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THE TOXICITY OF FUSARIUM MYCOTOXINS ENNIATIN AND MONILIFORMIN

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ABSTRACT

The red mold *Fusarium*, is one of the most prevalent fungal pathogens, infecting small-grain cereals in temperate regions of the world. In Scandinavia, *F. avenaceum, F. culmorum, F. poae,* and *F. sporotrichioides* infections are most common, but in recent years, possibly due to climatic changes, *F. graminearum* and *F. langsethiae* infections have increased, as well. Most Fusarium species are capable of producing a variety of mycotoxins, possibly providing an edge over competing strains at the site of infection. Mycotoxins can evoke a broad range of toxic effects in humans and animals, including neurotoxicity, immunotoxicity, reproductive-, and developmental toxicity and carcinogenicity. The most toxic fusariatoxins include the tricothecenes, fumonisins and zearalenone. However, less studied mycotoxins, as enniatins (Enns) and moniliformin (MON) are frequently found in grain products, hence, causing a risk of a daily, low-level exposure of human and livestock, the significance of which is still unclear. The aim of this study was to provide further insights into the toxicity of Enns and MON, and into the mechanism of action of EnnB, monitored at gene expression level. Furthermore, the acute oral toxicity and repeated, low-dose, oral toxicity of MON in Sprague-Dawley rats was assessed, adapting OECD Guidelines 423 and 407.

We report clear toxic outcomes of EnnB *in vitro*, as it affected cellular energy metabolism by reducing ATP levels in cell cultures and cell proliferation, already at low concentrations (below 10 μM) in the cell lines Balb 3T3 and HepG2. EnnB exposure slightly increased the proportion of early apoptotic cells, as well. Gene expression studies revealed alteration of energy metabolism, due to effects on gene expression of genes associated with mitochondrial organization and function and assembly of complex I of the electron transport chain.

Moniliformin (MON) did not induce significant toxicity in vitro. However, high doses of MON (50 mg/kg b.w.) caused acute toxicity in rats, seen as cardiovascular changes and respiratory distress, resulting in death. According to the OECD Globally Harmonized System, the toxicity of MON could be classified into category 2, and a LD_{50} cut of value of 25 mg/kg b.w. was determined. Long term, low dose exposure affected mainly the innate immunity, by reducing the phagocytic activity of rat neutrophils in all tested groups (3 - 15 mg/kg b.w.). Hence, we suggested a LOAEL of 3 mg/kg b.w. for MON. The rats of the lowest dose groups (3-6 mg/kg b.w.) remained clinically healthy. Two rats of five in the highest dose group (15 mg/kg b.w.) showed similar signs as in the acute oral toxicity test and died. Excretion kinetics revealed that MON is rapidly excreted in urine, in less than 6 h, but only 1-2% was found in feces. This indicates that the urinary excretion is the main route

for elimination and biliary excretion has a minor role. Furthermore, MON is unlikely to accumulate in the body, being highly hydrophilic.

I hope that these results could contribute to risk assessment of moniliformin and enniatins in the future, as toxicity studies on moniliformin and enniatin are scarce.

LIST OF ORIGINAL PUBLICATIONS

I Jonsson, M., Jestoi, M., Anthoni, M., Welling, A., Loivamaa, I., Hallikainen, V., Kankainen, M., Lysøe, E., Koivisto, P., Peltonen, K. Fusarium mycotoxin enniatin B: cytotoxic effects and gene expression profile. Toxicology in vitro 34 (2016) 309-320.

II Jonsson, M., Jestoi, M., Nathanail, A., Kokkonen, U-M., Anttila, M., Koivisto, P., Karhunen, P., Peltonen, K. Application of OECD Guideline 423 in assessing the acute oral toxicity of moniliformin. Food and Chemical Toxicology vol 53 (2013): 27-32.

III Jonsson, M., Atosuo, J., Jestoi, M., Nathanail, A., Kokkonen, U-M., Anttila, M., Koivisto P., Lilius E-M., Peltonen, K. Repeated dose 28-day oral toxicity study of moniliformin in rats. Toxicology Letters 233 (2015) 38–44.

RESEARCH INPUT AND AUTHORSHIP OF ARTICLES I-III

I The author planned and performed most of the analyses and carried out statistical handling. Rat whole genome expression analyses were performed at the Functional Genomics Unit, University of Helsinki. The author wrote most parts of the article and was the corresponding author in the publication.

II The author took part in the planning and organization of the study and performed a part of the dosing of the test animals and sample collection, collected data and carried out statistical handling. The author wrote main parts of the article and was the corresponding author in the publication.

III The author took part in the planning of the study, collected data and performed some of the statistical analyses. The author wrote main parts of the article and was the corresponding author in the publication. This publication has been included as a part of the PhD thesis of PhD Janne Atosuo (University of Turku, 2015). Authors contributed equally to this work.

ABBREVIATIONS

ADME	Absorption, distribution, metabolism, excretion
ATP	Adenosine triphosphate
BEA	Beauvericin, mycotoxin
b.w.	Body weight
CYP	Cytochrome Protein, hemoproteins
DNA	Deoxyribonucleic
DON	Deoxynivalenol, mycotoxin
EC_{50}	Half maximal effective concentration
ED_{50}	Median effective dose, dose causing effect in 50% of population
Enn	Enniatin, mycotoxin
ESI	Electrospray ionisation
ETC	Electron transport chain
FDR	False discovery rate
FITC	Fluorescein isothiocyanate, fluorochrome
GHS	Globally Harmonized System
GI	Gastro- intestinal tract
GMP	Guanosine monophosphate
GO	Gene Ontology, classification term in bioinformatics
GTP	Guanosine triphosphate
HILIC	Hydrophilic interaction chromatography
Hiv	Hydroxyisovaleric acid
HT-2	HT-2 Toxin, mycotoxin
LD_{50}	Dose lethal to 50% of animals
LOAEL	Lowest observed adverse effect level
MAPK/ERK pathway	MAP/Extracellular signal–Regulated Kinases, intracellular signaling molecules
MON	Moniliformin, mycotoxin
MS	Mass spectrometry
NMR	Nuclear magnetic resonance spectroscopy,
OECD	Organisation for Economic Cooperation and Development
p-value	Probability value
q-value	A p-value adjusted for FDR
Redox	reduction–oxidation reaction
RNA	Ribonucleic acid

RT-qPCR	Quantitative reverse transcription Polymerase chain reaction
T-2	T-2 Toxin
TCA cycle	Tricarboxylic acid cycle
TDI	Tolerable daily intake level
UPLC	Ultra performance liquid chromatography
Q-Tof	Quadrupole-time-of-flight

1. INTRODUCTION

The kingdom Fungi includes mushrooms, molds, and yeasts and is divided into seven phyla. Three phyla produce motile asexual zoospores (with flagella): the aquatic fungi *Chytridiomycota,* the *Blastocladiomycota,* which are parasites of eukaryote, and the anaerobes *Neocallimastigomycota*, which inhabit the digestive systems of herbivores. The remaining four phyla are nonmotile: the *Glomeromycota*, forming mycorrhizae with plant roots, the *Ascomycota,* which are sac fungi including morels, truffles, filamentous fungi, and yeasts, the *Basidiomycota* (most mushrooms), and the *microsporidia*, which are unicellular parasites of animals (Hibbett et al., 2007). Fungi are heterotrophs, using organic compounds such as carbohydrates, fats, and proteins as energy sources by secreting enzymes to the environment. The cell wall, which is composed of chitin crosslinked by glucan and proteins, distinguishes fungal cells from animal cells, which lack a cell wall, and plant cells, in which the cell wall is composed of mainly cellulose, hemicellulose, pectin, and lignin.

For thousands of years, fungi have been used by humans as food and in food production and preservation via fermentation (beer, wine), bread baking, and cheese production. Presently, the use of fungi has widely expanded, mainly due to bioengineering techniques, from the large-scale production of some chemicals (e.g. citric acid, enzymes), medicine production (antibiotics, statins, immunosuppressants), and use in scientific studies, especially yeasts in genetics, to new food products such as meat replacements like Quorn, – a mycoprotein produced by *Fusarium venenatum* (Hamlyn, 1997).

Despite a wide range of useful effects, several fungi are pathogens of the kingdoms Plantae and Animalia, causing severe economic losses and health hazards. Human mycoses range from superficial skin infections such as pityriasis versicolor (caused by *Malassezia furfur*), affecting skin pigmentation, and *Candida albicans*, mainly affecting skin and mucus membranes, to invasive, life-threatening infections, often beginning as primary pulmonary infections that disseminate to other organs, e.g. the brain, as in coccidioides meningitis, histoplasmosis, aspergillosis, and cryptococcosis (Baron, 1996).Fungal plant pathogens infecting agriculturally important plants cause severe economic losses globally and also pose a potential health hazard to humans and livestock, by producing mycotoxins. Annual losses of up to $ 1.5 billion have been estimated to be due to mycotoxin contamination of grain in the United States (Schmale and Munkvold, 2009). The most significant plant pathogenic fungal species are considered to be *Magnaporthe oryzae*, infecting rice, *Botrytis cinerea* (gray mold), with a broad host range, *Puccinia* spp., infecting grain, and *Fusarium* spp., with several subspecies infecting different plants (Dean et al., 2012). In Europe,

Fusarium infections are the most prevalent ones in grains, causing plant diseases such as crown rot and head blight in cereal grains (wheat, barley and oats), as well as, root-, stem-, and ear rot of maize and vascular wilt in several plant species, including greenhouse vegetables and fruits (Cerkauskas and Brown, 2001). Several fungal species, including most *Fusarium* species, as well as species of *Aspergillus*, *Penicillium,* and *Clavicpes* are capable of producing a variety of secondary metabolites, i.e. mycotoxins, possibly providing an edge over competing strains at the site of infection (Desjardins, 2006, Jestoi, 2005, Desphande, 2002). Some of these mycotoxins have serious consequences for animal productivity and animal and human health, increasing the negative economic impacts even further. One of the best-known groups of mycotoxins is the hepatotoxic and carcinogenic aflatoxins (B1, B2, G1, G2, M1, M2), produced by *Aspergillus flavus* and *A. parasiticus*. They are common findings in spices, nuts, and grains. Brewery products, cheese, and soy sauce are occasionally contaminated with the nephrotoxins ochratoxin (A, B, C) and citrínin produced by some *Penicillum* and *Aspergillus* species. *Claviceps purpurea* can contaminate grain with ergot alkaloids and cause ergotism, a serious condition affecting the central nervous system and causing gangrene in the limbs due to vasoconstriction (Desphande, 2002). Patulin, a mycotoxin produced by *Penicillum expansum*, some *Aspergillus* species, and *Paecilomyce*s in moldy fruits and vegetables, is known to affect the immune system (Sorenson, 1986; Desphande, 2002) just to mention a few.

Following exposure to and entrance of a xenobiotic (e.g. mycotoxin) into the body, the elimination or detoxification of the foreign substance often occurs via biotransformation reactions. These reactions take place in most tissues (e.g. skin, lungs, GI tract), but the main organ involved is the liver. In general, hydrophilic, polar chemicals can be excreted more rapidly via urinary or biliary/fecal routes than lipophilic chemicals, and hence, biotransformation reactions that modify the toxin in order to increase water solubility, thus take place first. These functionalization reactions (" phase I reactions") include oxidation, reduction, and hydrolysis reactions and the addition of polar functional groups (Desphande, 2002). The enzymes involved (e.g. cytochrome-P450 monooxygenases, epoxide dehydrolases, alcohol- and aldehyde dehydrogenases) are mainly located in the endoplasmic reticulum of (liver) cells. In some cases, this is sufficient to remove the foreign substance via urine or bile. However, xenobiotics, especially lipophilic substances, often undergo even further transformations in order to reduce toxicity or enhance excretion. In these conjugation reactions ("Phase II reactions") enzymes conjugate xenobiotics with amino acids (glutamine), glutathione, sulfates, or sugars (glucuronic acid), or induce the methylation of amino acids, thiol- and hydroxyl groups to further increase water solubility, excretion, and detoxification (Desphande, 2002). For some chemical carcinogens and xenobiotics, the biotransformation reactions may result in a more toxic end product. For instance, aflatoxin B1 (AFB1), produced

by *A. flavis* and *A. parasiticus*, can be converted into several different types of metabolites, most of which are less toxic. However, oxidation reactions by two P450 enzyme family members in liver cells, the CYP1A2 and CYP3A4 in mammals (Eaton and Groopman, 1994), and CYP 2A6 in chickens (Diaz et al., 2010), can lead to the formation of aflatoxin 8,9-epoxide (AFBO), which is reactive with nucleic acids (DNA). Aflatoxin epoxides react with DNA, especially with the base guanine, followed by insertion of the molecule between bases in the DNA helix and hence, disturbance of the polymerase enzyme function. This leads to the replacement or loss of bases of the DNA chain (Tropp, 2011), resulting in mutagenesis and carcinogenicity and increasing the risk of liver cancer even at low levels during long-term exposure (Desphande, 2002, Ch11). AFBO, in turn, can be hydrolyzed to AFB1 8,9- dihydrodiol and cause cytotoxic effects in liver cells (Diaz et al., 2010, Eaton and Gallanger, 1994). High-level exposure to aflatoxin B1 causes acute toxicity and liver damage in many species, including humans, with a 75 mg/kg b.w. dose being lethal and having an LD_{50} value in monkeys of 0.56 mg/kg b.w. (Desphande, 2002).

Natural toxins, such as *Fusarium* mycotoxins, are in practice impossible to eradicate from food and feed, as molds are always present in growth and storage environments. However, the concentrations, occurrence, and carry over to food and feed can be reduced by improving agricultural practices and storage facilities and through governmental and international legislation, defining maximum permitted levels for selected mycotoxins in food and feed. In the European Union (EU), the Commission of the European Communities is responsible for risk management legislation and policy regarding food additives, substances, and contaminants in food, and defines the regulations and legal standards together with the European Parliament and member states. The Scientific Committee for Food (SCF) advises the Commission regarding the directives (Desphande, 2002). The European Food Safety Authority (EFSA) provides scientific risk assessment based on published scientific- and epidemiological data. The scientific opinions of EFSA form the basis for European policies and legislation. EFSA provides safety assurance for the studied xenobiotic or chemical by assessing dose-response relationships and determining threshold values for the xenobiotic, such as the NOAEL (no observed adverse effect level). NOAEL is divided by a safety factor of normally 100 to yield presumably safe levels for consumption: the TDI (tolerable daily intake level) or ADI (acceptable daily intake) expressed, for example, as mg/kg body weight (Desphande, 2002, ch 6). For instance, maximum levels for the *Fusarium* trichotecene deoxynivalenol (DON) are set at 1250 μg/kg in unprocessed cereal grain and 750 μg/kg in flour, and the TDI for DON is 1 μg/kg b.w. For the *Fusarium* mycotoxins T-2/HT-2, the TDI has been estimated to be 100 ng/kg b.w (EFSA, CONTAM, 2011). Maximum levels for the sum of aflatoxins are set at 5 μg/kg for rice and maize and 2-8 μg/kg for different nuts (EC regulation No 1881/2006, EUR-Lex, 2006).

Climatic and environmental conditions strongly impact on the composition of the infective fungal species, as well as their mycotoxin production. Climate change, with increases in atmospheric CO_2 levels, rising average temperatures and changes in humidity and rain quantity, are likely to have an impact on the fungal plant disease risk, as fungal species are mostly dependent on humidity for infection. It is difficult to predict the outcome of climate change, as the studies are long term and a number of changing factors can contribute to the results, e.g. new cultivars, new pesticides, changes in planting dates, tillage, changes in vectors or plant pathogen diversity. Simulated climate change experiments with elevated temperatures, precipitation, or CO_2 enrichment treatments are used to reveal trends among plant pathogens or so-called "fingerprints of climate change", and are an ongoing process (Garrett et al., 2009). Some long- term studies have revealed correlations between plant pathogens and climate change. The fear is that plant pathogens will invade new areas or develop increased virulence. Hannukkala et al. (2007) compared the incidences of potato late blight epidemics, caused by the oomycete or "water mold" *Phytophthora infestans* and climate change during 1933–1962 and 1983–2002 and determined that the onset of potato late-blight epidemics in Finland started 2-4 weeks earlier during the latter time period and that late-blight had also invaded new areas further north (north of latitude 65°N). Another issue that might be linked to climate change is the finding that *Fusarium graminearum*, common in central Europe and previously less abundant in Scandinavia, has increasingly been found to infect Scandinavian grain since 2000. Also, the new invader *F. langsethiae*, mainly infecting oats, which was first found in Norway in the 1990´s and was also present in Germany, Britain, and Denmark, has now spread throughout Scandinavia (Torp and Langseth, 1999, Yli-Mattila, 2010). Even though it is extremely difficult to predict the impact of climate change on (fungal) plant pathogens, it is clear that new challenges and changes will appear, highlighting the need for further studies.

The science of mycotoxicology includes everything from occurrence data from fields, epidemiologic studies, toxicity studies *in vivo* (acute-, subacute- and chronic) and *in vitro*, to the assessment of the mechanism of action of bioactive molecules such as mycotoxins. In epidemiological studies, biomarkers such as metabolites and DNA adducts can be scanned from urine, blood, and milk samples in different populations. For example, the aflatoxin B1-N7 guanine adduct, The O-demethylated aflatoxin B1 metabolite AFP1, DON, and de-epoxy DON are used as urinary biomarkers of exposure and can be used to detect a correlation between the toxic outcome and exposure (e.g. hepatocarcinoma vs. aflatoxin exposure) in a population (Ross et al.,1992, Eaton and Gallanger, 1994).

Recently, the tendency in toxicology has shifted from *in vivo* testing towards animal-free assays, not only due to the high costs of animal tests, but also on the grounds of public opinion and legislative changes, e.g. the EU ban on animal testing in the cosmetic sector from 2013. However, predicting the *in vivo* relevance of *in*

vitro assays is currently a challenging, if not impossible task. Therefore, *in vivo* assays are still essential and unavoidable in toxicity assessment. *In vitro* methods provide excellent tools in studies on the mechanism of action. A wide range of *in vitro* cell viability tests are available on the market, with several different end points, including cell membrane integrity, cell energy (ATP production, mitochondrial effects), oxidative stress (glutathione levels, redox status), apoptosis, cell proliferation, and protein synthesis. Fluorometric/luminometric methods are worth mentioning, since they are rather economical, rapid, and easy to perform. However, mechanistic studies often also require the use of extensive research methods, including enzyme activity assays and flow cytometric assays etc. Gene expression tests can also be a valuable tool to gain further information on the mechanism of toxic insult and possible biotransformation reactions. However, they are often costly and laborious and require special equipment. There are multiple ways to study gene expression, from microarrays producing large amounts of data covering whole genomes, to studies on single genes using real-time PCR methods or northern blotting.

One important issue in *in vitro* testing is the choice of cells used. The traditionally used immortal cell lines have often lost features characteristic of the cells in corresponding tissues, and the toxic responses might therefore greatly differ from *in vivo* studies. One solution has been the use of co-cultures of cell lines or cell types, or the use of primary cells or tissue slices (e.g. precision-cut liver slices). However, these methods are difficult to apply in high-throughput screening. Recently, 2D sandwich cultures and 3D cell culture techniques (hanging drop cultures), which better simulate tissues and organs, have been established in which cell polarity and differentiated functions are better retained (Rimann and Graf-Hauser, 2012,Fraczek et al., 2013,Tung et al., 2011). Even 3D high-throughput applications for co-cultured primary cell types are nowadays possible, such as 96- well format arrays of spheroids. The use of primary cells is not, however, problem free: higher costs, the rapid loss of primary cell specific features, high "batch variation", and the short lifetime of cultures (only days) restrict their use. Another problematic issue, especially concerning human primary cells, is their availability. One solution to overcome this problem in the future would be the use of human induced pluripotent stem cells (Sengupta et al., 2014).

The outcome of gene expression studies in *in vitro* assays may also be distinct from *in vivo* models, which is good to keep in mind. According to Boess et al., (2003) mRNA expression profiles in rodent *in vivo* studies versus *in vitro* setups markedly differed when comparing precision-cut liver slices, primary hepatocytes, and hepatic cell lines with animal settings. Precision-cut liver slices showed expression profiles most similar to those in animal studies, while in primary cells the profile differed somewhat more, and hepatic cell lines were very divergent from *in vivo* models.

Recently, organ-on-chip systems have been developed to mimic organ-specific changes on microscale cell culture platforms. In these applications, several fluid

chambers and channels are connected to each cell culture well, enabling controlled fluid flow and culture conditions. The use of tissue slices, cell lines, primary cells, co-cultures of different cell types, and even stem cells is becoming possible in these models. One goal is to develop a human on- a- chip model, with cells from several organs cultured on one microplatform, where the fluidic system would mimic the blood circulation *in vivo* (Luni et al., 2014), possibly even further reducing the need for *in vivo* studies in the future.

2. REVIEW OF THE LITERATURE

2.1 The genus *Fusarium*

The red mold *Fusarium* belongs to the sac fungi, *Ascomycota*, characterized by the formation of an ascus – the site of sexual reproduction, nuclear fusion and meiosis. Meiosis results in the formation of haploid ascopsores, which germinate into new fungi. Both homothallic (self-fertilizing) and heterothallic species (two mating types) are represented among the genus. For most strains, sexual reproduction is uncommon in nature and rather provides a means for genetic recombination and diversity during environmental changes (Brown and Proctor, 2013). Most *Fusarium* species reproduce asexually via the mitotic formation of conidia (micro and/or macrospores). In some strains, such as *F. avenaceum, F. poae, and F. culmorum,* reproduction is solely asexual (anamorphs) (Kerényi et al., 2004). Asexual reproduction is a continuous process in favorable environmental conditions, and the fusoid-shaped conidia are produced in slimy clusters of conidiophores called sporodochia, from which the conidia are spread by splash dispersal (Brown and Proctor, 2013). Ascospores are more resistant to extreme temperatures and humidity than conidia and are produced during extreme environmental conditions. However, for *F. graminearum (G. zeae*), the airborne spread of ascospores is common during the onset of plant disease outbreaks.

Sexual reproduction possibly starts due to pheromone stimulation, causing the cytoplasms of the female hyphae (ascogonium) and the male element (micro-, macroconidium or hyphae) to fuse. From the ascogonium, the walls of a fruiting body, the perithecium, are formed. Inside these walls, dikaryotic ascogonium hyphae develop into asci (sac) and haploid ascospores due to nuclear fusion, meiosis, and mitotic division. Ascopores are discharged through an opening (ostiole) of the perithecium and are efficiently spread into new areas by the wind (Brown and Proctor, 2013). Grains are particularly sensitive to *Fusarium* infections during flowering, as the anthers are exposed. The openings for the exchange of oxygen and carbon dioxide in leaves, the stomata, also provide an ideal site of initial infection. More than 90 species of *Fusarium* have been characterized, and this number is increasing due to better taxonomic tools, including genetics. (Brown and Proctor, 2013, Torp and Nirenberg, 2004). To make it even more challenging, a group of *Fusarium* species, the *Gibberella fujikuroi* species complex, has two names: *Fusarium* for the anamorphic stage and *Gibberella* for the sexual phase. Hence, the telemorph (sexual) phase of *Fusarium graminearum* is called *Gibberella zeae* and the telemorph of *F. verticilloides (*formerly *F. moniliforme)* is *Gibberella moniliforme*.

2.2 Fusarium infections in Europe

Common *Fusarium* species infecting grain in Europe include *F. graminearum, F. culmorum, F. poae, F. avenaceum, F. sporotrichoides, F. verticilloides,* and *F. tricinctum*, but the dominant species in warm and temperate areas of Europe is *F. graminearum* (Yli-Mattila, 2010, Pasquali et al., 2016, Osborne and Stein, 2007). *F. poae* has a preference for cooler climates and is mainly present in northern areas. In Scandinavia, *F. avenaceum, F. culmorum, F. poae,* and *F. sporotrichioides* infections are most common, but in recent years, possibly due to climatic changes, *F. graminearum* and *F. langsethiae* infections have increased, especially in oats (Hietaniemi et al., 2016, Parikka et al., 2014). In Finland, spring cereals appear to be more susceptible to *Fusarium* infections than winter cereals (Hietaniemi et al., 2016).

2.3 *Fusarium* mycotoxicology

Most *Fusarium* species are capable of producing a variety of mycotoxins, possibly providing an edge over competing strains at the site of infection (Bottalico, 1998, Desjardins, 2006, Jestoi, 2005). Climatic and environmental conditions strongly impact on the composition of the infective species and the mycotoxin production. Some of these natural toxins have shown acute toxicity (toxicoses) in animals and humans, especially the most studied mycotoxins, the trichothecenes, zearalenone, and fumonisins. The toxic effects of the mycotoxins are multifold, including neurotoxicity, immunotoxicity, reproductive, and developmental toxicity and carcinogenicity (Desjardins, 2006). In 2005, the European Union set maximum levels for the trichothecene DON (1.2-1.7 mg/kg in unprocessed grain) and zearalenone (0.1 mg/kg in unprocessed grain) in cereals and cereal products, including maize and maize products (Commission Regulation (EC) No 856/2005 of 6 June 2005). Recommendations for maximum levels of DON in feed (8 mg/kg grain and 12 mg/kg maize) were also established. However, *Fusarium* species produce a number of other less studied secondary metabolites, such as moniliformin (MON) and enniatin (Enn), recurrently detected in cereal grain and grain-based food and feed in Scandinavian countries (Jestoi, 2005,Uhlig et al., 2006,Uhlig et al., 2004). Although Enn and MON are common contaminants of grain, there is only limited knowledge of the acute and chronic toxicity and absorption-, distribution-, metabolism-, and excretion (ADME) and mode of action of these two fusariotoxins. There is, hence, a need for further assessments of the consequences of their exposure for human and animal health.

2.3.1 Fumonisins

Fumonisins are long-chain alcohols, with structural similarity to sphinganine (Desjardins, 2006). They are produced by polyketide synthase (PK) enzymes, as are many secondary metabolites, pigments, and antibiotics, and other mycotoxins such as fusarin and aflatoxin, as well. Polyketide enzymes join carboxylic acids to make long carbon chains. Fumonisin synthase is encoded by the FUM1 gene and several other genes in the FUM1 gene cluster. The procedure resembles human fatty acid production, and both fatty acid synthases and PK contain similar domains: the acetyl carrier protein, acyl- or malonyl transferase, and the keto-reductase domain. Due to their structural similarity, fumonisins are thought to inhibit the enzyme ceramide synthase, catalyzing the reaction from sphinganine fatty acyl-CoA (acyl-coenzyme A) to ceramide, and toxicity is thought to result from the accumulation of sphinganine in cells. (Merrill et al., 1993, Schmelz et al., 1998).

Ceramides are components of sphingomyelin, which makes up the myelin in nerve cell axons and is part of the cell membrane lipid bilayer. Hence, fumonisins have caused symptoms in the central nervous system, mainly in horses and mules, causing a condition known as equine leukoencephalomalachia, which was first reported in the US in 1900 and later in south America (1945, 1985-90), China (1957 and 1981), South Africa (1972,1976,1981) and Spain (1996). The horses and mules suffered from symptoms such as loss of coordination, tremors, paralysis, and convulsions. Pathological studies revealed hemorrhaging and liquefaction of brain white matter. The condition was ultimately lethal for thousands of horses and occurred after feeding with moldy maize that was infected with *F. verticilloides* and contained high levels of fumonisin (Desjardins, 2006). Fumonisins have also caused health effects in humans and other animal species, such as pulmonary edema, cyanosis, and death in more than 1000 pigs in 1989 in Iowa, US. They have been associated with human esophageal cancer in the Transkent area of South Africa since 1955, and with an increased number of neural tube defects (spina bifida) along the Rio-Grande river area in Mexico in 1990-91 (Desjardins, 2006).

Fumonisins are not produced by trichothecene producers, but are commonly produced among *F. verticilloides, F. proliferatum,* and the *Gibberella fujikuroi* species complex (Desjardins, 2006).

2.3.2 Trichothecenes

Many fungal genera, such as *Fusarium, Stachybotrys,* and *Cephaosporium,* are capable of producing thrichothecenes (McCormick, 2011). Trichothecenes are tricyclic sesquiterpenes ($C_{15}H_{24}$), with a C-12,-13 epoxide ring (Desjardins, 2006). Sesquiterpenes are composed of 3 isoprene units [C_5H_8, CH_2=C(CH_3)-CH=CH_2]. Epoxides have an oxygen molecule between two carbon atoms and are generally electrophilic, carcinogenic compounds. All trichothecenes are derived

from trichodiene, formed by the enzyme trichodiene synthase from farnesyl pyrophosphate. The first *Fusarium*-produced trichothecene discovered was diacetoxyscirpenol, isolated from *F. scirpi* in 1961. Subsequently, more than 200 trichothecenes have been isolated (Desjardins, 2006).

Trichothecenes are divided into 4 groups, types A to D, mainly according to C-8 substitutions. Type A trichothecenes, (T-2/HT-2 toxins) have a hydroxyl or an ester (O-alkyl, e.g. methyl (-O–CH_3)) at position C-8, whereas type B (DON, nivalenol) has a keto group (C=O). Type C has a C-7-8 epoxide group. Type D differs from the others, as it has an additional ring structure between C-4 and C-15 (McCormick, 2011). Type B trichothecenes are subdivided even further into two distinct chemotypes: producers of DON and its acetylated forms 3-ADON and 15-ADON, and producers of nivalenol and 4-ANIV. Northern *F. graminearum* strains prefer the production of 3-ADON, rather than 15-ADON, in contrast to the southern strains (Yli-Mattila, 2010).

Trichothecenes act by inhibiting the protein synthesis of the host, and the epoxide ring is needed for protein synthesis inhibition. (Desphande, 2002). Trichothecenes bind to the 60S ribosomal unit, hence inhibiting the action of the enzyme peptidyltransferase, forming the peptide bond between the amino acids at the aminoacyl tRNA site (A site) and the peptidyl tRNA site (P site) on the ribosomes. (Rotter, 1996)

The most important trichothecenes associated with human toxic outbreaks are T-2 /HT-2 toxin, nivalenol (NIV), and DON. Trichothecenes are the most studied *Fusarium* mycotoxin group, as they have been associated with serious human and animal mycotoxicoses. Cases of a condition called alimentary toxic aleukia were reported in Kazakhstan and Russia from 1932 to 1945, causing symptoms such as nausea, vomiting, and, diarrhea, a decreased number of blood cells, especially leucocytes, skin rashes, and necrotic lesions of the GI tract in patients who had consumed overwintered, moldy grains. The grains were later discovered to have contained T-2 toxins due to *F. sporotrichoides,* and *F. poae* infections. Another human toxicosis associated with trichothecenes is “Akakabi-byo”, or “drunken bread” syndrome, caused by the mycotoxins nivalenol and DON, in Japan in 1933. Patients who had consumed red-colored, moldy grain, mainly infected with *F. graminearum,* had symptoms such as nausea, vomiting, and diarrhea, as well as, neurological manifestations such as headache, trembling, euphoria, and hallucinations (Desjardins, 2006). Toxic outbreaks in animals, due to the consumption of feed infected with *F. graminearum* and containing high levels of DON have also been reported, first in the USA in 1928. Swine, fed with moldy barley, showed symptoms such as feed refusal, vomiting and weight loss (Desjardins, 2006).

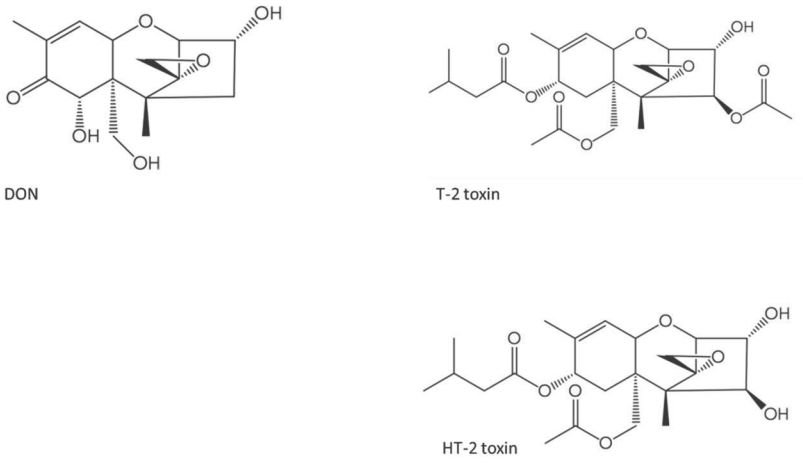


Figure 1. Structure of the type B-trichothescene deoxynivalenol (DON) and type A-trichothescenes T-2 and HT-2, kindly provided by Dr. Alexis Nathanail.

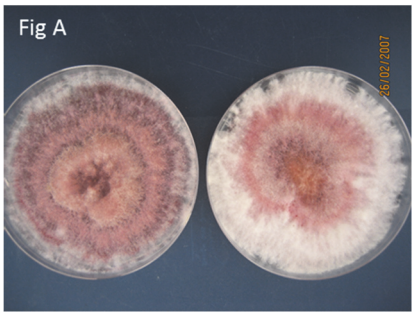
Among *Fusarium* species*, F. graminearum, F. culmorum, F. sporotrichoides, F. poae, and F. langsethiae* are known trichothecene producers (Desjardins, 2006). *F. langsethiae and F. sporotrichoides* are common T-2/HT-2 producers in oats, whereas DON and NIV are widely produced by *F. graminearum* and *F. culmorum* (Bottalico, 1998). In Finnish grain, the DON producer *F. culmorum* outnumbers *F. graminearum* infections, and *F. langsethiae* appears to be the main HT-2/T-2 producer, especially in oats (Hallikainen et al., 2009).

2.3.3 Zearalenone

Zearalenone is a resorcyclic acid lactone (cyclic ester), globally found at low levels in maize and grain (barley, oats). It is not acutely toxic, but induces estrogenic effects at higher doses (~10 μg/g feed), as it binds to estrogen receptors (ERα, ERβ) on several cell types. It causes a condition called estrogenic syndrome in farm animals (especially pigs) and rodents, which display symptoms such as enlarged mammary glands, atrophy of the ovaries or testes, infertility, and reduced offspring weight. It has also been associated with precocious puberty in children in Puerto Rico in the 1980´s. Zearalenone is mainly produced by *F. graminearum, F. culmorum*, and *F. crookwellense*, and is often coincident with trichothecenes (DON) (Desjardins, 2006).

2.3.4 Moniliformin

The *Fusarium* mycotoxin MON, is produced by a number of *Fusarium* species, most belonging to the sexual state (teleomorph) *Gibberellae* (Schütt et al., 1998) (Fig 2A). It is a sodium or potassium salt of cyclobutane 1-hydroxycyclobut-3, 4-dione (Springer et al., 1974)(Fig 2B).



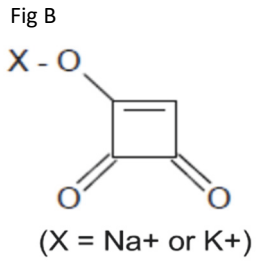


Figure 2 A *Fusarium avenaceum*, producing monilifomin, growing on potato dextrose agar.

Figure 2 B Moniliformin, a sodium or potassium salt of squeric acid (hydrocyclobutane dione), is formed from two acetate units by oxidation and hydration reactions. The figures were kindly provided by Dr. Päivi Parikka (Fig 2A) and Alexis Nathanail (Fig 2B).

MON has a polar nature and is hence water soluble and weakly acidic (pKa = 1.72) (Steyn et al., 1978). No enzymes or genes have been associated with MON synthesis. It is, however, formed from two acetate units by oxidation and dehydration reactions (Franck and Breipohl, 1984).

The exact mechanism of toxicity of MON is still unclear. However, studies on the effects of MON on oxidation rates of substrates involved in cell energy metabolism have shown that oxidation of pyruvate and α-ketoglutarate, in particular, but also citrate and iso-citrate were inhibited in isolated rat mitochondria by low concentrations (< 10 μM) of MON (Thiel, 1978, Pirrung and Nauhaus, 1996). Thiel demonstrated that MON inhibits the conversion of pyruvate to acetyl-CoA, and α-ketoglutarate to succinyl-CoA, and inhibits the tricarboxylic acid (TCA) cycle, and hence the energy metabolism of the cell. Gathercole et al. (1986) reported that MON reduced the activity of the enzyme pyruvate dehydrogenase and proposed that because of the structural similarity, MON competes with pyruvate for complex formation with thiamin pyrophosphate (co-factor) and hence the binding site of the enzyme. MON could therefore inhibit the incorporation of pyruvate into the TCA cycle (Gathercole et al., 1986). This could explain the respiratory stress, myocardial effects, and even the mortality of test animals caused by MON (Kriek et al., 1977, Thiel, 1978, Burka et al., 1982, Gathercole et al., 1986, Nagaraj et al., 1996, Morgan

et al., 1999, Engelhardt et al., 1989, Abbas et al., 1990). MON may also interfere with the polyol pathway, converting glucose to sorbitol and sorbitol to fructose, by the inhibition of aldose reductase (Deruiter et al., 1993). Moreover, MON inhibits the action of glutathione peroxidase and glutathione reductase, resulting in elevated levels of free radicals, which can oxidize unsaturated lipids in biological membranes and cause changes in permeability (Chen et al., 1990).

Even though MON has not caused any natural mycotoxicoses in animals or humans, it has shown toxicity in experimental animal studies. MON was first described in 1973 by Richard Cole, who isolated it from the seeds of corn plants suffering from leaf blight caused by the isolate *F. moniliforme Sheldon*. Cole et al. (1973) found this purified water-soluble molecule to be toxic to 1-day-old cockerels with an LD_{50} of 4.0 mg/kg b.w., causing death within 24 h. High doses (40 mg/kg b.w.) caused death in only 45 min. In cockerels exposed to ≥ 6.25 mg MON that survived for more than 2 hours, hemorrhaging was observed in the proventriculus, the small and large intestine, and the skin. However, high doses, that had caused death in less than 2 h, did not give rise to any histological changes. Furthermore, Cole et al. (1973) reported phytotoxic effects on wheat, corn and tobacco plants, with inter-nodal shortening, necrosis, and inter-vein chlorois following single MON doses of 200 μg/ml and 2000 μg/ml, respectively, sprayed onto seedlings.

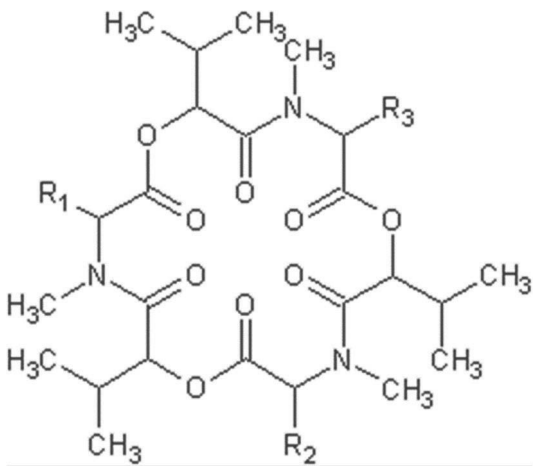
Later studies have confirmed the toxicity of MON in several animal species: ducklings, chicks, turkeys, rats, mice, and mink (Engelhardt et al., 1989, Kriek et al., 1977b, Morgan et al., 1999). In a 14-day feeding trial by Engelhardt et al. (1989), poultry (chicks, turkeys) and ducklings showed clinical signs such as dyspnea and cyanosis after receiving feed inoculated with extract of *F. moniliforme var. subglutinans* containing MON. Histological changes could only be seen in the heart and liver. Myocardial degeneration (swollen muscle fibers, vacuolation of sarcoplasm, lysis of myofibrils) and necrosis were reported, especially in the left ventricle and interventricular septum. Vacuolization, swelling, and necrosis of hepatocytes were also detected. MON has been found to cause bradycardia in broiler chickens in only 15 min post-dosing i.v. with 1 mg/kg b.w. MON, and death to 3 of the 7 tested chickens in 50 min (Nagaraj et al., 1996). Similar signs have been reported in mice, rats, and mink, and the toxicity appears to be targeted especially at the cardiac muscle. Lili et al. (1991) reported that MON reduced the mean arterial and left ventricular systolic pressure and increased ventricular diastolic pressure in Wistar rats after an i.v. MON dose of 4.5 mg/kg b.w. In the same study, a perfused dose of 10 mol/l MON reduced the contractile force of isolated Wistar rat hearts by 52 % (Lili et al., 1991). Sensitivity to MON, however, varies between animal species. The acute oral LD_{50} for MON in rats and mice is approx. 10 times higher than for avian species, as the acute oral LD_{50} for MON is 41.6 – 50 mg/kg b.w. in rats (Kriek et al., 1977b) and 47.6 mg/kg b.w. in mice (Burmeister et al., 1979) compared with 4.0 mg/kg b.w. in chickens (Cole et al., 1973) and 3.68 mg/kg b.w. in ducklings

(Kriek et al., 1977b). The mink seems to be the most sensitive species (LD_{50} = 2.2-2.8 mg/kg b.w., i.p. dosing) (Morgan et al., 1999). *In vivo* studies have confirmed that acute MON toxicity is mainly due to effects on cardiac muscle and liver, and death is induced by cardiac failure.

MON has been found at high concentrations (> 400 mg/kg , Poland) in maize (Logrieco et al., 1993) and is commonly present at low levels (0.81 and 0.95 mg/kg wheat in Finland and Norway, respectively) in grain in Northern Europe (Jestoi, 2005, Uhlig et al., 2004, Jestoi et al., 2004). It is produced by several *Fusarium* strains, including *F. proliferatum, F. nygamai, F. subglutinans, F. thapsinum, F. avenaceum, F. tricinctum, and F. fujikuroi* (Desjardins, 2006).

2.3.5 Enniatins and beauvericin

Enniatins (Enns) and beauvericin (Bea) belong to a group of cyclic hexadepsipeptides that act as ionophores, transporting alkali metal cations (K^+, Cs^+, Na^+, Rb^+, Li^+, Fr^+) and small molecules (Ca^{2+}, NH_4^+) through biological membranes (Ovchinnikov et al., 1974, Hilgenfeld and Saenger, 1982). The core structure is an 18- membered ring, with alternating N-methylated amino acids (N-Met AA, in EnnA = isoleucine, EnnB = valine, EnnC = leucin, Bea = phenylalanine) and hydroxyisovaleric acids (Hiv), joined by ester and amide bonds (Hilgenfeld and Saenger, 1982). The macromolecule resembles a disc, where the oxygen atoms of the carbonyl groups (of the ester bonds) and the nitrogen atoms (of the amide bonds) interact with the cations via ion-dipole bonds (Ovchinnikov et al., 1974, Jestoi, 2005, Hilgenfeld and Saenger, 1982). Enns and Bea interact with cations as either 1:1 complexes, where 1 cation is bound to 1 macromolecule, or by forming “sandwich structures”, where 2 Enn or Bea molecules surround 1 cation (2:1 complex), making up a lipophilic outterior (due to isopropyl, N-methyl or phenyl groups) and a polar interior, where the cation is bound. Even 3:2 complexes have been proposed to exist (Ivanov et al., 1973, Ovchinnikov et al., 1974).



	R1	R2	R3
EnnA	sec-butyl	sec-butyl	sec-butyl
EnnA1	sec-butyl	sec-butyl	iso-propyl
EnnB	iso-propyl	iso-propyl	iso-propyl
EnnB1	iso-propyl	iso-propyl	sec-butyl
Bea	phenylmethyl	phenylmethyl	phenylmethyl

Figure 3. The structure of beauvericin (Bea) and enniatins (Enn).

The 18-membered ring structure of Enns and Bea (only ester- and amide bonds of N-Met-Amino acids and Hydroxyisovaleric acid shown). In the sandwich structure, the cation (K+, Na+) is trapped between two Enn or Bea molecules by pole-dipole bonds between the oxygen molecules of ester or amide groups or mixtures of them, pointing to the polar interior of the sandwich molecule (Ovchinnikov et al., 1974). The figure was kindly provided by Dr. Marika Jestoi.

Biosynthesis of Enns is due to a non-ribosomal multienzyme, enniatin synthetase, encoded by the gene *esyn1*. Nonribosomal peptide synthesis is a multistep condensation reaction, and nonribosomal multienzymes contain three types of domains: adenylation domains for substrate activation, peptidyl carrier domains, and condensation domains, where peptide bonds are formed (Liuzzi et al., 2017). The enniatin molecule is made up of three dipeptidols that are condensed, and the linear molecule obtains its cyclic form at the cyclization cavity (Hornbogen et al., 2002). Bea is produced in a similar way by beauverisin synthetase. The ESYN1 multienzyme has both a peptide synthase function and an N-methyltransferase function, and contains two active sites/modules: the D-hydroxyisovaleric acid (D-Hiv) activation module and the L-amino acid (L-valine for EnnB) activation module, with an N-methyltransferase domain (Hornbogen et al., 2002). The two modules form a dipeptidol from D-Hiv and L-amino acid (L-val for EnnB), which

is transferred to a thiol acceptor on the enzyme. Allelic variation in the amino acid-adenylation domain determines the chemotype (Enn producer versus Enn + Bea producer) of the strain, i.e. which amino acid is to be joined (Liuzzi et al., 2017).

Enns have shown insecticidal effects against the blowfly (*Calliphora ertyhrocephala*), dosed as 1 µl injections into the haemolymph in the dorsum of the thorax. containing EnnB, EnnB1, and EnnA killed 28-60% of adult flies. EnnA and especially Bea injections were effective against mosquito larvae (*Aedes* a*egypti*), killing 37% and 86% of the larvae in 48 h, respectively (Grove and Pople, 1980). Furthermore, *F. avenaceum* hyphae and hyphal extracts, containing EnnA and A1 proved to be lethal to eastern spruce budworms at concentrations of 1% and 0.04% W/V in a meridic diet, respectively killing 56% and 24% of the larvae (Strongman et al., 1988).

Like MON, Enns have not caused clinical outbreaks in farm animals or humans. In *in vivo* studies, a low level or no toxicity has been observed. In a 28-day subchronic feeding study on Wistar rats, with a daily oral dose of 20.91 mg/kg b.w. EnnA, no adverse effects were detected (Manyes et al., 2014). The LD_{50} of fusafungine, an antibiotic mixture of Enns, which has been used as oral or nasal inhalation spray to treat upper respiratory tract infections, was as high as 350 mg/kg b.w. for mice (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2014). Fusafungine is an anti-inflammatory compound that inhibits cytokine (IL-10) and tumor necrosis factor gamma (TNF-ɣ) production of peripheral blood leukocytes and reduces the production of superoxide anion by alveolar macrophages, modulating the acute inflammatory reaction (German-Fattal, 2001). In a clinical trial, 41 patients with follicular pharyngitis received fusafungine and 40 received a placebo mixture for 7 days. Fusafungine had an anti-inflammatory effect, as 73% of fusafungine-treated patients recovered vs. 51% of the placebo group (Pandraud, 2002).

As highly lipophilic, Enns have been shown to bioaccumulate in egg yolk (Jestoi et al., 2009), and low levels have been recovered from broiler meat, liver, and skin in a poultry feeding trial (CODA-CERVA, 2011). In a study by Rodríguez-Carrasco (2016), SCID mice intraperitoneally injected with 5 mg/kg b.w. EnnB showed no toxic signs or histopathological changes. However, EnnB was recovered from all tissues (especially liver and fat) and serum, but not from urine, providing additional proof of Enn bioaccumulation. EnnB and Bea also accumulated in human cervical cancer xenograft mouse models, supporting the idea that they could act as potential anticancer drugs, as previously reported *in vitro* and *in vivo*. A combination of EnnB and sorafenib (kinase inhibitor) synergistically increased the apoptotic cell ratio in cervical cancer cell lines KB-3-1, CaSki, C4-I, and HTB-31 by increasing apoptotic cell death via the mitochondrial pathway. Moreover, *in vivo* (SCID mouse xeno-transplantation model) EnnB/sorafenib treatment reduced tumor volume and weight and increased the proportion of apoptotic cells to 50 % in the tumor tissues. Immunoblotting analyses (western blot) revealed that EnnB inhibited

the ERK signaling pathway, associated with cell proliferation, and the synergistic effects were due to changes in p38 MAPK signaling (stress stimuli) (Rodríguez-Carrasco et al., 2016). Another action of Enns (and the related mycotoxin Bea), is the modulation of the ATPase function (efflux) of two ATP-binding cassette (ABC) transporters (ABCB1 and ABCG2) involved in multidrug (e.g. cancer drug and antibiotics) resistance. This could be an advantage in drug therapy, as Enns may prevent drug efflux out of cells. The ATP-driven efflux pumps in the intestinal epithelial cells, form a barrier against drugs by pumping them back to the gut lumen. Enn and Bea exposure inhibited the efflux of pheophorbide (PhA) and rhodamin 123 in lung carcinoma A549 cells and leukemia HL60/Vinc cells, and led to their accumulation in the cells by inhibiting ABCB1 and ABCG2 efflux pump function (Dornetshuber et al., 2009b). In *Candida albicans*, Enns also inhibited the efflux pump CDR1 associated with fluconazole resistance (Lee et al., 2001). Enns may thereby act as chemosensitizers and enhance the absorbance of drugs (e.g. cancer drugs and antibiotics) from the intestine.

Despite their low toxicity *in vivo*, Enns have shown toxicity *in vitro* in several cell lines. The toxic response is thought to be due to the cation-selective sandwich formation and transport across biological membranes, resulting in altered ion concentrations in the cell (Hilgenfeld and Saenger, 1982, Hornbogen et al., 2002, Ovchinnikov et al., 1974). Enns caused lysosomal disruption in Caco-2 cells (Ivanova et al., 2012), lysosomal damage in RAW 267.4 macrophages, and cell-cycle arrest in the G0/G1 phase of the cell cycle (Devreese et al., 2013, Dornetshuber et al., 2007, Gammelsrud et al., 2012), and reduced the endocytic capacity of macrophages exposed during maturation (Ficheux et al., 2013). Anton Tonshin reported mitochondrial dysfunction and a loss of mitochondrial membrane potential in isolated rat liver mitochondria, neural cells (Paju), murine insulinoma cells, and boar sperm cells, which might affect cellular energy production (Tonshin et al., 2010). Enns also altered cell signaling by binding calmodulin and inhibiting calmodulin dependent 3,5 - cyclic nucleotide phosphodiesterase, hydrolyzing cyclic-AMP and cyclic-GMP (the second messengers in cells), and transmitting the signal from extracellular molecules, e.g. hormones, glucagon, and adrenaline, into the cell (Mereish et al., 1990). MAPK (p38) induction by EnnB, leading to increased cell survival due to the stress response, was also reported by Dornetshuber-Fleiss et al. (2014). Enns have additionally induced apoptosis in the H4IIE hepatocyte cell line by increasing caspase 3/7 activity and causing nuclear fragmentation (Wätjen et al., 2009). However, EnnB was reported to cause cell death by necrotic cell death in Caco-2 cells, rather than inducing apoptosis (Ivanova et al., 2012).

Enns are seldom produced among trichothecene-producing strains, but are produced by species of the *F. oxysporum* species complex. Common Enn producers include *F. avenaceum, F. proliferatum, F. tricinctum, and F. chlamydosporum* (Desjardins, 2006).

3. AIMS OF THE STUDY

Several fungal species infecting agricultural plants, including most *Fusarium* species, produce secondary metabolites, mycotoxins, some of which have serious consequences for animal productivity and animal and human health. As mycotoxins are in practice impossible to eradicate from food and feed, their toxic effects and safe consuming levels are of most importance. *Fusarium* mycotoxins MON and Enns are common findings in Scandinavian grain and as their toxic impact is still unclear no TDI or maximum levels have been established.

This dissertation study formed a part of the Akatox project, funded by the Academy of Finland. The aims of the study were to provide further insights into the toxicity of Enns and MON and into the mechanism of action of EnnB.

1. *In vitro* toxicity of both mycotoxins EnnB and MON was studied with an emphasis on cellular energy metabolism, cell proliferation, and apoptotic cell death in mouse embryo fibroblast Balb 3T3 cells and human hepatocellular carcinoma HepG2 cells.

2. EnnB was further assessed to give an idea of the toxic mechanisms of action, monitored at the gene expression level in rat primary hepatocytes.

3. The acute oral toxicity and repeated dose oral toxicity of MON in rats was assessed using OECD Guidelines 423 and 407, respectively. The acute oral toxicity study was performed to confirm the levels of toxicity (LD_{50} level), clinical signs and target organs of MON and to help in determining the exposure levels in the following subacute oral toxicity study. The subacute oral toxicity study aimed at defining the possible toxic outcome of longer-term exposure and to propose a lowest adverse effect level (LOAEL) for MON. Furthermore, in both studies, the excretion kinetics of MON was monitored.

4. MATERIALS AND METHODS

4.1 *In vitro* toxicity of EnnB and MON

The mouse fibroblast Balb 3T3 cell line, clone A31 (CCL-163, ATCC, USA), sensitive to xenobiotics and the human hepatocellular carcinoma HepG2 cell line (HB-8065, ATCC, USA), commonly used in toxicity tests, were chosen for the bioassays to monitor the various cytotoxic effects of EnnB and MON, which enabled a comparison of the toxic effects between different cell types. Cryopreserved rat primary hepatocytes (RTCP 10, Gibco, Life Technologies, USA) were used to examine the changes in gene expression profile implicating mechanism of action, as primary cells are considered to simulate the *in vivo* conditions better than cell lines, which are transformed and have often lost many of the properties of "parental cells", such as the production of metabolic enzymes.

The effect of EnnB on cell viability was investigated using luminometric bioassays. The ToxiLight Bioassay (LT07-217, Lonza, Rockland, USA) measures plasma membrane integrity, detecting the leakage of the cytoplasmic enzyme adenylate kinase (AK) through the cell membrane into the culture medium. AK phosphorylates ADP to form ATP, which is measured using the bioluminescent firefly luciferase reaction. The ViaLight Plus bioassay (LT37-620, Lonza, Rockland, USA) was used to study the effects of the mycotoxins on energy metabolism (cellular ATP levels). The ATP content of lysed cells is measured using the firefly enzyme luciferase, cellular ATP, and added luciferin to produce light. The emitted light intensity is linearly related to the ATP content of the cell culture. The luminescence was measured using a Victor3 Multilabel Plate Reader 1420 (Perkin Elmer, Belgium). The cells were grown in standard conditions (37 °C, 5% CO_2, humid atmosphere) on white, flat, clear-bottomed, 96-well plates (Viewplate-96TC, Perkin-Elmer) at a density of 10 000 cells/well and were exposed for 24 h to different toxin concentrations in growth medium (100 µl/well) ranging from 1.5 µM to 100 µM EnnB or 250 µM to 1200 µM MON. Both experiments were performed according to the manufacturer´s protocols using the same cell samples. The data were collected from a minimum of three independent experiments, with 3 to 5 replicates.

The cytotoxic effects of EnnB and MON on cell proliferation were investigated by monitoring changes in the levels of DNA synthesis, using a chemiluminescent cell proliferation ELISA immunoassay (Ref 1166915001, Roche Diagnostics GmbH, Mannheim, Germany). This assay measures the quantity of BrdU (bromodeoxyuridine)-labeled DNA precursors, incorporated into newly synthesized DNA instead of thymidine by dividing cells. The amount of synthesized DNA is detected by a quantitative cellular enzyme immunoassay, using monoclonal

antibodies against BrdU. The chemiluminescence (rlu/sec), produced by luminol, correlates with the level of DNA synthesis in the cell. Balb 3T3 cells, grown in standard conditions and plated on black, flat, 96-well plates (CellBIND, Corning, USA) at a density of 3000 cells/well, were exposed to 3-12 μM EnnB or 100 -1000 μM MON in growth medium for 24 h . HepG2 cells were only exposed to 1-10μM EnnB for 24 h in standard conditions. The test was performed according to the manufacturer´s instructions.

The last endpoint, apoptotic cell death, was studied by means of fluorescent staining of cells and flow cytometric analyses. In the early stages of apoptosis, cells translocate phosphatidylserine (PS) from the inner surface of the plasma membrane to the cell surface. PS on the outside of the plasma membrane was detected by staining cells with FITC- labeled Annexin V protein (Annexin V-FITC / PI double-staining kit, K101-100, Biovision). The kit includes Annexin V-FITC for detecting apoptotic cells and propidium iodide (PI), for staining the nuclei of necrotic cells. Intact cells remain unstained. Balb 3T3 cells, were cultured in standard conditions on 6-well plates (CatNo140685, Nunc), 450 000 cells/well, and were exposed to 11 μM, 22 μM and 45 μM EnnB or 800 μM MON in culture medium for 24 h. As a positive control, camptothecin concentrations of 10 μM and 20 μM were used, and the growth medium served as a negative control. The proportion of pre-apoptotic cells, necrotic cells and intact cells was measured by flow cytometric analysis using a BD FACSAria II flow cytometer (Becton, Dickinson and Company) and BD FACSDiva software.

Gene expression studies

Rat primary hepatocytes (RTCP 10, Gibco, Life Technologies, USA) were maintained and cultured according to the manufacturer´s instructions using optimized thawing medium (CHRM, APScineces, USA), plating medium (CHPM, APScineces, USA) and maintenance medium (William's E medium, without phenol red, Cat A1217601, supplemented with 4% cocktail B, Cat A13448, Gibco, Life Technologies, UK). The cells were plated at a density of 2000 viable cells/well on collagen-coated 6-well plates (Thermo Scientific, NY USA) and cultured (12 h) in standard conditions in order to attach to the collagen surface. The cells were then exposed for 1 and 4 h in standard conditions to 0 μM, 1 μM, 10 μM or 20 μM EnnB in maintenance medium. The doses represented a negative control, a low toxin level, the EC_{50}, and a high level of toxin. RNA was isolated using the RNeasy Plus Mini Kit (Cat. No. 74134, Qiagen). Genome-wide expression analysis of the exposed rat primary hepatocyte samples was carried out by the Functional Genomics Unit at the University of Helsinki in April 2013, using rat total genome RaGene 2.0 ST expression arrays (Ref 520784, Affymetrix, USA) and the Affymetrix standard protocol. 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. The Gene Ontology (GO) enrichment analysis of the transcriptome was performed using Blast2GO software (Conesa et al., 2005) and the hierarchical cluster analysis (Euclidean distance) was performed using the TMeV Multiple Experiment Viewer 4.9.0 software (Saeed et al., 2003).

Seven genes were chosen on the basis of the microarray experiment for further expression analysis. The results of selected genes were confirmed by RT-qPCR, using KiCqStart® SYBR Green qPCR reagents and BioRad CFX96 equipment (Sigma-Aldrich). The rat SDHA gene served as a reference gene. The rat primary cells were treated as in the array experiments and the experiment was repeated twice. Three technical repeats were used for each gene. The relative quantification was analyzed by the Pfaffl method (Bio-rad, 2006). The experiment was performed twice.

4.2 *In vivo* toxicity of MON

A single-dose, 14-days acute oral toxicity study was carried out according to OECD Guideline 423, with some minor modifications. This method enables the classification of test compounds into different categories of toxicity and estimation of the LD_{50} for the toxins (Fig. 2 in article II). Male Sprague-Dawley rats aged 9-10 weeks were used in this study. A low dose of 5 mg/kg b.w. and a high dose of 50 mg/kg b.w. were administered to the rats by gavage, followed by clinical observations, collection of urine and fecal samples, gross necropsy and histological examination of tissue samples, and mass spectrometric excretion studies of the urine and fecal samples, analyzed using a Waters ACQUITY UPLC™ (Milford, MA, USA) system coupled to a Micromass Q-Tof Premier™ (Waters, Manchester, UK) and HILIC column.

Subacute toxicity studies on MON were performed by adapting OECD Guideline 407. Contradictorily to the OECD Guideline, no female rats were used to minimize the amount of animals. In this experiment, 5 dose groups (3, 6, 9, 12, and 15 mg/kg b.w.), each consisting of 5 Sprague-Dawley rats, were exposed to MON by gavage for 28 days. In addition, a negative control group and two satellite groups (dosed with 12 and 15 mg/kg b.w. MON) were included. The two satellite groups were kept alive for an additional 14 days to detect possible delayed toxic effects and to follow up recovery. Clinical observations were carried out twice daily and urine and fecal samples were collected at set time points following mass spectrometric excretion studies. Blood samples were collected once a week and prior to euthanasia. Analysis of blood parameters (red blood cell counts, white blood cell counts, haemoglobin,

haematocrit, platelet counts) were performed at the University of Helsinki, Faculty of Veterinary Medicine, Department of Equine and Small Animal Medicine. In addition, a luminol-amplified chemiluminescence (CL) assay was performed to study neutrophil activity. Gross necropsy and histological examination of tissue samples were performed for all test animals.

All experiments were conducted under a permit from the National Animal Experiment Board of Finland.

Table 1. Studies, compounds and models.

Compounds	**Study**	**Model of study**	**Article**
EnnB, isolated from *F. orthoceras var ennatum*, A.G. Scientific, INC, USA. The purity was demonstrated to be ≥ 90-99% (HPLC analysis).	Bioassays	Balb 3T3, clone A31 (CCL-163, ATCC, USA) Human hepatocellular carcinoma cell line HepG2 (HB-8065, ATCC, USA)	I
EnnB, isolated from *Gnomonia errabunda*, Sigma-Aldrich, USA. The purity was demonstrated to be ≥ 95% (HPLC analysis).	Gene expression studies	Cryopreserved rat primary hepatocytes (RTCP10, Gibco, Life Technologies, USA)	I
Synthetic potassium salt of MON Provided by Dr. Ilkka Kilpeläinen and Dr. Pirkko Karhunen, Dept of Chemistry, University of Helsinki.	Acute oral toxicity study	Sprague-Dawley rat (male) Harlan Laboratories Inc.	II
Synthetic potassium salt of MON Provided by Sigma-Aldrich, Bangalore, India	Repeated-dose 28-day study	Sprague-Dawley rat (male) Harlan Laboratories Inc.	III
Moniliformin sodium salt from *Fusarium proliferatum*, M5269, Sigma-Aldrich, USA. Purity was studied by HPLC analysis.	Bioassays	Balb 3T3, clone A31 (CCL-163, ATCC, USA) Human hepatocellular carcinoma cell line HepG2 (HB-8065, ATCC, USA)	unpublished data

Table 2. Methods and assays

***In vitro* Methods**	**Assay**	**Article**
Cell membrane integrity Adenylate kinase (AK) leakage from cells	ToxiLight assay LT07-217, Lonza, Rockland, USA	I
Cytotoxicity ATP production of cell lysates	ViaLight assay LT07-221, Lonza, Rockland, USA	I
Cell proliferation BrdU incorporation into DNA	BrdU Elisa, chemiluminescent, Ref 1166915001, Roche Diagnostics GmbH, Mannheim, Germany	I
Apoptosis Flowcytometer measurements	Annexin V-FITC/PI double staining kit Cat # K101-25, Biovision	I
Gene expression studies RNA isolation	RNeasy Plus Minikit, Cat. No. 74134, QIAGEN QIAshredder columns Cat. No. 79654, QIAGEN.	I
Gene expression studies Microarray method:	Rat total genome RaGene 2.0 array Ref 520784, Affymetrix, USA Ambion WT Expression Kit for Affymetrix GeneChip Whole Transcript Expression Arrays Part Number 4425209, Rev. C 09/2009, Applied Biosystems	I
RT-qPCR method RNA isolation cDNA synthesis	RNeasy plus mini kit Cat. No. 74134,Quiagen ReadyScriptTM cDNA Synthesis Mix Sigma-Aldrich Predesigned KiCqStart SYBR green primers, Sigma-Aldrich KiCqStart® SYBR Green qPCR Ready MIX for BioRad CFX Sigma-Aldrich, KCQS00	I
***In vivo* methods**		
Sprague-Dawley rat housing		II, III
Acute oral toxicity study	OECD Guideline 423	II
Repeated-dose, 28-day, oral toxicity study,	OECD Guideline 407	III
Necropsy and histopathology	Evaluation of macroscopic changes Light microscopy of haematoxylin and eosin stained, paraffin embedded tissue samples.	II, III
Excretion	UPLC-Q-Tof-MS analysis of moniliformin in urine and fecal samples	II, III
Immunotoxicity	Neutrophil activity chemiluminescence assay III	III

5. RESULTS

5.1 *In vitro* toxicity of EnnB and MON

5.1.1 Light microscopy

Light microscopy of the cell lines Balb 3T3 and HepG2 cells, exposed to concentrations of EnnB ranging from 3 μM to 60 μM, did not show clear morphological changes typical of cell death - neither apoptosis nor necrosis. Instead, the cells remained intact, and exposures to ≥ 10 μM EnnB lowered the cell density and visibly increased cell size, compared to negative control cells. Furthermore, mitotic cells (with rounded morphology) were less abundant in the EnnB exposed cell culture than in the negative control, also indicating a decrease in cell proliferation (Fig4 A and B).

Light microscopic studies also revealed contradictory effects of EnnB on rat primary hepatocytes compared to studies conducted on the cell lines (Balb 3T3 and HepG2). Even short-term and low-level (4 h, 10 μM) exposure of rat primary hepatocytes to EnnB, resulted in rounded rat primary cells with a granulated cell morphology and detachment of cells from the collagen-coated culture plates, ending in the release of cell contents in a necrotic manner (Fig. 4. C and D), indicating a clearly different outcome depending on the cell type.

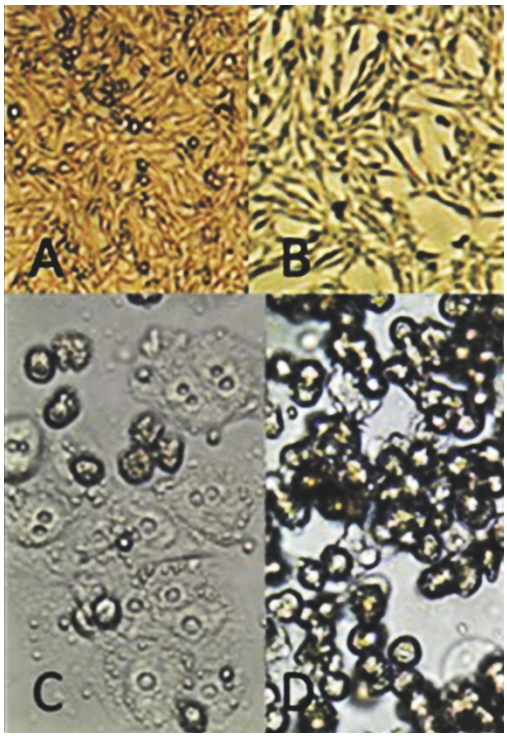


Figure 4. Light microscopic images of untreated mouse Balb 3T3 cells (A) and cells exposed to 11 µM EnnB for 24 h (B). The exposed cells remained intact, but stopped proliferating, giving rise to a less dense cell culture compared to the negative control. Untreated rat primary hepatocytes before (C) and after exposure to 10 µM EnnB for 210 min (D). Primary hepatocytes detached from the collagen-coated culture plate. 20x objective, EVOS® XL Imaging System, Thermo Fisher Scientific.

5.1.2 Cell viability: cell membrane integrity, cell energy production, and cell proliferation

In Balb 3T3 cells, no leakage of the cell membranes could be detected post exposure to EnnB or MON, tested by measuring AK levels in the growth medium (Fig. 5A and B). Cell viability was further confirmed by flow cytometric studies, which indicated that most Balb 3T3 cells remained intact after a 24-h exposure to 11 µM, 22 µM, and 45 µM EnnB, as the ratio of intact cells to damaged cells was comparable to that of unexposed control cells (86 % intact cells in negative control versus 96.6 % at the 11 µM EnnB exposure level and 84.3 % at 45 µM EnnB).

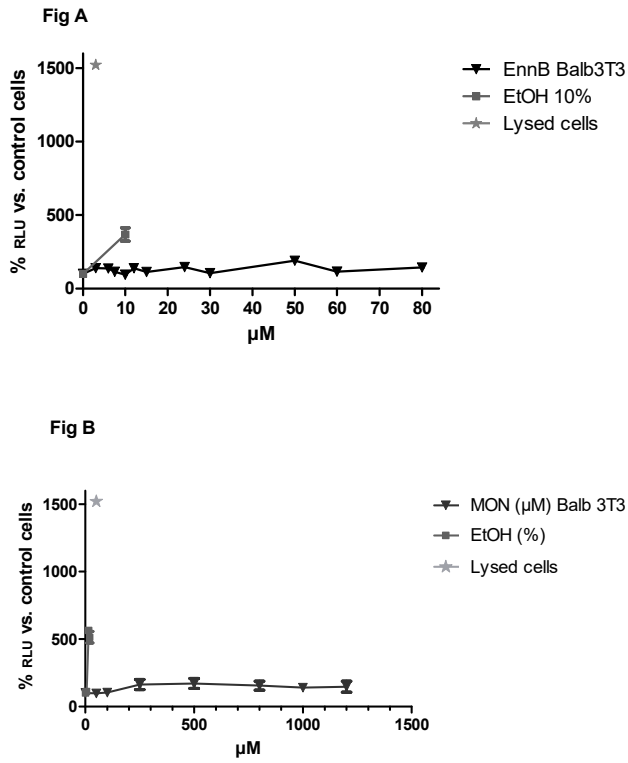


Figure 5. Plasma membrane integrity of Balb 3T3 cells, measured by adenylate kinase (AK) leakage from the cell cytoplasm, was not affected by the tested concentrations of EnnB (Fig 5A) or MON (Fig 5 B) after a 24-h exposure period. Ethanol was used as a positive control for cellular damage and unexposed cells as a negative control. A lysed cell sample represented the maximal measureable effect. Relative light units (RLU) are directly proportional to the amount of AK in growth media.

However, a clear, dose-dependent reduction in ATP production and cell proliferation was seen after a 24-h EnnB exposure in both cell lines (Balb 3T3 and HepG2), resulting in a steep decline in ATP levels (Fig 6A) and in *de novo* DNA synthesis at low concentrations (Fig 7A). In ATP production, the ED_{50} value for EnnB was 8.41 µM for Balb 3T3 cells and 2.87 µM for HepG2 cells. In DNA synthesis, the corresponding ED_{50} values were 4.24 µM (Balb 3T3 cells) and 0.50 µM (HepG2 cells). HepG2 cells proved to be more sensitive to EnnB exposure than Balb 3T3 cells, although Balb 3T3 cells are generally considered a very sensitive cell line to xenobiotic insults. However, the toxic impact in both cell lines lay within the same concentration range.

MON exposure, on the other hand, did not appear to have any effects on the cell membrane integrity (Fig 5B) or ATP production in the cell lines (Fig 6B), although very high doses of MON (ED_{50} = 800 µM) had an inhibitory effect on cell proliferation (Fig 7B).

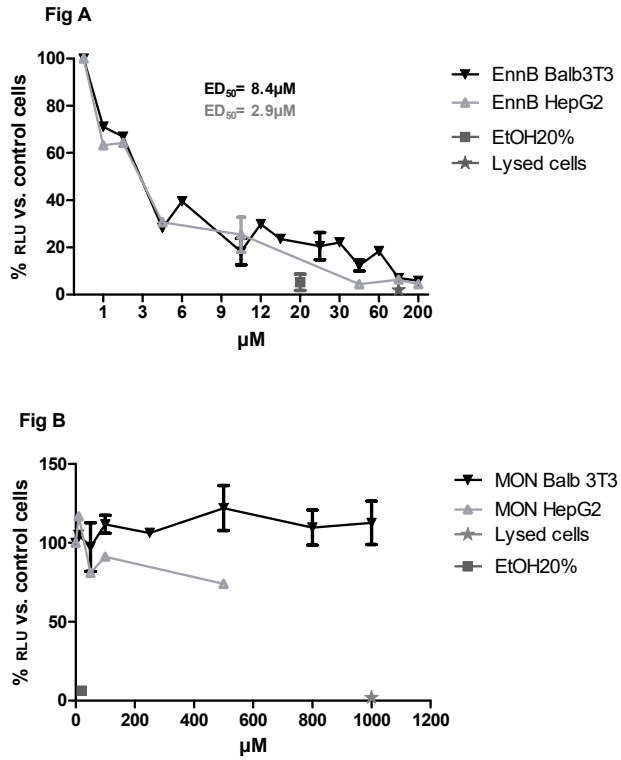


Figure 6. A 24-h EnnB exposure resulted in a drop in ATP production in Balb 3T3 cells and HepG2 cells, with ED50 values of 8.4 ± 0.76 µM and 2.9 ± 0.77 µM, respectively (Fig 6A). MON exposure did not affect cell energy production in either of the cell lines (Fig 6B). Ethanol was used as a positive control and unexposed cells as a negative control. A lysed cell sample represent the maximal measureable effect. Relative light units (RLU) are directly proportional to the amount of ATP in cell culture.

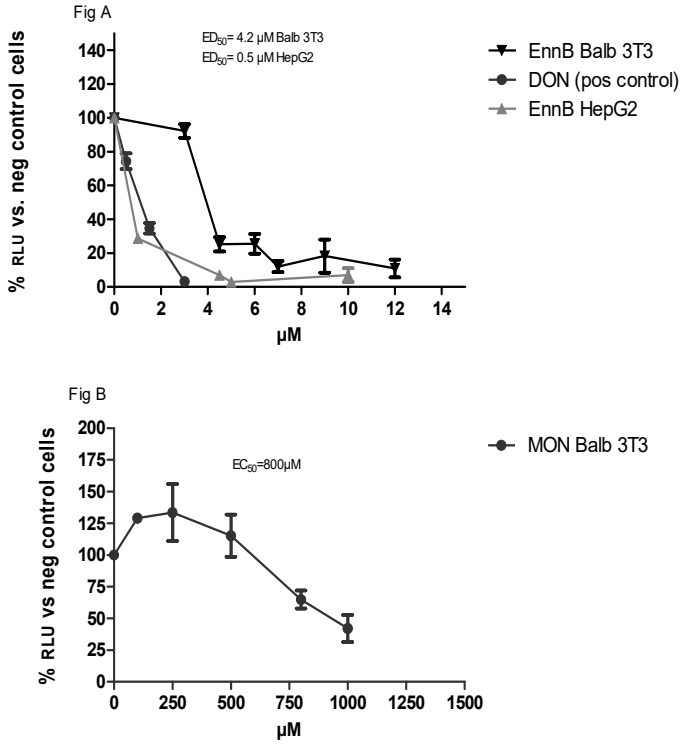


Figure 7. The effect of a 24-h EnnB exposure can be seen as a steep decline in *de novo* DNA synthesis, measured as BrdU incorporation into DNA. Relative light units (RLU) are directly proportional to the amount of BrdU incorporated in cell DNA (cells in S-phase). ED_{50} values for EnnB of 4.2 ± 0.06 µM in Balb 3T3 cells and 0.5 ± 0.09 µM in HepG2 cells could be determined (Fig 7 A). MON reduced cell proliferation, but at very high concentrations (> 500 µM), with an ED_{50} value of 800 µM in Balb 3T3 cells (Fig 7 B). Deoxynivalenol (DON) served as a positive control and untreated cells as a negative control.

5.1.3 Apoptosis

The ability of EnnB to induce apoptotic changes in Balb 3T3 cells was investigated with Annexin V-FITC/PI staining of early- and late apoptotic, as well as, necrotic Balb 3T3 cell populations followed by flow cytometric measurements (Fig. 8). A 24-h EnnB exposure moderately increased the proportion of early apoptotic cells in a dose-dependent manner from 0.3% (negative control) to 1.25% (11 µM), 2.5% (22 µM), and 4.4% (45 µM), respectively (Fig. 9). The positive control, camptothecin, had a similar pro-apoptotic effect, increasing the early apoptotic cell population to 1.85% (10 µM) and 3.0% (20 µM). A rising trend could also be seen in the late apoptotic/necrotic cell population, although it was not statistically significant. A 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 µM (data not shown). EnnB apparently had an apoptotic effect on Balb 3T3 cells, comparable to the positive control camptothesin. On the other hand, the majority of cells were unaffected (96.6% for 11 µM, 91% for 22 µM, and 84.3% for 45 µM EnnB), at levels comparable to the unexposed control cells (86%), suggesting that apoptosis is not the main event in EnnB-induced toxicity in the tested concentrations. Furthermore, gene expression studies (section 5.2) confirmed that genes central to apoptotic events (e.g. caspase-3, -8, -9, Tradd, Bid, Apaf-1, Aifm) were down regulated or unchanged in EnnB treated cells.

MON did not induce apoptosis in the tested cells, even at high exposure levels (600 µM and 1 mM), as the proportion of the early apoptotic cell population after 24 h of treatment (0.7 % and 0.4%, respectively) was close to the negative control (0.3%).

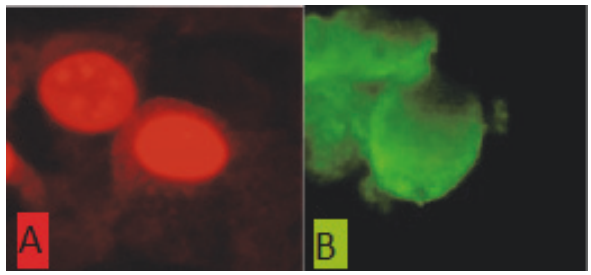


Figure 8. The Annexin-V FITC/PI staining kit (Biovision) stains the phosphatidylserine (PS) molecules on the outside of the plasma membrane of apoptotic cells green (Annexin V-FITC) and propidium iodide (PI) stains the nucleus of necrotic cells red. Intact cells remain unstained. In figure 8, apoptotic (green) (Fig 8B) and necrotic (red) (Fig 8A) Balb 3T3 cells are presented. The cells were exposed to 22 µM EnnB for 24 h and stained with the Annexin V-FITC/PI double stain kit. The proportion of apoptotic vs. necrotic cells was determined by flow cytometric measurements.

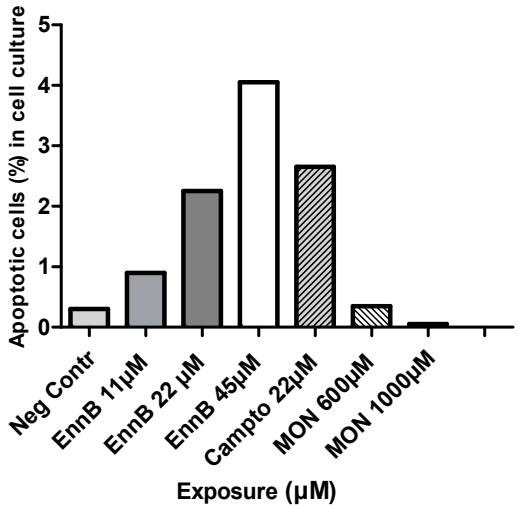


Figure 9. Proportion on apoptotic cells in Balb 3T3 cell cultures post a 24 h exposure. Exposure to enniatin B (EnnB) had a tendency to increase the early apoptotic cell populations compared to the negative control cells. EnnB caused pro-apoptotic changes at the same dose and of the same level as the positive control camptothecin. MON did not affect the apoptotic cell population compared to the negative control cells. The doses used in this test were determined from previous *in vitro* toxicity tests.

5.2 Gene expression studies on EnnB

To give an insight to the mode of action of EnnB, a whole genome gene expression study (Affymetrix gene chip array) on rat primary hepatocytes was conducted. As MON induced cytotoxic effects *in vitro* only at extremely high concentrations (in DNA synthesis, ED_{50} = 800 µM), gene expression studies with MON were not carried out.

5.2.1 Microarray data

Genome-wide microarray analysis was carried out using Affymetrix gene chip technology. Rat primary hepatocytes were exposed for 1 and 4 h in standard conditions to 0 µM, 1 µM, 10 µM, or 20 µM EnnB, representing a negative control, a low toxin level, the ED_{50}, and a high level of toxin, respectively. Exposure to the low dose of EnnB (1 µM) did not affect the overall gene expression of rat primary hepatocytes compared to the negative control, which received only fresh growth medium. On the contrary, a 4-h exposure to 10 µM or 20 µM EnnB resulted in an altered gene expression profile. The 10 µM concentration compromised the normal expression levels most, with 4.8% (985) of the genes in the rat total genome array significantly down regulated and only 0.89% (179 genes) significantly up regulated.

In total, only 5.7 % of the total rat transcriptome was significantly changed after exposure to EnnB. A limit to the log2 fold change (a "100 % "change in expression) and a q value ≤ 0.05 were considered significant.

The smallest dose (1 μM EnnB) upregulated the expression of only 2 genes: an unknown gene and Sipa1/2 (signal-induced proliferation-associated 1-like protein 2), also known as SPAR2, which is involved in GTPase signal transduction, activating the small GTPase Rap, reported to regulate spine morphology in hippocampal neurons (Spilker, 2008).

Enrichment analysis of microarray data

A gene ontology enrichment analysis (Fisher's exact test), using Blast2GO software was performed on the array data to examine whether the differently expressed genes are overrepresented with regard to specific biological processes (enriched gene ontology (GO) terms). A false discovery rate (FDR) value of 0.01 was used as a cut-off value. The most enriched down regulated genes belonged to GO terms mainly involving catabolic and metabolic processes and the GO term mitochondrion organization (GO: 0007005, FDR ≤ 8.4 E-03). These GO terms are listed in Table 1, article I. Some very modest enrichment was also seen in genes associated with apoptotic processes (apoptotic mitochondrial changes), cell signal transduction (TOR signaling pathway, ERBB Signaling pathway) MAPK-activated stress cascade, and iron-sulfur cluster assembly. Because FDR values were higher than the limit for statistical significance, which is FDR ≤ 0.01, they were not analyzed further in this work.

No statistically significant enrichment in functional categories of up regulated genes could be detected. Modest enrichment (FDR ≥0.01) was discovered in some GO terms, namely the protein kinase C-activating G-protein coupled receptor signaling pathway, which increases cytoplasmic Ca^{2+} levels in the cytosol and is important in several cell signaling pathways; negative regulation of hexokinase activity, which is involved in glycolysis and glycogen production; negative regulation of glucokinase activity, with glucokinase acting as a "glucose sensor" in glycogen production, and the GO term chromatin organization (Table 1, article I). As the data on these particular GO terms were not statistically significant, further studies were not carried out.

Cluster analysis of microarray data

As the bioassays had shown significant reduction in ATP production in the cell lines, and the GO term mitochondrial organization was statistically significantly enriched among downregulated genes, it was chosen for further studies. The GO term mitochondrial organization includes 37 down regulated genes, which are

summarized in Table 2, article I. The GO term, however, consists of more than 300 genes (both up- and down regulated in our study), involved in mitochondrial assembly, the arrangement of constituent parts, or disassembly (The EBI, 2015). Hierarchical clustering analysis (using the Euclidean distance and TMeV 4.9.0 software) was used to find the genes that "are expressed in the most similar way". The expression pattern of the low dose (1μM) was similar to the negative control, and only the high EnnB doses (10 μM and 20 μM) appeared to affect the expression patterns of the genes, compared to the negative control. The hierarchical cluster analysis revealed three clustered gene sets, two of which consisted of down-regulated genes and one of upregulated genes (Fig 10; see also Fig 7 in article I). Within the downregulated clusters, two gene families seemed to have several gene members included: the Ndufs gene family with the three genes Ndfus1, Ndufs4 and Ndufs8, and the Timm gene family with the genes Timm8b, Timm9, and Timm21 represented. The Ndufs family of genes are NADH dehydrogenase Fe-S proteins, and form parts of the iron-sulfur fragment of complex I (GeneCards, 2015) - the first enzyme in the electron transport chain in the mitochondrial inner membrane, leading to ATP production (Fig. 12). The other family of clustered, downregulated genes, the Timm gene family, are involved in the transport of proteins across the mitochondrial intermembrane space. In the original array data, a trend of downregulation was seen in many genes belonging to both families. However, only Ndufs4 gene, Timm21 and Timm8b reached the (self-set) threshold for statistical significance (log2 fold change ± 1 or q-value ≤ 0.05, Figure 8 in article I). RT-qPCR performed on a few selected genes, including Ndufs4 and Timm21, confirmed the results of the microarray, indicating downregulated relative expression if exposed (Fig. 9, article I).

In contrast to the cell proliferation bioassay results performed on the cell lines Balb3T3 and HepG2, the microarray gene expression study did not indicate any changes in the expression of the key proteins involved in cell proliferation in rat primary hepatocytes (Table 3, paper I). Cyclin-dependent kinases, 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. Furthermore, there was no enrichment among functional categories involving 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 therefore did not provide a means to efficiently study this endpoint in our experimental settings.

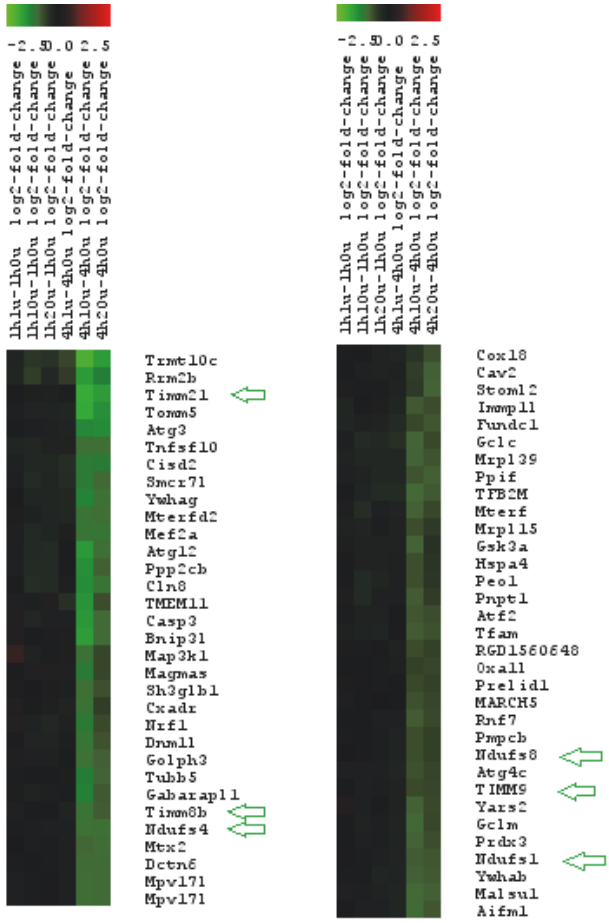


Figure 10. Cluster analysis of genes involved with the GO term mitochondrial organization, following a 4-h exposure of rat primary hepatocytes to 0 µM, 1 µM, 10 µM or 20 µM EnnB. Hierarchical cluster analysis using the Euclidean distance revealed two clusters, with downregulated expression patterns (green) in the high doses, containing several genes of the Ndufs and Timm gene families (Ndufs1, 4, 8 and Timm 8b, 21 , 9), suggesting an effect of EnnB on the function of the two gene families. Green arrows indicate the genes belonging to same clustered gene families (Ndufs or Timm).

The third endpoint of our *in vitro* studies was apoptosis. According to flow cytometric studies, EnnB slightly increased the early apoptotic cell populations of the Balb 3T3 cell line. However, in primary hepatocytes, microarray studies did not reveal any significant enrichment of functional categories (GO terms) in up- or down regulated genes, indicating that apoptosis is not associated with EnnB toxicity in rat primary hepatocytes. Only borderline enrichment among down regulated genes was observed (GO terms apoptotic mitochondrial changes, FDR ≤ 3.3 E-01, and regulation of apoptotic DNA fragmentation, FDR ≤ 6.4 E-01). Also the expression of central genes involved in apoptosis (e.g. caspase-3, -8, -9, Tradd, Bid, Apaf-1, Aifm) was down-regulated or unchanged compared to

the unexposed control in microarray studies (Table 4, article I). The statistically significant down-regulation of caspase-3 and Aifm - the main executing molecules in apoptosis - further excludes apoptosis as the mechanism of toxicity of EnnB (the downregulation can also be seen in Fig 10). In addition, 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, compared to negative control cells, suggesting the inhibition of apoptotic cascades. The data were confirmed by RT-qPCR studies on caspase genes, which did not show significant changes in expression levels compared to the unexposed control (Fig. 10, article I). Together, these results indicate that apoptotic cell death is not involved in the cytotoxicity of EnnB in rat primary hepatocytes.

5.3 *In vivo* toxicity of MON

5.3.1 Acute oral toxicity of MON

We carried out an oral acute toxicity study (a single-dose, 14-day observation period) and determined the acute toxicity levels, clinical symptoms and histopathology of the target organs of exposed animals. In addition, the excretion of MON in urine and feces was investigated. The process is a stepwise procedure, using three, 9- 10 week old rats of the same sex at each step. Acute toxicity was determined based on the mortality or moribund status of the animals (Fig 11). Our starting dose (high dose) of 50 mg/kg b.w. MON, was chosen on the basis of existing *in vivo* data

All three male rats in the high-dose group (50 mg/kg b.w.), died at 48, 60, and 83 min post-administration. Signs of toxicity were decreased activity, altered body position, respiratory and cardiovascular changes, as well as muscular weakness. Decreased activity was already observed 5-10 min post exposure. The rats were lying with flaccid tails and eyes slightly open. Unsteady movements and gasping for air were recorded. Two of the rats, showed spasmodic jumping preceding death. The rats also had a bleached eye color and cardiovascular changes (faint heart beats (palpated) in 2 rats, cardiac arrhythmia in 1 rat) in the late phase of the symptoms.

In necropsy, two of the three rats displayed macroscopic changes that included unevenly contracted hearts with a dimpled surface and marked congestion in the liver. The gastrointestinal tracts were empty. The third rat had normal contents in the rectum, but an otherwise empty gastrointestinal tract and no macroscopic changes. Microscopically, mild multifocal edema and lymphocytic infiltration in the heart muscle was seen in all three animals. No specific changes were reported in the other organs.

As the outcome of the 50 mg/kg b.w. dose was the death of all three rats, the next dose to be tested was 10 x lower, i.e. 5 mg/kg b.w. This dose was tested twice.

None of the six tested rats in this low dose group showed any clinical signs of toxicity and all gained weight until the end of the study. In necropsy, no macroscopic or microscopic changes were found and the stomach and intestinal tracts were full of normal contents.

Control animals (1 rat per dose) received 5 or 50 mg/kg b.w. of potassium chloride (KCl) in water to ensure that the clinical signs were due to MON, as a potassium salt of MON was used in this study. The control animals remained healthy throughout the study and gained weight normally. No histopathological changes were reported. According to the OECD guideline, our data indicate that MON belongs to category 2 in the Globally Harmonized System (GHS) of classification, with an LD_{50} cut-off value of 25 mg/kg b.w (Fig 11).

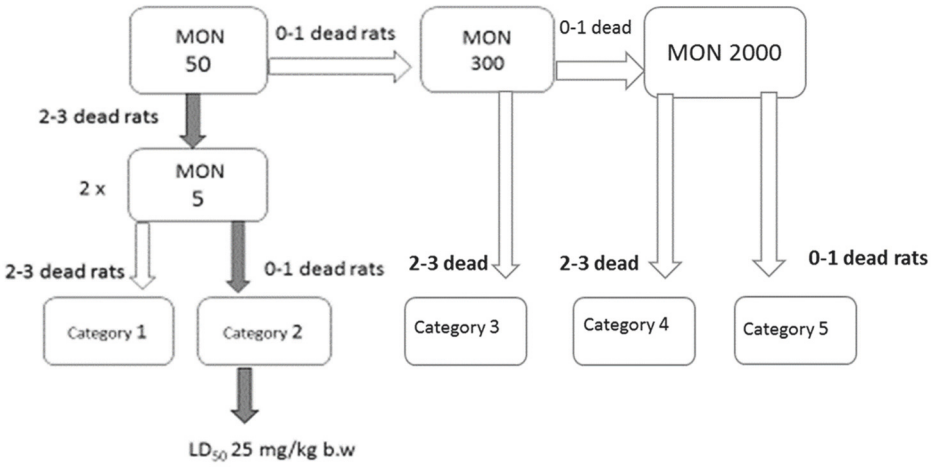


Figure 11. The stepwise approach used in the acute toxicity test. Three animals (rats) are used per step and the mortality of the starting dose determines the next fixed doses, and finally the category of toxicity of a substance. Dark gray arrows indicates the test procedure in our study on MON toxicity and the determination of an LD50 value of 25 mg/kg b.w. The figure is adapted from OECD Guideline 423.

To further assess the doses to be used in the subacute study, three rats were, respectively, dosed with 40 mg / kg b.w., 25 mg/ kg b.w, or 10 mg/kg b.w. MON. The single rats dosed with 40 and 20 mg/kg b.w., displayed similar signs as the rats tested with the high dose (50 mg/kg b.w. MON), with decreased activity, altered body position, gasping for air, and cardiovascular changes, followed by death at 75 min and 60 min, respectively, post dosing. The third rat, dosed with 10 mg/ kg b.w., showed decreased activity, altered body position, respiratory changes, trembling, and piloerection for 3 hours post-dosing. However, the rat recovered in 48 h, after which it was euthanized and a full necropsy was performed, especially to determine adverse effects on the heart tissue.

The rats dosed with 25 mg/kg b.w. and 40 mg /kg b.w. had marked congestion in the liver, but no other specific microscopic changes were present. Both rats had an otherwise empty gastrointestinal tract, but the one dosed with 40 mg/kg b.w. had normal contents in the rectum. The rat dosed with 10 mg/kg b.w. MON had acute purulent pneumonia, which was not likely to have been caused by MON, but no other macroscopic or microscopic changes could be detected.

Excretion studies on MON were performed using an in-house validated liquid chromatography- quadrupole-time-of-flight mass spectrometry (UPLC-Q-Tof-MS) method and a hydrophilic interaction chromatography column (HILIC) (section 2.5, article II). Urine and fecal samples were collected at pre-defined time points from surviving animals (5 mg/kg b.w. dose) until the end of the study. MON was rapidly excreted into urine, with the highest amounts detected only 6 hours post-dosing and most MON was excreted within the first 24 h. At 48 h post-dosing MON was no longer detectable in urine. The excretion in feces was very low (< 1% of administered dose), indicating that excretion via the biliary pathway is not significant.

5.3.2 Repeated dose oral toxicity study of MON

To further examine the toxic impact of synthetic MON (as a potassium salt) *in vivo,* and particularly the health effects of repeated oral low dose exposure in rats, we applied an adaption of OECD Guideline 407 on Sprague–Dawley rats, for 28-days. We used five dose groups (exposed to 3, 6, 9, 12 or 15 mg/kg b.w. MON by gavage), each consisting of five, 9-10 week old, male Sprague Dawley rats. We also used two satellite groups, dosed with 12 and 15 mg/kg b.w. MON, to examine possible delayed toxic responses and recovery. These rats were kept alive for 2 weeks after the completion of the study. Control rats only received filtered tap water. Three animals of each tested dose group were housed in metabolic cages, to collect urine and feces samples, and the rest of the rats were maintained in standard plastic cages. Urine and fecal samples were collected at set time points daily and blood samples once a week. Clinical assessment took place twice a day. A full post mortem necropsy was performed on all rats.

Four rats out of 40 died and all of these belonged to the two highest dose groups: two rats in the 15 mg/kg b.w., showed decreased activity and somnolence, and died of acute heart failure at days 4 and 21. These two rats had pulmonary congestion and leukostasis, but otherwise normal findings at necropsy. One of them had slightly blood stained contents in the small intestine. One rat in the 12 mg/kg b.w. group and one rat in the 15 mg/kg b.w. satellite group died or was euthanized due to respiratory distress caused by dosing by gavage and showed pulmonary congestion, but no other specific changes were found at necropsy.

One rat in the 9 mg/kg b.w. group was reported to show symptoms similar to the two rats that died of cardiac failure (somnolence, withdrawal, decreased activity) but

survived until the end of the study and had no macroscopic or microscopic changes, as was the case for all the other rats that survived (3, 6 and 9 mg/kg b.w. doses). All rats in the lower dose groups (3, 6 and most 9 mg/kg b.w. MON) remained clinically healthy and gained weight until the end of the study.

No statistically significant changes in feed or water consumption, body weight, relative organ weights (thymus and spleen weights in relation to body weight) or blood parameters could be detected in any of the tested groups or the negative control group (Fig 1, article III)

The impact of MON on the innate immunity was assessed as changes in neutrophil activity of the blood samples, using a novel chemiluminescence assay developed at the University of Turku by Drs. Atosuo and Lilius (article III). At the end of the study (day 28), the phagocytic activity of the neutrophils was significantly reduced in all dose-groups, compared to the negative control group. On average the activity was reduced to 48 ± 8 % and was not dose dependent, with a severe reduction also recorded in the lowest dose group (3 mg/kg b.w.) (Fig 2, article III). MON did not, however, cause changes in neutrophil or total leucocyte counts. Interestingly, the reduction in neutrophil activity continued in the satellite groups, even after cessation of exposure (to 28 %), suggesting sustained damage to neutrophils.

Excretion studies were performed with the same in-house validated liquid chromatography quadrupole-time-of- flight- mass spectrometry (UPLC-Q-Tof-MS) method used in the acute oral toxicity study. Urine and fecal samples were collected daily and all the urine samples of the first week and samples of the 1st, 3rd, and 6th day of the following weeks were analyzed. The excretion to urine was constant between the three first weeks and the mean excretion ranged between 29 – 31.5 %. During the 4th week, excretion was reduced by another 10% - 20.2% of the dosed MON.

Our repeated 28-day oral toxicity study indicated that MON is acutely toxic to rats and causes mortality by heart failure and symptoms of acute toxicity at doses greater than 9 mg/kg. At lower doses, MON causes no clinical signs of toxicity. However, it does affect innate immunity, reducing the phagocytic activity of neutrophils, even at the lowest tested dose. Our data allow us for the first time to propose a LOAEL value of 3 mg/kg b.w. for MON. MON is rapidly excreted and independently of the dose into urine, proven by both the acute and repeated dose study. However, only 20.2-31.5 % (mean excretion) of the dosed MON was excreted as the parent compound.

6. DISCUSSION

Food and feed safety is nowadays taken for granted in the EU and other industrialized countries due to efficient surveillance programs and legislation. However, globalization and climate change have increased the risk of exposure to harmful chemicals, particularly to natural toxins and other substances via food consumption, bringing new challenges to food safety. The aim of this study was to elucidate the toxicity and mechanism of toxicity of two *Fusarium* mycotoxins, EnnB and MON, both common findings in Finnish grain and grain products. Although Enns and MON have not been reported to be involved in any human outbreaks, harmful effects on humans and livestock cannot be ruled out, especially on a long-term and low-dose basis, as toxicity data are very limited.

In vitro, the study focused on three main aspects of toxicity: 1) cellular energy production 2) cell proliferation and 3) cell death. Furthermore, the mode of action of EnnB was pursued by means of gene expression, conducting a rat whole-genome gene expression study. Rat primary hepatocytes were used, as they are considered to better correspond to *in vivo* studies than immortalized cell lines. The *in vivo* studies focused on the oral toxicity of MON, as it has frequently been found at low levels (up to 810 μg/kg in wheat) in Finnish grain(Jestoi, 2005), and might pose an unidentified risk of a daily low-level exposure in the Finnish population. Hence, an acute oral toxicity test and a repeated-dose oral toxicity test on rats were performed, as no animal studies had been conducted using synthetic MON. The few existing animal studies had been performed using MON, purified from fungal cultures, possibly including remains of other mycotoxins, as well (Kriek et al., 1977, Abbas et al., 1990).

6.1 Toxicity and mechanism of action of EnnB

Enns have been reported to be cytotoxic in a wide variety of cell lines *in vitro* over the years. Gammelsrud et al. (2012) reported immunotoxic responses and reduced lysosomal activity in mouse RAW 267.4 macrophages. Lysosomal disruption and increased necrotic cell death in human colon adenocarcinoma Caco-2 cells (Ivanova et al., 2012) and inhibition of enzyme activity (e.g. acyl-CoA: cholesterol acyltransferase (ACAT) in rat liver microsomes have also been reported (Tomoda et al., 1992). Surprisingly, the toxicity of Enns *in vivo* has been low, with an LD_{50} value as high as 350 mg/kg b.w. for a mixture of Enns, called Fusafungine (Berdy, 1980). One explanation could be a rapid biotransformation involving CYP450 enzymes and a fast elimination *in vivo* (Fæste et al., 2011). Being highly lipophilic

compounds, Enns could, however, accumulate in the body, possibly causing toxicity over time. Indeed, Enns have been shown to bioaccumulate in eggs (Jestoi et al., 2009) and have been recovered, at low levels, from broiler meat, liver, and skin (CODA-CERVA, 2011-2012).

Enns are known to form sandwich-structured dimers, transporting cations (especially K^+) across biological membranes (Ovchinnikov et al., 1974, Kamyar et al., 2004), and the toxic impact is thought to be due to an altered ion concentration in cells. However, the exact mechanism of action is still unclear.

Our data point to an alteration in the energy metabolism of mouse Balb 3T3 fibroblasts, human hepatocellular carcinoma HepG2 cells, and in rat primary hepatocytes. ATP levels in the cell cultures of the cell lines (Balb 3T3, HepG2) were significantly reduced at relatively low (< 10 μM) concentrations, indicating toxicity. The reduction could be due to a reduced cell number in the cultures compared to the negative control cells, possibly due to a decrease in cell proliferation, as no cell lysis was observed (by AK-bioassay or light microscopy). Furthermore, the results of the gene expression study on rat primary hepatocytes indicated that EnnB alters the ATP production of cells due to effects on the ETC. The enrichment analysis of microarray data (from primary hepatocytes) revealed that genes involved in metabolic and catabolic processes were generally down regulated and enrichment was also seen in the downregulated genes belonging to the GO term linked with "mitochondrion organization". This could refer to mitochondrial dysfunction and consequently energy deprivation in cells. No enrichment among any up regulated genes was detected. Further studies on the impact of EnnB on the mitochondrion was conducted by means of hierarchical cluster analysis of the 300 genes (both up- and downregulated) belonging to the GO term "mitochondrion organization". Three gene clusters could be distinguished: two consisting of downregulated genes and one of upregulated genes. In the downregulated clusters, two gene families had several members included - the Ndufs gene family, with 3 members (Ndufs1, 4, and 8) and the Timm gene family, with two members (Timm21 and 8b). The Ndufs gene family members are involved in cellular respiration (ATP production), as they are subunits of the mitochondrial electron transport chain (ETC) protein complex I (Breuer et al., 2013, Mimaki et al., 2012). Complex I is the largest enzyme complex of the ETC, consisting of 45 subunits, and its functions include binding and oxidizing NADH and transferring (2) electrons to the next carrier of the ETC, ubiquinone (Fig. 12). The energy derived from the electron transfer is used to pump protons (H+) into the intermembrane space in mitochondria.

Complex I is divided into the N-module, oxidizing NADH, the Q-module, transferring the electrons to ubiquinone, and the P-module, translocating protons to the mitochondrial intermembrane space, producing the proton gradient used by ATP synthase in ATP production (Mimaki et al., 2012). The gene products of the Ndufs genes are NADH-dehydrogenase iron-sulfur protein subunits (Breuer et al.,

2013, Gene Cards, 2015). The iron-sulfur protein cluster of complex I and flavin mononucleatide are responsible for the transfer of the electrons to ubiquinone (Garrett and Grisham, 1999). Ndufs1 gene product makes up the largest subunit of complex I and is a part of the N-module, responsible for the binding and oxidation of NADH, whereas Ndufs8 is a part of the P-module, pumping protons into the intermembrane space (Mimaki et al., 2012). The Ndufs4 gene encodes an 18 kDa subunit, phosphorylated by intracellular cAMP, leading to the maturation and assembly of complex I (Breuer et al., 2013). Complex I deficiency patients, with mutations in the Ndufs4 gene, develop an early-childhood fatal Leigh-like syndrome, leading to lesions in different parts of the brain. In the fibroblasts of these patients, an altered mitochondrial membrane potential, NADH levels, and changes in mitochondrial morphology have been reported (Breuer et al., 2013).

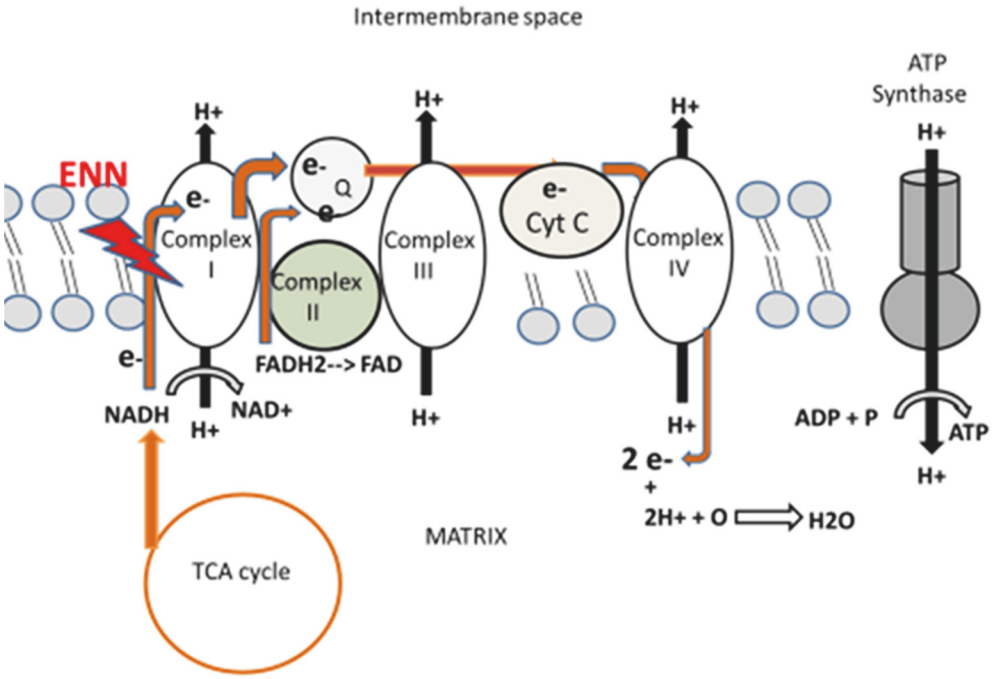


Figure 12. The gene expression data indicate that EnnB affects the electron transfer chain (ETC) of the mitochondrion, suggesting reduced cellular energy (ATP) production in vitro. The expression of some genes belonging to the Ndufs gene family, forming a part of the iron-sulfur fragment of complex I, the first enzyme of the ETC, was down regulated (Ndufs 1, 4, and 8), possibly interfering with the normal assembly and functioning of complex I and hence the electron transport chain and ATP production. Downregulation of Timm21, belonging to the other clustered gene family, might further contribute to the dysfunction of complex I, as the gene product is a translocase of the mitochondrial inner membrane and is needed for complex I assembly.

The product of the Timm21 gene, belonging to the other family of clustered genes, is a translocase of the mitochondrial inner membrane and is required for the assembly of complex I and complex IV (Gene Cards, 2015). Down regulation of Timm21 further contributes to the idea of EnnB causing adverse effects on complex I and electron transfer. The Timm 8b gene product guides membrane proteins through the intermembrane space, as they are embedded in the inner membrane of the mitochondrion (The NCBI, 2015). RT-qPCR studies performed to confirm array data findings also revealed a trend of down- regulation in both the Ndufs and Timm gene families. In particular, downregulation of the Ndufs family of genes and Timm21, could indicate that EnnB exposure may alter the function of the mitochondrial electron transport chain (ETC) due to effects on complex I function and assembly, and hence, dramatically reduce ATP production, resulting in energy depletion of the cell. This could provide an explanation for the rapid necrotic death of the rat primary hepatocytes and the drastic drop in ATP production in the cell lines (Balb 3T3, HepG2). Our results are in line with studies by Tonshin et al. (2012), who reported that 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.

Enns 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). A loss of mitochondrial membrane potential was also discovered in intact Paju- and Min-6 cells (Tonshin et al., 2010), and Caco-2 cells (Ivanova et al., 2012) causing necrotic cell death. In sperm cells, Enns caused a partial depolarization of 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).

A number of chemicals and drugs act by altering mitochondrial function. However, the mechanism of action might be very different depending on the chemical. The effects can be directly targeted at the DNA transcription of ETC complexes or at the inhibition of enzyme activity of the ETC and glycolysis or beta-oxidation. Chemicals can also act as oxidative phosphorylation uncouplers, as does the proton ionophore 2, 4-dinitrophenol, carrying protons across the inner membrane, decreasing the H+ gradient across the mitochondrial inner membrane, and hence leading to the formation of heat instead of the synthesis of ATP (Modica-Napolitano et al., 2003). Enhancement of free radical production might lead to imbalanced antioxidant levels and damage the enzymes of the ETC and other cell components (Neustadt and Pieczenik, 2008, Modica-Napolitano et al., 2003). Oxybarbiturates (Amytal), the neuroleptics haloperidol and risperidone, as well as the pesticide rotenone, are examples of chemicals inhibiting the action of complex I, thereby reducing electron transfer and oxidative phosphorylation (ATP production) (Neustadt and Pieczenik, 2008, Alridge and Partker, 1960, Li et al., 2003). Haloperidol and risperidone

inhibit the NADH-CoQ reductase activity of complex I (Modica-Napolitano et al., 2003), whereas rotenone inhibition of complex I leads to elevated ROS production (especially superoxide).

High ROS concentrations can cause further mitochondrial damage and have also been reported to be linked to increased levels of apoptotic cell death (Li et al., 2003). However, Dornetshuber et al. (2009a), reported that Enns do not cause oxidative stress in HL-60 leukemia cells or cervix carcinoma KB 3-1 cells, and Ivanova et al. (2012), reported increased necrosis in Caco-2 cells induced by EnnB, rather than cell death by apoptosis. In fact, chemicals that only inhibit complex I function (NAHD oxidation) do not necessarily reduce ATP levels as drastically as EnnB in our study, as complex II still functions by delivering electrons from $FADH_2$ (succinate) to the ETC. Hence, The decrease in ATP levels in the cell line cultures (Balb 3T3 and HepG2) could be explained by reduced cell number and a possible effect on ATP production, indicated by studies on EnnB exposed primary hepatocytes, with an altered ATP production via effects on the ETC Complex I transcription.

EnnB has also been reported to lower the mitochondrial inner membrane potential, which could lead to dysfunction of ATP synthase (Tonshin et al., 2010, Ivanova et al., 2012). Thus, EnnB may affect the energy metabolism of the cell (mitochondria) by a) affecting ionophoric properties (cation transport), leading to disruption of the mitochondrial inner membrane potential, and b) altering the assembly and function of the ETC complex I, possibly due to reduced transcription of the Ndufs family of genes and Timm21. This could limit electron transfer and inhibit the maintenance of the proton (H+) gradient in the intermembrane space by the ETC. Together, these two effects can lead to dysfunction of ATP synthase and drastically reduce ATP levels in the cell.

Another of our findings was that EnnB exposure altered cell proliferation in the cell lines (Balb 3T3 and HepG2), possibly as a response to energy deprivation. The cells remained intact but DNA synthesis and dividing of cells ceased. In contrast, the primary hepatocytes, died via necrotic cell death already at concentrations as low as 10 μM EnnB and hence, seemed to be more sensitive. The use of higher doses (> 200μM) could have finally resulted in necrotic cell death also in the cell lines (Balb 3T3 and HepG2). One reason for the different outcome of primary hepatocytes and cell lines could be a higher energy demand of primary cells, as they still have high enzyme activity, some of which may be lost in the transformed cell line HepG2 and absent in Balb 3T3 cells. Therefore, energy deprivation by EnnB might lead to the death of primary cells following exposure to lower toxin concentrations than in cell lines.

Biotransformation involving CYP450 enzymes could provide another explanation to the differences in toxic responses of cell lines vs primary cells and *in vivo* settings. Fæste et al. (2011) discovered 12 different metabolites of EnnB (M1-M12), in exposed rat -, dog-, and human microsome preparations, resulting from oxidation

or demethylation reactions. Furthermore, the half –life of EnnB in the preparations was short, only 12-32 min. The biotransformation of EnnB was inhibited most (62%) in the human microsome preparation by blocking (phase I) P450 enzyme CYP3A4, present mainly in liver cells. As the HepG2 cell-line has been reported to have a weak P450 gene expression and activity compared to human hepatic primary cells (Gerets et al., 2012), biotransformation of EnnB would not occur effectively, hence leading to cytotoxic effects (Cell cycle arrest and reduced ATP levels) by EnnB in the cell lines. In *in vivo* settings, biotransformation reactions and elimination of EnnB from the body occurs fast and efficiently, hence, preventing toxic insults and being less toxic *in vivo* than *in vitro*. In primary cells biotransformation reactions via P450 enzymes might be effective, however, a well known fact of xenobiotic metabolism is that some of the metabolites are very reactive and can be more toxic to the target than the actual toxin itself. If reactive metabolites are formed, primary cells may not be able to eliminate them, leaving the metabolites inside the cell or in the culture medium, possibly causing some of the cytotoxic effects.

Low levels of EnnB had a strong impact on cell proliferation in Balb 3T3 and HepG2 cells (ED_{50} = 4 μM and 0.5 μM, respectively). The effect was even stronger than for ATP production, but still lay within the same concentration range. The differences might be due to the different sensitivity of the two test methods. The mitotic cell cycle is a complex and highly regulated process ending in the mitotic division of a cell into two daughter cells. Cyclin-dependent kinases and regulators are mainly responsible for the cell cycle control (For a review see Lewin, 2000). Cyclin-dependent kinases (Cdk) are made up from a catalytic kinase and a regulatory cyclin, needed for activity and substrate specificity. The different stages of the cell cycle in animal cells are controlled by different kinase subunits and cyclins. For example, Cdk4, 6–cyclin D dimers are involved in the regulation of the G1 phase, the first phase of the interphase of the cell cycle, whereas Cdk2–cyclin E or A are active in the S phase (= DNA replication) (Lewin, 2000). Our data on reduced cell proliferation are in line with the results of Gammelsrud et al. (2012), from RAW 267.4 macrophages, reporting reduced mitochondrial metabolic activity and reduced cell proliferation due to cell cycle arrest in the G0/G1 phase, with a concomitant decrease in cyclin D1 levels in exposed cells. D cyclins are short lived and their synthesis is inhibited if growth factors are not present, and low cyclin D levels might be the signal for cells to enter the G0 phase if growth conditions are altered (Lewin, 2000). Cell cycle arrest by EnnB in the G2 phase in Caco-2 cells was also reported by Ivanova et al. (2012). Hence, ATP depression due to the effects of EnnB on ETC complex I, might be the explanation to the decreased cell proliferation (DNA synthesis) in the cell lines Balb 3T3 and HepG2.

Gene expression studies on rat primary hepatocytes did not confirm any changes in the key proteins of the cell cycle compared to the negative control, except for cyclin B, which was down regulated. Cyclin B is only active during mitosis and is

extensively produced during late interphase (G2/M). No significant enrichment (GO terms) among up- or downregulated genes involving cell proliferation was detected. This result was, however, expected, as primary cells do not proliferate unless stimulated by growth factors (e.g. platelet derived growth factor) and are not a good tool to study cell proliferation. However, our study confirmed that the toxicity of high doses of EnnB (greater than 10 μM) is not due to arrest in cell proliferation in rat primary hepatocytes, but rather an event of necrotic cell death, manifested by swelling of the cells and their detachment from the cell culture plate, most likely due to energy deprivation caused by mitochondrial dysfunction (effects on complex I).

The last endpoint we focused on was programmed cell death, or apoptosis. In contrast to necrosis, apoptosis does not cause an inflammation reaction in tissues. Instead apoptotic cells undergo typical morphological changes, such as nuclear fragmentation, chromatin condensation, DNA fragmentation, and cell membrane blebbing. A number of chemicals increase apoptotic cell death, and it is not unusual that low levels of chemicals or toxins induce apoptosis, but higher doses lead to death via necrosis (see review by Lewin, 2000, and by Elmore S., 2007,). Two separate apoptotic pathways exist: the death receptor pathway and the mitochondrial pathway. Briefly, the death receptor pathway is activated as apoptosis stimulating fragment (Fas) or tumor necrosis factor (TNF) ligands binds to receptors on the cell surface and activate a series of cysteine-aspartic proteases (caspases) responsible for typical apoptotic changes in the cell. Activation of caspase-3, the main executioner caspase, finally activates DNA fragmentation factor 40 (DFF40), involved in DNA fragmentation and chromatin condensation (Widlak and Garrard, 2005). The contents of the apoptotic cells are packed in apoptotic bodies, which are phagocytosed by surrounding macrophages and parenchymal cells and degraded. An inflammatory reaction is avoided as cell contents is not released into the surroundings. Furthermore, the phagocytosing macrophages do not produce cytokines (Elmore S., 2007).

The mitochondrial pathway is triggered by certain stimuli (e.g. toxins, viruses, loss of growth factors) resulting in the formation of a mitochondrial permeability transition pore (MPT) and cytochrome C release from mitochondria, as well as SMAC proteins (second mitochondria-derived activator of caspases). Cytochrome C release also activates caspase-9 and caspase-3. The protein Aif (apoptosis inducing factor) is released from mitochondria to the nucleus and causes DNA fragmentation independent of caspases (Lewin, 2000, Elmore S., 2007).

Flow cytometric measurements of EnnB-exposed mouse fibroblast Balb 3T3 cells, revealed a slight increase in the early apoptotic cell population. The effect was comparable to the positive control camptothecin. The proportion of the pro-apoptotic cell population increased with the rising dose after 24 h and ranged from 0.3% (unexposed cells) to 4.4% (45 μM EnnB exposure). A 48-h exposure had similar effects. Furthermore, the proportion of late apoptotic or necrotic cells appeared to

increase as a function of the EnnB dose, although the change was not statistically significant. EnnB has previously been reported to induce apoptotic changes, such as caspase -3/7 activation in hepatoma H4IIE cells (Wätjen et al., 2009), caspase-7 activation in the KB-3-1 cell line, and nuclear fragmentation (Dornetshuber-Fleiss et al., 2014). However, Ivanova et al. reported necrotic cell death in Caco-2 cells rather than apoptosis (Ivanova et al., 2012). Our studies revealed a slight increase in pro-apoptotic cell populations in Balb 3T3 cells, although most cells survived.

The outcome of our gene expression studies on rat primary hepatocytes did not support apoptotic cell death. The morphological findings (swelling of cells, rupture of plasma membrane) already pointed towards necrotic cell death, and the gene expression results supported this, as no enrichment was detected among the up-regulated genes related to apoptosis. Furthermore, the only GO terms linked to apoptosis (apoptotic mitochondrial changes and regulation of apoptotic DNA fragmentation) were slightly (but not statistically significantly) enriched among down regulated genes. The central genes involved in apoptosis were not upregulated (e.g. caspase-3,-8,-9, TRADD, Bid, Apaf-1, Aifm), instead slightly down-regulated or unchanged compared to the negative control in the microarray experiment, providing further evidence against apoptotic cell death. RT-qPCR studies on selected central genes (caspase-3 and Aifm) also revealed down regulated expression. Furthermore, the c-JUN gene, involved in apoptosis inhibition via the TNF/NF-KB/IAP pathway, was marginally up-regulated (log2-fold change 0.8).

The significance of apoptotic cell death in the overall toxic response of Balb 3T3 cells to EnnB could be considered minor, as the increase in the early apoptotic cell population was less than5 % compared to the negative control and most cells survived. According to our results, cell death in primary hepatocytes was necrotic rather than apoptotic. However, the importance of apoptotic cell death in the toxic outcome of Enns propably differ between cell types, as confirmed by previous studies and according to the dose (Ivanova et al., 2012, Wätjen et al., 2009, Dornetshuber-Fleiss et al., 2014). It appears that Enns may cause both apoptosis and necrotic cell death, as shown in our study. This could be explained by the finding of Ivanova et al., (2012) who reported disruption of the lysosomal membranes in Caco-2 cells, which leads to the leakage of acidic hydrolases and cathepsins into the cytosol. Hydrolases digest cytoplasmic proteins vital to the cell and can lead to cell death by necrosis (Boya and Kroemer, 2008). Indeed, Ivanova et al. (2012) reported necrotic cell death of Caco-2 cells. However, it is possible that released cathepsins activate caspases and introduce apoptotic cell death (Boya and Kroemer, 2008).

Enns appears to introduce many different toxic manifestations in a variety of cells. High doses of Enns may cause direct toxic insult by inducing an imbalance in the ion concentration of the cytoplasm because of the ionophoric and lipophilic nature of the molecule (Ovchinnikov et al., 1974). The transport of high amounts of cations through the plasmalemma may lead to swelling and uncontrolled cell

death by necrosis. According to published data, Enns have been reported to cause lysosomal disruption (Ivanova et al., 2012), alteration of ATP-driven efflux pumps (Dornetshuber et al., 2009b) , cell cycle arrest (Dornetschuber et al., 2007, Devreese et al., 2013), a reduction in mitochondrial metabolism, the loss of membrane potential, and ATP production (Tonshin et al., 2010) , enzyme inhibition (Tomoda et al., 1992), apoptosis (Wätjen et al., 2009) and changes in calmodulin-dependent signaling (Mereish et al., 1990).

Our results indicated reduced cell proliferation and ATP production, possibly due to effects on the down regulation of central ETC complex I genes. Furthermore, EnnB slightly increased apoptotic cell death in the cell line Balb 3T3 and necrotic cell death in primary hepatocytes. Traditionally, Enn toxicity has been seen as a direct assault due to ionophoric properties, resulting in e.g. cytoplasmic ion balance or lysosomal disruption. However, reduced ATP production, due to effects on the mitochondrial inner membrane potential and ETC complex I, can account for an equally significant part of Enn toxicity, especially at lower concentrations, leading to other manifestations, such as reduced cell proliferation, cell cycle arrest, apoptosis, and necrosis.

6.2 Toxicity of MON *in vitro* and *in vivo*

MON is frequently found in grain at low concentrations (< 810 µg/ kg) in Europe, with the highest measured concentration in maize from Poland, 425 mg/kg (Logrieco et al., 1993). In a Finnish survey in 2001, 74% of all grain samples contained MON, although, at low concentrations (0 - 810 µg/kg grain), often associated with F. *avenaceum* infection (Jestoi, 2005, Jestoi et al., 2004). Hence, there is a risk of chronic human and animal exposure to low levels of MON. Despite the global and frequent distribution of MON in grain products, only a few *in vivo* studies have been conducted, and the significance of the exposure is unclear. To provide additional knowledge, we decided to examine the acute and subacute (repeated low dose) toxicity of a synthetic potassium salt of MON in male Sprague-Dawley rats, according to OECD Guidelines 423 and 407.

In this study, the acute oral toxicity and the subacute oral toxicity of MON in male Sprague-Dawley rats was studied by adapting OECD Guidelines 423 and 407. The OECD Guideline 423 toxicity study is a single-dose, 14-day acute oral toxicity study, aiming at minimizing the number of used animals, without compromising the reliability of the results. It is a stepwise procedure with the use of 3 animals of a single sex per step. The range of acute toxicity of the test substance depends on the mortality or moribund status of the animals. Both sexes are normally tested, however, in order to further reduce the number of rats used, we chose to test only males, as they were considered more sensitive than females in previous studies

by Kriek et al. (Kriek et al., 1977). This method enables the classification of test compounds into different categories of toxicity and to estimate the LD_{50} for the toxins (Fig. 2 in article II). Furthermore, the results were used to select exposure levels for a subsequent subacute toxicity study. The purpose of the subacute toxicity study (OECD Guideline 407) was to provide information on possible health effects arising from repeated-dose exposure to MON over a limited period of time (28-days). A 28-day period in rats is equivalent to 2-years in human lifetime (Agoston, 2017). The *in vitro* toxicity of MON was also assayed, mainly using the Balb 3T3 cell line, with the same end points as for EnnB – cell membrane integrity, ATP production, and cell proliferation (DNA synthesis).

Our data indicated clear acute toxicity of MON in rats, with an LD_{50} cut-off value of 25 mg/kg b.w. Additional studies that were performed to help refine the doses to be used in the subacute study, pointed towards an even lower LD_{50} value, ranging between 10-15 mg/kg b.w. Acute toxic signs (mainly cardiac and respiratory changes) were reported at doses of 25-50 mg/kg b.w. The tested low dose (5 mg/kg b.w.) did not induce clinical toxicity or histopathological changes.

The outcome of the subacute study (28-day repeated dose study) was in line with the acute study, as 2 rats of 5 in the highest dose group (15 mg/kg b.w.) died of acute heart failure and 1 rat of 5 in the 9 mg/kg b.w. dose group showed similar toxic signs, but survived the study. Low doses (3-6 mg/kg b.w.) did not cause acute clinical signs in the rats. No significant histopathological changes could be detected. In the literature, MON has been reported to cause heart muscle damage, ventricular arrhythmia and heart failure in rats (Kriek et al., 1977b, Zhao et al., 1993, Lili et al., 1991) providing support to our results. Kamyar et al. (2006) reported that MON reduced *in vitro* the contractile force of guinea pig papillary muscles, which normally prevent the mitral- and tricuspid valves from opening in the wrong direction during ventricular systole (Kamyar et al., 2006). No changes in action potentials or intracellular ion concentrations of the papillary cells were reported.

MON has been reported to reduce the energy metabolism of cells by inhibiting the action of the enzyme pyruvate dehydrogenase during glycolysis (Fig 13), hence preventing the oxidation of pyruvate to acetyl-CoA and its incorporation into the TCA cycle (Burka et al., 1982, Gathercole et al., 1986, Pirrung and Nauhaus, 1996). Burka et al. (1982) suggested that the inhibition is due to the structural similarity of MON and pyruvate, whereas Pirrung et al. (1996) proposed a reaction between the carbonyl of MON and the co-factor thiamine, as MON also inhibited other thiamine-dependent enzymes (yeast pyruvate decarboxylase, aceto-hydroxy acid synthase) (Pirrung and Nauhaus, 1996). The chemical arsenic also inhibits pyruvate dehydrogenase by binding to thiols (Samikkannu et al., 2003). MON was additionally reported to inhibit □-ketoglutarate dehydrogenase (Burka et al., 1982) of the TCA cycle (Fig 13). The energy deprivation caused by MON could explain the cardiac

manifestations, arrhythmia and death due to cardiac arrest, as cells with high energy demands would be most affected.

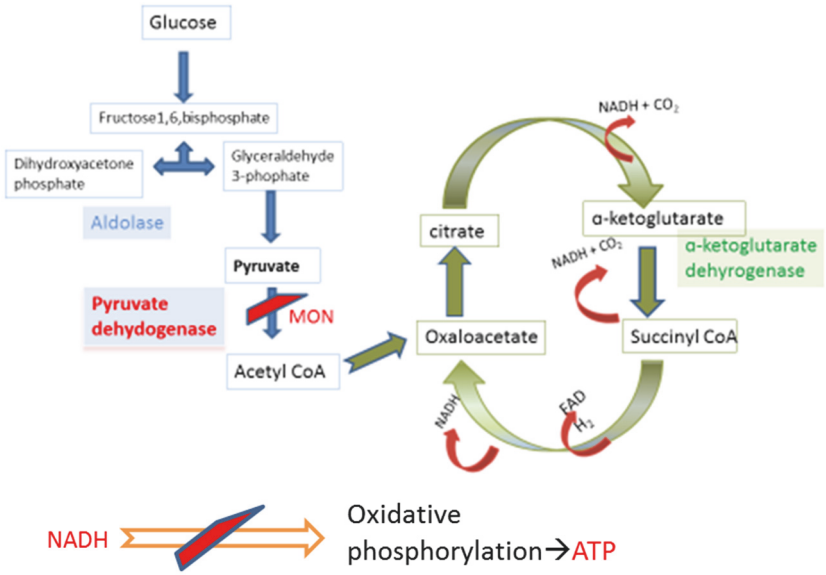


Figure 13. Moniliformin (MON) has been proposed to inhibit the oxidation of tricarboxylic acid (TCA) cycle intermediates by substituting pyruvate, leading to reduced amounts of NADH, which is normally used in oxidative phosphorylation and ATP production (Pirrung and Nauhaus, 1996, Burka et al., 1982, Gathercole et al., 1986). Hence, energy production in the cell could be altered and this would be likely to have the greatest effect on cells with a high energy demand, such as heart muscle cells.

The subacute study also demonstrated, somewhat unexpectedly, that MON alters innate immunity by reducing the phagocytosis of neutrophils by 50% in all dose groups, even in the lowest tested dose group of 3 mg/kg b.w., compared to the negative control. The reduction continued in the satellite group, even after dosing was ceased, suggesting a prolonged effect and increase in the susceptibility of the test animals to bacterial infections. Bacterial infections were, however, not detected possibly due to a clean and optimized environment (laboratory). Hence, we suggested a LOAEL of 3 mg/kg b.w. for MON. A similar reduction in phagocytic activity of neutrophils was found in myeloid leukemia patients, caused by reduced myeloperoxidase (MPO) activity (Anand et al., 2005). MPO converts superoxide into hypochlorous acid, responsible for bacterial destruction in the neutrophil phagosomes. As MON did not reduce the neutrophil or total leucocyte counts, a reduction in MPO activity could explain the reduction in phagocytic activity. However, further studies are needed to confirm the MPO activity reduction.

Excretion studies demonstrated that most of the administered MON was rapidly excreted (within 6 h) into urine, which was expected, as MON is a small hydrophilic molecule. Only traces could be detected in feces, suggesting effective absorption from the gut. However, only 42% of the dosed MON was detected in urine. The rest was probably metabolized or degraded and re-cycled, knowing that MON is synthetized from two acetate-CoA units by oxidation and dehydration reactions (Franck and Breipohl, 1984, Miller and Trenholm, 1994). The ring structure of MON may open and degrade to CO_2 (exhaled) and acetate.

Even though MON was found to be quite toxic *in vivo*, the *in vitro* findings were contradictory. MON did not induce toxic effects on the studied endpoints, cell membrane integrity or ATP production, and only very high concentrations (ED_{50} = 800 μM) reduced cell proliferation. However, toxicity of very high doses (> 2000 μM) cannot be excluded. One explanation could be that MON, being a hydrophilic compound, could not effectively enter the cell through the lipid bilayer of the cell membrane, but rather gained access into the body between cells. Gap junctions allow direct connections between the cytoplasms of two adjacent cells. Gap junction proteins, connexins, from adjacent cells form a hydrophilic channel between them, allowing ions and small molecules such as cyclic AMP (MW= 329 g/mol) and cGMP to pass from cell to cell (Kanaporis, et al.,2008). Gap junctions are especially important in cardiac muscles, being partly responsible for the co-ordinated heart beat (Smyth and Shaw, 2012, Juncueira, 2016). The molecular weight of MON is low enough to access connexons (90 g/mol) and perhaps it could somehow use gap junctions or other hydrophilic channels or receptors to enter cells, especially cardiac cells. In cell cultures, the cell density is lower and MON might not efficiently enter the cells and hence not be toxic. It is, however, clear that MON does enter the body quite effectively via the oral route and seem to affect the cardiac muscle cells most. Biotransformation reactions of MON are still unclear, hence, toxicity due to reactive metabolites cannot be ruled out.

7. CONCLUSIONS

EnnB exposure of cell lines Balb 3T3 and HepG2 induced cytotoxic effects in the form of altered cell proliferation and reduced ATP levels, at low concentrations (below 10μM). Concurrently, a slight increase in the proportion of early apoptotic cells could be seen in the cell line Balb 3T3. At the concentrations studied, most of the cells, however, survived and normal cell morphology was maintained. The toxic response to EnnB in rat primary hepatocytes was quite different, indicating necrosis rather than apoptosis. Gene expression studies in rat primary hepatocytes suggested an alteration of cell energy metabolism via effects on the function and assembly of complex I of the mitochondrial electron transfer chain (ETC). We propose that EnnB acts by causing energy deprivation due to alteration of the mitochondrial ETC, leading to a decrease in cell proliferation of cell lines and necrotic cell death in primary cells. This highlights the importance of recognizing the differences between cell lines, primary cells, organ slices and animals, especially when assessing the in vivo relevance of in vitro findings.

The outcome of the acute and subacute oral toxicity study on rats was that MON is acutely toxic to rats, with high doses inducing respiratory distress and severe cardiovascular changes, resulting in death. According to the OECD Globally Harmonized System, the toxicity of MON could be classified into category 2, with an LD_{50} cut-off value of 25 mg/ kg b.w. However, even doses between 10-25 mg/ kg b.w. caused severe toxicity. Subacute exposure to lower levels of MON (≤ 9 mg / kg b.w.) did not induce clinical signs. However, MON altered innate immunity by reducing the phagocytic activity of neutrophils, which already occurred at low exposure levels (≤3 mg/kg b.w.). Hence, we suggested a LOAEL of 3 mg/kg b.w. for MON. MON was rapidly excreted into urine (in 6-24 h) and being a polar substrate was unlikely to accumulate in the body. In contradiction to *in vivo* studies, MON did not induce toxicity *in vitro* in the cell lines Balb 3T3 and HepG2 at tested, rather high concentration levels. As *in vivo* toxicity mainly were due to cardiac changes, further studies on mechanism of toxicity, using a cardiac myocyte model would be well-grounded.

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