occurred at low exposure concentrations (below 10 μ M). Concurrently, cell proliferation ceased and the proportion of early apoptotic cells slightly increased. Most of the cells, however, survived and cell morphology remained normal. The toxic response to EnnB in rat primary hepatocytes was quite different. The rat hepatocyte morphology was altered, as the cells swelled, the plasma membrane was disrupted, and the cell contents were released, indicating necrosis. Gene expression studies revealed altered mitochondrial organization and dysfunction of the mitochondrial electron transfer chain due to the effects of enniatin B on complex I function and assembly. We propose that EnnB acts by causing energy deprivation due to mitochondrial alteration in cells, leading to diverse outcomes in different cell types: a decrease in cell proliferation of cell lines vs. necrotic cell death in primary cells. This highlights the importance of recognizing the differences between cell lines and primary cells, especially when assessing the relevance of *in vitro* to *in vivo* findings.

Conflict of interest

The authors have declared no conflict of interest.

Transparency document

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