

**Effect of ochratoxin A on toxicity and gene expression in  
hepatocytes and kidney tubulus cells – role of dietary  
antioxidants**

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# TABLE OF CONTENTS

SUMMARY.....	-I-
ZUSAMMENFASSUNG.....	-II-
ABBREVIATIONS.....	-III -
CHAPTER ONE	
GENERAL INTRODUCTION.....	-1-
CHAPTER TWO	
EFFECT OF VITAMIN E ON CYTOCHROME P450 mRNA LEVELS IN CULTURED HEPATOCYTES (HEPG2) AND IN RAT LIVER (PUBLICATION 1).....	-14-
CHAPTER THREE	
EFFECT OF VITAMIN E AND POLYPHENOLS ON OCHRATOXIN A-INDUCED CYTOTOXICITY IN LIVER (HEPG2) CELLS (PUBLICATION 2).....	-23-
CHAPTER FOUR	
OCHRATOXIN A-INDUCED CYTOTOXICITY IN LIVER (HEPG2) CELLS: IMPACT OF SERUM CONCENTRATION, DIETARY ANTIOXIDANTS AND GLUTATHIONE- MODULATING COMPOUNDS (PUBLICATION 3).....	-29-
CHAPTER FIVE	
OCHRATOXIN A IMPAIRS NRF2 DEPENDENT GENE EXPRESSION IN PORCINE KIDNEY TUBULUS CELLS (PUBLICATION 4).....	-38-
CHAPTER SIX	
OCHRATOXIN A LOWERS mRNA LEVELS OF GENES ENCODING FOR KEY PROTEINS OF LIVER CELL METABOLISM (PUBLICATION 5).....	-47-
CHAPTER SEVEN	
GENERAL DISCUSSION.....	-71-
LEBENS LAUF.....	-80-
DANKSAGUNG.....	-81-
APPENDIX.....	-82-

## Summary

The mycotoxin ochratoxin A (OTA) exhibits a wide range of toxic effects in animals and humans including hepato-, nephro-, neuro-, and genotoxicity. The toxicity mechanisms of OTA are not yet fully understood. It has been suggested that inhibition of protein synthesis, disturbance of mitochondrial function, and oxidative stress are involved in OTA mediated toxicity.

The studies presented in this thesis aimed to counteract OTA cytotoxicity in HepG2 cells by various antioxidants such as vitamin E and dietary flavonoids, for example epigallocatechin gallate and quercetin. Furthermore, the effects of N-acetylcysteine (NAC) and buthionine sulfoximine (BSO), modulators of intracellular glutathione (GSH) levels, on OTA cytotoxicity were investigated.

Neither vitamin E nor flavonoids protected HepG2 cells from OTA induced cell death. This lack of protection by antioxidants might be due to an insufficient access of the antioxidants to the intracellular sites of reactive oxygen species (ROS) production and/or suggests a minor role for ROS in OTA mediated hepatotoxicity. In agreement, neither NAC nor BSO affected OTA induced cytotoxicity, suggesting that GSH may not be pivotally involved in the protection from acute OTA toxicity in HepG2 cells.

A second aim of this thesis was to study the gene modulatory activity of OTA in HepG2 cells using Affymetrix GeneChip® technology. OTA reduced mRNA levels of genes encoding for proteins that are centrally involved in energy metabolism, such as phosphoglycerate kinase 1, isocitrate dehydrogenase 3 (NAD<sup>+</sup>) beta, and uncoupling protein 2. All mitochondrial genes affected by OTA were at least twofold down regulated. A particularly strong down-regulation was observed for mRNA and protein levels of the insulin-like growth factor binding protein 1, which has been demonstrated to counteract p53 mediated apoptosis in mitochondria. Overall, the data presented in this thesis suggest that OTA may mediate its cytotoxicity in liver cells partly by lowering mRNA levels of genes crucial for energy homeostasis.

## **Zusammenfassung**

Das Mykotoxin Ochratoxin A (OTA), das vor allem Getreide kontaminiert, wirkt auf vielfältige Weise giftig für Mensch und Tier: OTA ist unter anderem leber-, nieren-, neuro-, und genotoxisch. Die Toxizitätsmechanismen von OTA sind bis heute nicht vollständig geklärt. Es wird vermutet, dass eine Hemmung der Proteinsynthese, eine Funktionsstörung der Mitochondrien sowie oxidativer Stress zur Toxizität von OTA beitragen.

Die experimentellen Studien, die in dieser Arbeit vorgestellt werden, hatten zum Ziel, der durch OTA induzierten Toxizität in HepG2 Leberzellen mit verschiedenen Antioxidantien wie beispielsweise Vitamin E und Flavonoiden, z.B. Epigallocatechingallat und Quercetin, entgegenzuwirken.

Darüber hinaus wurden die Effekte von N-Acetylcystein (NAC) und Buthioninsulfoximin (BSO) auf intrazelluläre Glutathion-Gehalte und OTA induzierten Zelltod systematisch untersucht. Weder Vitamin E noch die untersuchten Flavonoide schützten HepG2 Zellen signifikant vor Zelltod durch OTA. Der fehlende Schutz durch Antioxidantien könnte durch eine möglicherweise eher untergeordnete Rolle reaktiver Sauerstoffspezies (ROS) in der durch OTA verursachten Lebertoxizität oder durch einen unzureichenden Zugang der Antioxidantien zu den für die ROS Produktion entscheidenden zellulären Bildungsorten sein. Außerdem beeinflussten weder NAC noch BSO den Grad der Viabilität der mit OTA behandelten Zellen, was darauf schließen lässt, dass GSH in der Leber nicht zentral in die akute Toxizität von OTA involviert ist.

Ein weiterer Schwerpunkt dieser Arbeit bestand darin, mittels Genchip-Technologie die genregulatorische Aktivität von OTA in HepG2 Zellen zu untersuchen. Der vorherrschende Effekt von OTA war eine Erniedrigung von mRNA Spiegeln von Genen, die für Schlüsselproteine des Energiestoffwechsels kodieren, wie zum Beispiel Phosphoglyceratkinase 1, Isocitratdehydrogenase 3 (NAD<sup>+</sup>) beta und Uncoupling Protein 2. Alle untersuchten mitochondrialen Gene, die OTA beeinflusste, wurden mindestens zweifach in ihren Expressionswerten erniedrigt. Ein besonders starker Abfall des mRNA und Protein-Gehaltes wurde für das Insulin-like Growth Factor Binding Protein 1 beobachtet, für das gezeigt wurde, dass es die apoptotischen Effekte von p53 in Mitochondrien antagonisiert. Insgesamt deuten die Befunde dieser Arbeit darauf hin, dass die Zytotoxizität von OTA in Leberzellen, zumindest teilweise, durch eine Erniedrigung der mRNA Gehalte von Genen vermittelt werden wird, die für die zelluläre Energiegewinnung von essentieller Bedeutung sind.

## Abbreviations

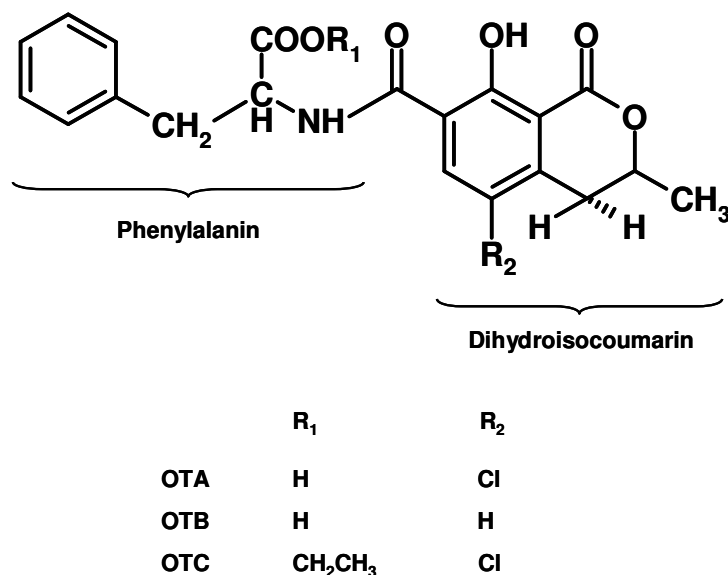
AGE	Advanced Glycation End-Product
ATP	Adenosine Triphosphate
BAK	BCL2-antagonist/killer
BEN	Balkan Endemic Nephropathy
BHT	Butylated Hydroxytoluene
BME	Bovine Mammary Epithelium
BSO	Buthionine Sulfoximine
CAT	Catechin
CYP450	Cytochrome P450
DAI	Daidzein
DNA	Deoxyribonucleic acid
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EC	Epicatechin
EGCG	Epigallocatechin Gallate
EQU	Equol
FRAP	Ferric Reducing Ability of Plasma
$\gamma$ -GCS	$\gamma$ -Glutamylcysteinyl Synthetase
GEN	Genistein
GSH	Glutathione
GST	Glutathione-S-Transferase
GSTO	Glutathione-S-Transferase omega
IARC	International Agency for Research on Cancer
IDH3B	Isocitrate Dehydrogenase 3 (NAD <sup>+</sup> ) Beta
IGFBP1	Insulin-like Growth Factor Binding Protein 1
JECFA	Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives
LD <sub>50</sub>	Median Lethal Dose
LOEL	Lowest Observed Effect Level
MDA	Malondialdehyde
NAC	N-Acetyl Cysteine
NADH	Nicotinamide Adenine Dinucleotide
NRF2	Nuclear Factor, Erythroid Derived 2, Like 2
OTA	Ochratoxin A
PFK	Phosphofructokinase
PGK1	Phosphoglycerate Kinase 1
P53	Protein 53

PPP3CB	Protein Phosphatase 3 (Formerly 2B), Catalytic Subunit, Beta Isoform (Calcineurin A Beta)
PTWI	Provisional Tolerable Weekly Intake
QUE	Quercetin
RNA	Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
ROS	Reactive Oxygen Species
ROSAC	Rosmarinic Acid
SCF	Scientific Committee on Food
SOD	Superoxide Dismutase
TEAC	Trolox-Equivalent Antioxidant Capacity
$\alpha$ -TOC-P	$\alpha$ -Tocopherol Phosphate
$\alpha$ -TTP	$\alpha$ -Tocopherol Transfer Protein
UCP2	Uncoupling Protein 2

## **CHAPTER ONE**

## General Introduction

The ochratoxins belong to the mycotoxins which comprise a large group of secondary metabolites of fungi. To date, nearly 400 mycotoxins are known and generally categorized into different groups based on their structural similarities. An overview of selected mycotoxins, their main producers, occurrence and toxicological effects, is given in Table 1. Ochratoxins, together with aflatoxins, fumonisins, trichothecenes, and zearalenones, belong to the most common types of mycotoxins that may cause severe health problems in both domestic animals and in humans. To date, three ochratoxins are known, namely ochratoxins A, B, and C (OTA, OTB, OTC), which chemically consist of a dihydroisocoumarin moiety linked to phenylalanine (Figure 1). The most toxic ochratoxin is OTA, which was first isolated in 1965 from *Aspergillus ochraceus* [1]. OTA is heat stable [2] and slowly cleared from the body [3].



**Figure 1** Chemical structure of ochratoxins

**Table 1 Overview of selected mycotoxins**

<b>Toxin</b>	<b>Main Producers</b>	<b>Main Occurrence</b>	<b>Main Effects</b>	<b>Reference</b>
Aflatoxins	<i>Aspergillus flavus</i> , <i>Aspergillus parasiticus</i>	peanuts, cereals, figs, milk (carry over)	hepatotoxic, carcinogen	[4]
Alternariol	<i>Alternaria alternata</i> , <i>Alternaria solani</i>	fruits, vegetables, tabac, sorghum, nuts	mutagene	[5]
Deoxynivalenol	<i>Fusarium culmorum</i> , <i>Fusarium graminearum</i>	cereals	induces vomiting, possibly immuno- suppressive	[6]
Fumonisin	<i>Fusarium moniliforme</i> , <i>Fusarium proliferatum</i>	mainly maize	possibly carcinogen, genotoxic in cultured cells	[7]
Ergot alkaloids	<i>Claviceps purpurea</i>	cereals	„ergotism“: convulsions, nausea, and diarrhea	[8]
<b>Ochratoxins</b>	<b><i>Aspergillus ochraceus</i>, <i>Penicillium verrucosum</i></b>	<b>cereals, maize, grapes, beans, nuts etc.</b>	<b>nephro-, hepatotoxic, cancerogen, mutagen, immunosuppressive etc.</b>	<b>[9]</b>
Patulin	<i>Penicillium claviforme</i> <i>Penicillium expansum</i> <i>Penicillium griseofulvum</i>	apples, other fruits	haemorrhagic, oedematous, cancerogen (in animal studies)	[10, 11]
Trichothecene	<i>mainly Fusarium species</i> , <i>also Cephalosporium etc.</i>	cereals	dermatotoxic, immunosuppressive, leading to nausea and diarrhea	[12, 13]
Zearalenon	<i>various Fusarium species</i> ,	cereals, nuts	estrogenic, possibly leading to infertility	[14]

OTA is produced by several *Aspergillus* and *Penicilium* species and mainly contaminates cereals [15, 16], but also legumes, oilseeds, grapes [17], beans, spices and dried fruits, to name a few [18]. Due to a carry over effect OTA can also occur in blood [19-21], meat [22] and milk [23].

Over the last decades numerous animal and cell culture studies showed that OTA exhibits a wide range of toxic effects: OTA has been associated with nephrotoxicity [24, 25], hepatotoxicity [26, 27] neurotoxicity [28], genotoxicity [29, 30], teratogenicity [31, 32], and immunosuppression [33, 34]. It has also been reported that OTA may cause cancer in rats [35], mice [36] and trout [37]. The fact that OTA toxicity differs strongly between species can partly be explained by the differences in half-lives of OTA and a species-specific metabolism [38]. Ruminants, for example, are relatively resistant to the acute toxic effects of OTA due to extensive degradation of OTA to its less toxic metabolite ochratoxin alpha by microorganisms in the rumen [39, 40]. In contrast, OTA is highly toxic in monogastric species such as rodents, poultry and pigs [9]. Oral LD<sub>50</sub> values of OTA are 20 mg/kg for young rats and 3.6 mg/kg for chickens [41]. Based on similarities to the OTA-induced porcine nephropathy [24], OTA has been linked to a severe human kidney disease called Balcan endemic nephropathy (BEN) [20]. Contrary, data of OTA intake and corresponding serum concentrations, as compared to data in laboratory rats, suggest that human OTA exposure might be too low to cause nephrotoxic lesions as observed in BEN [42]. Other studies underline the risks of possible chronic toxic effects of OTA; in this context it has been recently hypothesized that long-term exposure to low doses of OTA may even lead to an earlier onset of parkinsonism [43].

The International Agency for Research on Cancer (IARC) has classified OTA as a putative human carcinogen [44]. The toxicity of OTA in food has been evaluated by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA), and by the European Commission's Scientific Committee on Food (SCF) [45]. The JECFA established a provisional tolerable weekly intake (PTWI) of 100 ng/kg body

weight based on the **Lowest Observed Effect Level (LOEL)** for renal effects in pigs. The SCF recommended reducing OTA exposure as much as possible and preferably below a value of 5 ng/kg body weight per day.

While several mechanisms for OTA toxicity have been proposed, its precise mode of action is currently not fully understood. In the 1970's, it was shown that OTA disturbs the mitochondrial respiration in isolated rat liver mitochondria [46] and reduced the enzyme activity of NADH-tetrazolium reductase and succinate dehydrogenase in tubular cells of pigs [47]. In the 1980's, it was demonstrated that OTA inhibits protein biosynthesis by competition with phenylalanine in the phenylalanine-tRNA acylation reaction [48], and over the last two decades many studies also revealed an increase of reactive oxygen species (ROS) production and lipid peroxidation due to OTA [49-51]. It is well-known that ROS can damage lipids [52], proteins [53], and nucleic acids [54], thus possibly being a starting point of OTA induced toxicity. Therefore, one of the objectives of the present study was to test whether OTA toxicity can be counteracted by antioxidants such as vitamin E and polyphenols. Vitamin E was used, because it is the most important chain breaking lipid-soluble antioxidant *in vivo* [55]. However, also non-antioxidant properties of vitamin E including signal transduction and gene regulation, have been demonstrated over the last years [56].

We have shown in a preliminary experiment in HepG2 liver cells (Chapter two) that vitamin E did not affect mRNA levels of cytochrome P450 (CYP450) enzymes which are believed to be involved in OTA metabolism [57]. Thus vitamin E is not interfering with phase I enzymes and therefore may be a potential candidate for counteracting OTA induced cytotoxicity.

Vitamin E is not a single substance but a generic term for a group of eight natural compounds, namely  $\alpha$ -,  $\beta$ -,  $\gamma$  and  $\delta$ - tocopherols and -tocotrienols [56, 58]. Of these eight isoforms,  $\alpha$ -tocopherol exhibits the highest biological activity and is preferentially incorporated into lipoproteins and secreted into plasma facilitated by the  $\alpha$ -tocopherol transfer protein ( $\alpha$ -TTP) [59].  $\alpha$ -Tocopherol has been demonstrated to function as a potent inhibitor of lipid peroxidation by scavenging peroxy radicals [60, 61]. Furthermore, there has been evidence for a 10% reduction in OTA induced cell mortality of bovine mammary

epithelium (BME-UV1) cells by  $\alpha$ -tocopherol [62]. Therefore we have chosen  $\alpha$ -tocopherol as a test substance which may antagonize OTA-induced oxidative stress in hepatocytes.

Additionally, we tested several flavonoids for their potency to counteract OTA induced cytotoxicity in cultured HepG2 cells. Flavonoids are a large group of antioxidant polyphenols, that can be further divided into subclasses such as flavones, isoflavones, flavanols (catechins), flavonols, flavanones, anthocyanins, and proanthocyanidins [63]. We used catechin (CAT), epicatechin (EC), and epigallocatechin gallate (EGCG) since they have been described as inhibitors of radical reactions in human plasma [64]. Furthermore, the isoflavones genistein (GEN), daidzein (DAI) and equol (EQU) have been shown to attenuate oxidative DNA damage induced by advanced glycation end-products (AGEs) in rats [65]. The flavonol quercetin (QUE) has been reported to protect HepG2 cells against tert-butyl hydroperoxide mediated oxidative stress [66]. Finally, rosmarinic acid (ROSAC) has been shown to decrease OTA induced cytotoxicity in HepG2 liver cells [67]. The effect of vitamin E and polyphenols on OTA induced cytotoxicity are described in chapter three.

However, we did not only try to protect HepG2 cells from OTA induced cytotoxicity by radical scavenging antioxidants, but also by treatment of the cells with N-acetylcysteine (NAC), a precursor of the tripeptide glutathione (GSH), which is the most important water soluble cytosolic antioxidant and a substrate of many detoxifying enzymes in liver and kidney [68, 69]. As controls, we exposed the cells to buthionine sulfoximine (BSO) which is well-known to deplete cellular GSH by inhibiting the rate limiting enzyme of GSH synthesis,  $\gamma$ -glutamylcysteinyl synthetase ( $\gamma$ -GCS) [70]. In addition, we determined total GSH levels in response to BSO, NAC, and OTA treatment (Chapter four).

Since little is known regarding the transcriptional alterations underlying OTA derived toxicity, another focus of our studies was to determine the impact of OTA on gene expression and transcription factor activity. In recent studies it has been reported that OTA may affect Nrf2 signal transduction pathways [71, 72]. Nrf2 is a basic leucine zipper transcriptional activator essential for the coordinated transcriptional induction of antioxidant and xenobiotic metabolizing enzymes in the kidney including  $\gamma$ -glutamylcysteinyl synthetase ( $\gamma$ -GCS) and

glutathione-S-transferase (GST). Therefore we investigated the effect of OTA on the mRNA levels of Nrf2,  $\gamma$ -GCS, and GST (Chapter five). So far, to the best of our knowledge, large-scale gene chip studies regarding the gene regulatory activity of OTA have only been performed in rat kidney cells [73]. However, much less information is available if and to what extent OTA affects gene expression in the hepatic tissue. This brought us to perform a large-scale gene expression profile of OTA exposed HepG2 liver cells using Affymetrix GeneChip technology aiming to identify molecular targets affected by OTA (Chapter six). Finally, the last chapter (Chapter seven) is a general discussion, in which our experimental data are critically discussed in the context of the international literature.

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## **CHAPTER TWO**

## Effect of Vitamin E on Cytochrome P450 mRNA Levels in Cultured Hepatocytes (HepG2) and in Rat Liver

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**Abstract.** *Vitamin E has been described in the literature as a regulator of gene expression. The gene-regulatory activity of vitamin E with regard to genes encoding cytochrome P450 (CYP) enzymes, which play a pivotal role both in the metabolism of xenobiotics and vitamin E, has not been conclusively characterised. The objective of the current study was, therefore, to elucidate the short- and long-term effects of natural and synthetic vitamin E on CYP gene expression using Affymetrix GeneChip® technology. To this end, HepG2 cells were incubated with 0, 10, 30, 80 and 300 µM RRR- $\alpha$ -tocopheryl acetate (natural vitamin E) or all rac- $\alpha$ -tocopheryl acetate (synthetic vitamin E) for 7 days and the mRNA of CYP genes was quantified. The expression of only one (CYP20A1) of 14 CYP genes with detectable mRNA levels was dose-dependently up-regulated. No differences in gene-regulatory activity were observed between RRR- and all rac- $\alpha$ -tocopheryl acetate. To study the role of vitamin E in CYP gene expression in vivo, Fisher 344 rats were randomly assigned to either a vitamin E-enriched (60 mg/kg RRR- $\alpha$ -tocopheryl acetate) or -deficient (1.7 mg/kg RRR- $\alpha$ -tocopheryl acetate) diet for 290 days. Neither in the vitamin E-enriched, nor in the vitamin E-deficient rats, were significant changes in the liver CYP, mRNA levels observed. In conclusion, our data indicated that vitamin E does not appear to modulate cytochrome P450 mRNA expression in HepG2 cells or in rats.*

$\alpha$ -Tocopherol, the predominant form of vitamin E *in vivo*, is the most important lipid-soluble, chain-breaking anti-oxidant in human plasma (1). In the past, studies have

focussed on the ability of vitamin E to prevent diseases that are believed to have an oxidative stress component, such as atherosclerosis, Alzheimer's disease and cancer (2-4). However, more recent findings have indicated that vitamin E also has other important functions that are independent of its anti-oxidant activity, such as the regulation of gene expression (5-8).

Vitamin E is not one particular substance, but a generic term for a group of lipid-soluble, chain-breaking anti-oxidants. The eight recognized natural vitamin E compounds consist of a chromanol head substituted with a 16-carbon side chain. They can be subdivided into tocopherols, with a saturated side chain and tocotrienols, with an unsaturated side chain with three isolated double bonds. The lower case greek letters  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  are assigned as prefixes according to the number and position of methyl groups attached to the chromanol head. Because the side chain of the tocopherols features three chiral centres at positions 2, 4', and 8', that can be in either R or S configuration, eight stereoisomers for each tocopherol are possible. In nature, tocopherols exist exclusively as RRR-stereoisomers, whereas synthetic vitamin E is an equimolar mixture of all eight stereoisomers, a so-called *all racemic* (*all rac*) mixture (9).

Upon ingestion, vitamin E generally follows the absorption and transport pathways of dietary lipids in the body. The metabolic degradation of vitamin E seems to occur in the liver, involving cytochrome P450 (CYP) enzymes (10, 11).

CYP enzymes constitute a large family of membrane-bound oxidative proteins. In liver cells, CYPs are located either in the inner membrane of the mitochondria or in the endoplasmic reticulum, where they metabolize a wide variety of endogenous compounds and xenobiotics. They are, however, also present in other tissues of the body including the mucosa of the gastrointestinal tract and kidney cells. In mammals, CYP enzymes are the most important enzymes of phase I metabolism. Additionally, they play an

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important role in the biosynthesis of steroids, fatty acids and bile acids. In humans, CYP3A4 and its closely related form CYP3A5 are the most abundant forms in the liver (from 30 to 60% of total CYP, depending on genetic background, food *etc.*). CYP3A4 metabolises more than 50% of the drugs currently used for therapy (12).

The initial step in the metabolism of tocopherols and tocotrienols is the hydroxylation of the terminal methyl group of the side-chain, followed by a step-wise shortening by  $\beta$ -oxidation. The resulting water-soluble carboxy-ethylhydroxychromans (CEHCs) are excreted in the urine. Both CYP4F2 and CYP3A4 were suggested to facilitate this initial  $\omega$ -hydroxylation and their inhibition resulted in a reduced production of CEHCs in various *in vitro* systems (13, 14).

High-throughput techniques for the analysis of changes in mRNA levels are powerful tools to study the gene-regulatory activity of vitamin E. These state-of-the-art techniques have been successfully used to identify genes that are differentially expressed in response to dietary vitamin E in various tissues including liver (5), testes (6), adrenal glands (15), cortex (16) and hippocampus (7). Although some studies indicate an induction of CYP mRNA by certain vitamin E isoforms (11, 17), the role of the vitamin on the expression of CYPs has not been studied in sufficient detail.

The aim of this trial was to perform a large-scale gene-chip experiment to determine the role of vitamin E on the gene expression of CYP enzymes both *in vitro* and *in vivo*.

## Materials and Methods

**Cell culture experiments.** HepG2 cells (ATCC HB-8065) were cultured in 6 cm dishes in DMEM medium (GIBCO-Invitrogen, Basel, Switzerland) with 10% NU serum™ (Becton Dickinson, Basel, Switzerland) containing 1% penicillin/streptomycin and no detectable amounts of vitamin E (detection limit 20 nM). *RRR*- and all *rac*- $\alpha$ -tocopheryl acetate (purity: 99.0-99.5 and 98.0-99.5 weight%, respectively, DSM Nutritional Products Ltd, Kaiseraugst, Switzerland) were dissolved in 100% ethanol to prepare stock solutions. Treatment media were prepared by the addition of *RRR*- or all *rac*- $\alpha$ -tocopheryl acetate (*RRR*- $\alpha$ -Tac or all *rac*- $\alpha$ -Tac, respectively) to the basic medium at the following final concentrations: 0, 10, 30, 80 and 300  $\mu$ M. The maximal ethanol concentration in the medium was 1%. Treatment media were aliquoted and stored at  $-20^{\circ}\text{C}$ . The vitamin E treatment was performed for seven days during the logarithmic growth phase of the cells. All treatment media were exchanged for fresh media every 24 h. All treatments were performed in quadruplicate dishes.

**Animals and diets.** Sixty recently-weaned male Fisher 344 rats (Charles River, Les Oncins, France) with an initial average weight of 50-60 g were randomly assigned to either a VE-containing diet (VE+) or to a control diet deficient in vitamin E (VE-) for 290 days. The VE+ diet contained 60 mg/kg *RRR*- $\alpha$ -tocopheryl acetate (DSM Nutritional Products Ltd), which corresponds to 80 IU per kg diet. The VE-diet contained 60 mg/kg of the vehicle (16% fish

gelatine, 8% micro gel E, 1% Sipernat 50) resulting in a final dietary concentration of 1.7 mg/kg *RRR*- $\alpha$ -tocopheryl acetate. The VE- and VE+ rats consumed the diets *ad libitum* and had free access to water. The rats were maintained under standard conditions at  $22\pm 1^{\circ}\text{C}$  with 12:12 h dark:light cycles. The Laboratory Animal Care Committees of F. Hoffmann-La Roche Ltd, Basel and the Veterinary Office of Basel-Stadt, Switzerland approved all animal protocols.

Beginning from day 17 of feeding, five animals per group were euthanised every 3 months under isoflurane anaesthesia by withdrawing blood from the *vena cava*. Livers were removed, rinsed in ice-cold phosphate-buffered saline (pH 7.4) and snap-frozen in liquid nitrogen. Samples were stored at  $-80^{\circ}\text{C}$  prior to RNA extraction.

### *Vitamin E concentrations in HepG2 cells and in rat liver and plasma.*

Adherent HepG2 cells were trypsinised, collected and washed three times with PBS containing 1% bovine serum albumin. The HepG2 cells or liver tissue, respectively, was saponified in a methanolic potassium hydroxide solution. The solution was diluted in 35% ethanol and extracted with hexane/toluol.  $\alpha$ -Tocopherol was quantified by isocratic HPLC analysis using a Lichrosorb™ Si 60.5 mm, 20x4 mm pre-column coupled to a Lichrosorb™ Si 60.5 mm, 125x4 mm column (Stagroma, Reinach, Switzerland) and 3% 1,4-dioxane in n-hexane as the mobile phase (flow rate  $\sim 1.6$  ml/min, pressure 35-55 bar). Fluorescence was measured at 330 nm after excitation at 295 nm.

The plasma samples were analysed for  $\alpha$ -tocopherol using a routine HPLC method. Briefly, the plasma proteins were precipitated using ethanol and tocopherol was extracted by liquid/liquid extraction with hexane. After centrifugation, an aliquot of the organic phase was chromatographed isocratically on a normal phase HPLC system.  $\alpha$ -Tocopherol was quantified by fluorimetry (excitation at 298 nm, emission at 326 nm).

### *Total RNA extraction, cRNA preparation and Affymetrix GeneChip® hybridization.*

The cells were washed three times with PBS and lysed with RTL buffer (Qiagen, Basel, Switzerland). Total RNA isolation was performed using RNeasy mini spin columns (Qiagen) and DNase digested on the columns (RNase-Free DNase Set, Qiagen) according to the manufacturer's description. cRNA preparation and Affymetrix GeneChip® (U133A) hybridisation were performed as described (5).

### *GeneChip® microarray expression and data analysis.*

Data processing was carried out using the RACE-A analysis tool (Roche Bioinformatics, Basel, Switzerland) as previously described (5). Briefly, the arrays were normalised against the mean of the total sum of Average Difference (AvgDiff) values across all arrays used. Mean average difference values (MeanAvgDiff) were calculated as the means of one experiment performed in quadruplicate. Possible outliers were identified using the procedure of Nalimov with a 95% confidence interval. Subsequently, mean change factors (Chgf) for each individual gene were calculated among the different treatment groups and control using pairwise comparisons and statistical significance was assessed by the Student's *t*-test with prior testing for the normal distribution of the data. The analysis of the experimental data obtained upon stimulation of the HepG2 cells with *RRR*- $\alpha$ -Tac or all *rac*- $\alpha$ -Tac was performed independently from each other. Confirmation of the gene expression data by RT-PCR was omitted because, in previously published

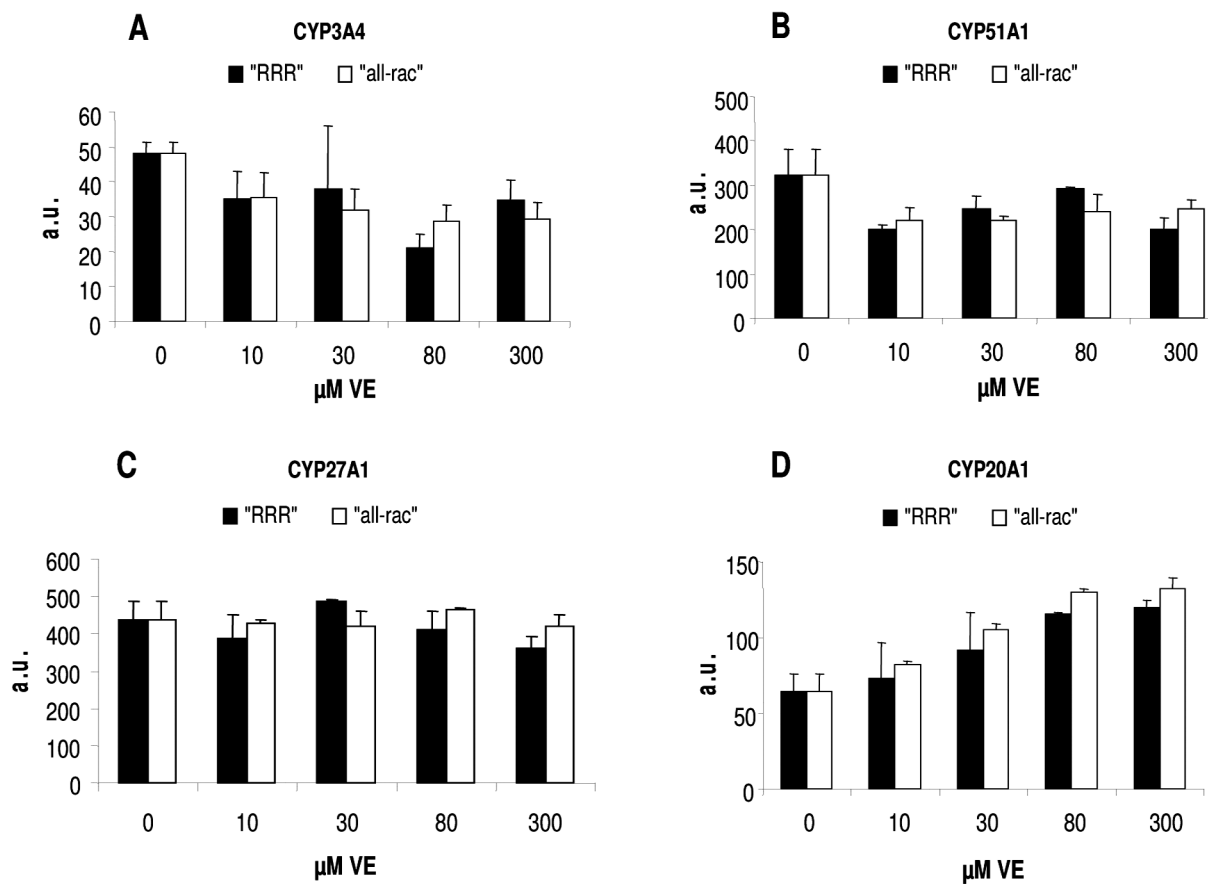


Figure 1. Cytochrome P450 mRNA levels in HepG2 cells – given in arbitrary units (a.u.) – after treatment with RRR- $\alpha$ -tocopheryl acetate ("RRR") or all rac- $\alpha$ -tocopheryl acetate ("all rac") at the concentrations given for 7 days. The data represent means  $\pm$  SD.

experiments performed on the same experimental animals, the GeneChip® data were always consistent with data confirmed by independent methods (5-7). A differential expression profile analysis between the two treatment groups was performed at four different time-points (T1=day 17, T2=day 91, T3=day 191 and T4=day 269 of feeding) over the 9-month study period in five individual liver samples.

## Results

**$\alpha$ -Tocopherol concentrations in HepG2 cells.** The incubation of HepG2 cells with media containing 0, 10, 30, 80 or 300  $\mu$ M RRR- or all rac- $\alpha$ -Tac for 7 days dose-dependently increased the intracellular content of free vitamin E. A plateau was reached at supplementation with 80  $\mu$ M vitamin E and a further increase in vitamin E did not cause higher intracellular vitamin E concentrations. There was no significant difference between the intracellular concentrations of RRR- and all rac- $\alpha$ -Tac (data not shown).

**CYP mRNA expression in vitro.** In the HepG2 cells, 14 out of the 44 CYP enzymes represented on the RNA microarray

were expressed. The highest expression levels of these 14 CYPs were observed for CYP27A1 and CYP51A1 (Figure 1). Four members of the CYP3A subfamily, namely CYP3A4 (Figure 1), CYP3A5, CYP3A7 and CYP3A43, as well as CYP4F3 and CYP4F12, showed low to moderate expression levels. CYP4F2 was only marginally expressed.

In response to vitamin E supplementation, only the mRNA levels of CYP20A1 dose-dependently increased (Figure 1). All other 13 CYPs with detectable mRNA levels showed no dose-dependency. Moreover, there was no substantial difference in CYP mRNA expression between treatment with RRR- $\alpha$ -Tac and all rac- $\alpha$ -Tac. A detailed list of the CYP mRNA levels for each vitamin E concentration is given in Table I.

**Body weight, plasma and liver vitamin E concentrations in rats.** No differences in weight gain and final body weight were observed between the rats fed VE+ and VE– diets. Rats fed the VE+ diet had significantly higher plasma levels ( $27.40 \pm 3.17$   $\mu$ mol/l, day 269) compared to the controls receiving the VE–

Table I. Cytochrome P450 mRNA levels in arbitrary units (a.u.) in HepG2 cells after treatment with RRR- $\alpha$ -tocopheryl acetate ("RRR") and all rac- $\alpha$ -tocopheryl acetate ("all rac") at the concentrations indicated for 7 days. Data represent means  $\pm$  SD.

CYP450 enzyme, HepG2	Function	VE form	mRNA expression (a.u. $\pm$ SD)				
			0	10	30	80	300 $\mu$ M VE
CYP1A2	metabolism of xenobiotics, drugs	"all rac"	45 (15)	67 (10)	56 (5)	74 (5)	63 (1)
		"RRR"	45 (15)	62 (21)	64 (12)	67 (1)	48 (9)
CYP2A6	metabolism of xenobiotics, drugs	"all rac"	72 (18)	61 (16)	54 (13)	44 (2)	43 (6)
		"RRR"	72 (18)	59 (11)	75 (3)	45 (13)	53 (2)
CYP2C9	metabolism of xenobiotics, drugs	"all rac"	66 (10)	36 (8)	34 (6)	42 (3)	35 (1)
		"RRR"	66 (10)	45 (24)	65 (6)	36 (4)	37 (1)
CYP3A4	metabolism of xenobiotics, drugs	"all rac"	48 (3)	35 (7)	31 (6)	28 (4)	29 (4)
		"RRR"	48 (3)	35 (7)	38 (17)	20 (4)	34 (5)
CYP3A5	metabolism of xenobiotics, drugs	"all rac"	36 (9)	23 (4)	23 (2)	38 (9)	24 (1)
		"RRR"	36 (9)	28 (6)	29 (6)	37 (3)	25 (4)
CYP3A7	metabolism of xenobiotics, drugs	"all rac"	38 (13)	25 (12)	27 (8)	26 (5)	23 (1)
		"RRR"	38 (13)	29 (7)	34 (10)	25 (1)	22 (5)
CYP3A43	metabolism of xenobiotics, drugs	"all rac"	45 (12)	45 (2)	40 (9)	46 (5)	34 (3)
		"RRR"	45 (12)	31 (11)	39 (2)	38 (6)	41 (2)
CYP4F3	arachidonic acid or fatty acid metabolism	"all rac"	37 (7)	18 (12)	23 (3)	26 (3)	20 (2)
		"RRR"	37 (7)	26 (9)	24 (4)	24 (8)	20 (5)
CYP4F12	arachidonic acid or fatty acid metabolism	"all rac"	77 (21)	39 (10)	36 (2)	61 (6)	59 (8)
		"RRR"	77 (21)	55 (12)	61 (7)	47 (7)	51 (12)
CYP11B1	steroid biosynthesis	"all rac"	35 (13)	13 (1)	21 (3)	21 (3)	15 (2)
		"RRR"	35 (13)	25 (4)	29 (5)	18 (6)	17 (4)
CYP20A1	unknown	"all rac"	64 (10)	82 (2)	105 (3)	130 (2)	132 (6)
		"RRR"	64 (10)	72 (24)	91 (25)	115 (1)	119 (5)
CYP24A1	vitamin D degradation	"all rac"	40 (8)	50 (1)	72 (10)	44 (8)	40 (7)
		"RRR"	40 (8)	43 (4)	52 (13)	56 (10)	45 (6)
CYP27A1	bile acid biosynthesis	"all rac"	435 (51)	430 (8)	420 (41)	463 (4)	418 (33)
		"RRR"	435 (51)	386 (63)	488 (1)	410 (49)	359 (33)
CYP51A1	cholesterol biosynthesis	"all rac"	323 (57)	219 (32)	220 (9)	239 (38)	246 (20)
		"RRR"	323 (57)	201 (9)	245 (31)	294 (1)	200 (24)

diet ( $0.20 \pm 0.09$   $\mu$ mol/l, day 269). Differences in dietary  $\alpha$ -tocopherol were also reflected by significant differences in its hepatic concentrations after 269 days (VE+:  $83.91 \pm 15.86$  nmol/g tissue, VE-:  $0.35 \pm 0.20$  nmol/g tissue) (5).

**CYP mRNA expression in vivo.** In rat liver, 34 out of the 44 CYP enzymes represented on the RNA microarray were expressed. The highest mRNA expression levels were observed for CYP2C7 and CYP2D3. CYP3A4 and CYP4F2 also

showed high expression levels (Figure 2). CYP1A1, CYP2A3, CYP2D18, CYP3A2, CYP3A9, CYP4A8, CYP11B3, CYP17 and CYP51 were only marginally expressed (data not shown). A detailed list of all the CYPs with moderate or high mRNA expression levels is given in Table II.

No significant differences in CYP mRNA concentrations were found at any time between rats deprived of or supplemented with vitamin E. Furthermore, no significant changes in mRNA levels were observed over time.

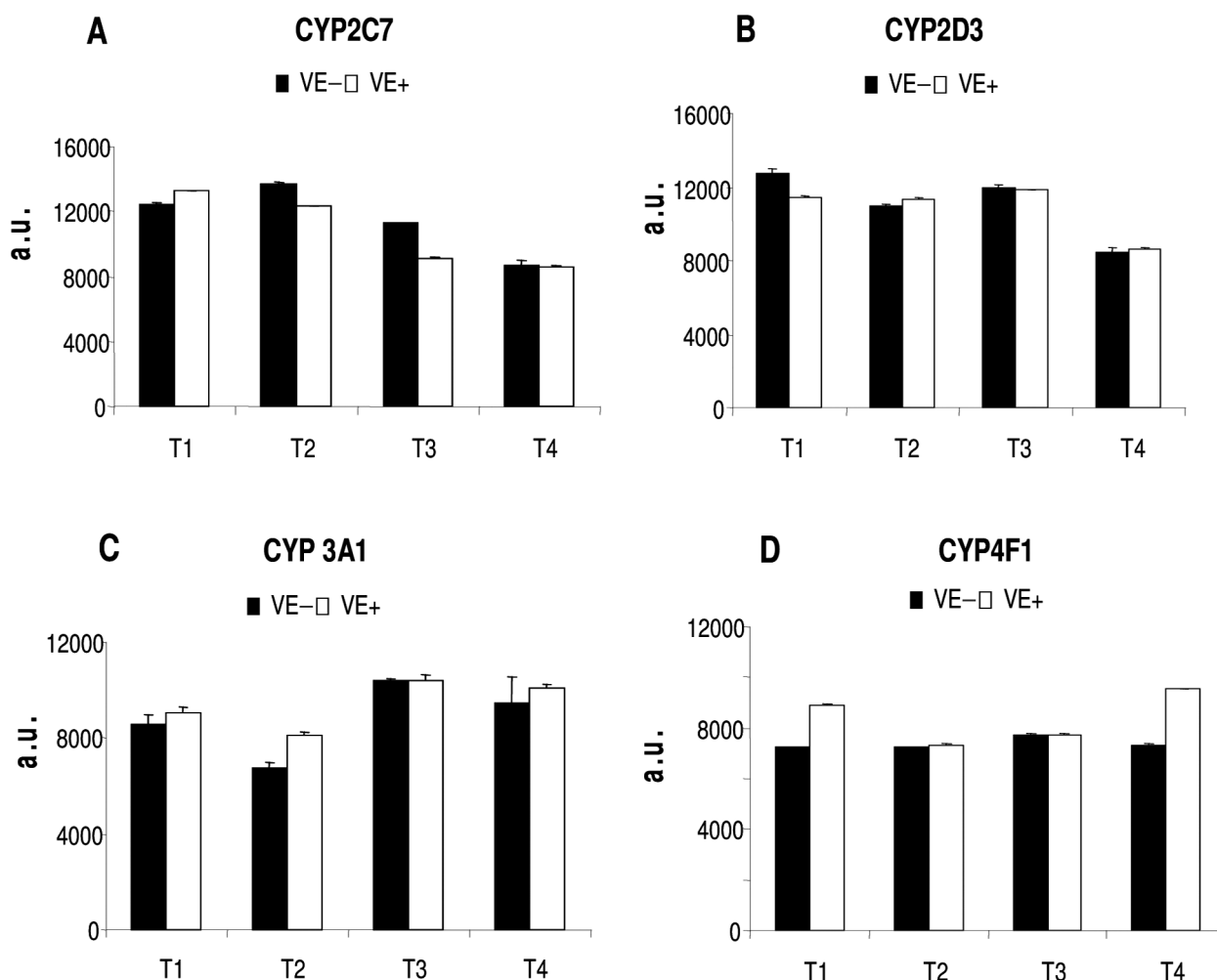


Figure 2. Cytochrome P450 mRNA levels in rat hepatocytes – given in arbitrary units (a.u.) – after treatment with a vitamin E-enriched diet (60 mg/kg RRR- $\alpha$ -tocopheryl acetate, VE+) or a vitamin E-deficient diet (1.7 mg/kg RRR- $\alpha$ -tocopheryl acetate, VE-) for 17 d (T1), 91 d (T2), 191 d (T3) or 269 d (T4). The data represent means  $\pm$  SD.

## Discussion

The objective of this study was to investigate the short- and long-term effects of vitamin E on CYP mRNA expression. To this end, HepG2 cells were supplemented with RRR- or all rac- $\alpha$ -tocopheryl acetate at concentrations of 0, 10, 30, 80 and 300  $\mu$ M. Only CYP20A1 mRNA was dose-dependently up-regulated by vitamin E *in vitro*. The function of CYP20A1 in humans is currently unknown. Its amino acid sequence has a 23% similarity to that of rat CYP17A1, which is known to be involved in sexual development during foetal life and at puberty (18, 19). CYP20A1 may play a role in the metabolism of human sex hormones. This is consistent with the discovery of vitamin E as an essential factor for successful reproduction in rats (20).

The remaining 13 CYP enzymes expressed in HepG2 cells showed no dose-dependent regulation by vitamin E. Previously, incubation of human HepG2 cells with a single dose of 50  $\mu$ M  $\gamma$ -tocotrienol, a different form of vitamin E, for 48 hours, resulted in a significant up-regulation of CYP3A4 and CYP3A5 mRNA (21). However,  $\gamma$ -tocotrienol is usually not detectable in human plasma. Supplementation with 250 mg tocotrienols/d for 8 weeks did not raise the plasma tocotrienol concentrations above 1 mM (22). Thus, the  $\gamma$ -tocotrienol concentrations used by Landes and co-workers are not physiologically achievable and the significance of their findings await confirmation or refutation in properly designed *in vivo* experiments. Moreover, in our study, the HepG2 cells were supplemented with vitamin E for 7 days, because we thought it important to keep the vitamin E concentrations

Table II. *Cytochrome P450 mRNA levels in arbitrary units (a.u.) in rat hepatocytes after treatment with a vitamin E-enriched (60 mg/kg RRR- $\alpha$ -tocopheryl acetate, VE+) or a vitamin E-deficient diet (1.7mg/kg RRR- $\alpha$ -tocopheryl acetate, VE-) for 17 d (T1), 91 d (T2), 191 d (T3) or 269 d (T4)). The data represent means  $\pm$  SD.*

CYP450 enzyme, rat		mRNA expression (a.u. $\pm$ SD)				
	Function	VE form	T1	T2	T3	T4
CYP1A2	may play a role in xenobiotic metabolism	VE- VE+	2910 (14) 2420 (16)	2202 (10) 1826 (39)	2098 (50) 1524 (14)	4583 (9) 2389 (40)
CYP2A1	steroid hormones 7-alpha-hydroxylase	VE- VE+	8842 (552) 7169 (735)	7758 (742) 6717 (48)	7268 (225) 7858 (431)	6883 (1543) 7324 (596)
CYP2A2	testosterone 15-alpha-hydroxylase	VE- VE+	6583 (59) 6307 (46)	5464 (19) 4816 (45)	5688 (39) 5367 (12)	7353 (46) 7470 (6)
CYP2B2	unknown	VE- VE+	3943 (37) 3598 (40)	887 (32) 1095 (35)	1529 (49) 1896 (20)	2007 (57) 2694 (40)
CYP2B3	oxidises a variety of compounds, including steroids, fatty acids, xenobiotics	VE- VE+	6958 (6) 6973 (46)	6868 (55) 7713 (38)	5258 (20) 8621 (4)	6260 (41) 6866 (38)
CYP2C6	may play a role in drug metabolism	VE- VE+	9635 (208) 8584 (384)	5583 (489) 4252 (258)	5374 (197) 7225 (441)	6885 (298) 7068 (431)
CYP2C7	a retinoic acid and testosterone hydroxylase	VE- VE+	12482 (41) 13257 (53)	13689 (94) 12374 (11)	11292 (27) 9144 (41)	8682 (327) 8596 (103)
CYP2C11	unknown	VE- VE+	6673 (363) 7126 (356)	10021 (872) 10949 (79)	7935 (461) 8516 (299)	4656 (1376) 5578 (723)
CYP2C13	metabolizes arachidonic acid	VE- VE+	7322 (9) 8921 (7)	5767 (6) 6074 (13)	6166 (3) 5509 (16)	4649 (60) 6034 (63)
CYP2C22	unknown	VE- VE+	6596 (48) 9940 (97)	3190 (60) 3823 (35)	4494 (57) 4627 (29)	2914 (63) 5757 (9)
CYP2C23	arachidonic acid epoxigenase	VE- VE+	9266 (41) 10562 (34)	10034 (18) 10174 (53)	11794 (65) 12254 (19)	12426 (82) 11044 (50)
CYP2D3	debrisoquine 4-hydroxylase	VE- VE+	12763 (277) 11433 (80)	10947 (166) 11349 (64)	11975 (167) 11863 (29)	8467 (215) 8617 (93)
CYP2D4	debrisoquine 4-hydroxylase	VE- VE+	1323 (163) 1139 (336)	1364 (81) 1305 (243)	1332 (50) 1153 (46)	754 (380) 705 (89)
CYP2D5	debrisoquine 4-hydroxylase	VE- VE+	6495 (80) 7849 (57)	6068 (57) 5884 (5)	6690 (36) 6652 (15)	7071 (60) 8954 (32)
CYP2F4	unknown	VE- VE+	588 (16) 534 (16)	720 (27) 636 (7)	732 (15) 836 (18)	1117 (12) 996 (25)
CYP2J3	unknown	VE- VE+	1505 (10) 1900 (21)	1963 (19) 1602 (4)	1316 (6) 1201 (6)	1051 (100) 1494 (52)
CYP3A1	unknown	VE- VE+	8549 (433) 9025 (276)	6776 (220) 8143 (139)	10376 (111) 10392 (227)	9472 (1082) 10079 (187)
CYP3A18	catalyses 16 beta- and 6-alpha hydroxylations of testosterone	VE- VE+	3965 (34) 3980 (29)	4531 (55) 3808 (68)	3875 (43) 5410 (36)	4086 (27) 3839 (18)

*continued*

Table II. *continued.*

CYP450 enzyme, rat		mRNA expression (a.u.±SD)				
	Function	VE form	T1	T2	T3	T4
CYP4A1	lauric acid omega-hydroxylase	VE- VE+	1484 (65) 1400 (35)	1121 (65) 1096 (108)	934 (50) 767 (65)	2860 (56) 3005 (62)
CYP4F1	plays a role in metabolism of arachidonic acid	VE- VE+	7250 (13) 8933 (51)	7244 (7) 7330 (55)	7694 (52) 7743 (9)	7305 (86) 9531 (45)
CYP4F4	plays a role in metabolism of arachidonic acid	VE- VE+	1699 (27) 1728 (12)	1381 (7) 1347 (3)	1439 (6) 1186 (11)	2018 (39) 2016 (11)
CYP4F6	catalyses the conversion of leukotriene B(4) into 19-hydroxy- and 18-hydroxy-leukotriene B(4); plays a role in inflammation	VE- VE+	1586 (462) 1770 (833)	1658 (133) 1794 (264)	1371 (437) 2219 (216)	1683 (217) 2220 (26)
CYP7A1	cholesterol 7- $\alpha$ - monooxygenase	VE- VE+	664 (15) 675 (16)	854 (23) 803 (20)	1746 (36) 2063 (12)	780 (58) 237 (23)

stable over time and to establish intracellular steady state concentrations of vitamin E (23), whereas in most studies reported in the literature, the incubation periods were only 24 or 48 h (14, 21). Thus, the previously reported changes of CYP expression may be transient effects.

In our *in vitro* model, no significant differences were found in CYP gene expression between the natural and synthetic forms of vitamin E. This is in agreement with our previously published data showing that *RRR*- and all *rac*- $\alpha$ -tocopherol share identical transcriptional activity in HepG2 cells (23).

Feeding male rats over a period of 9 months with diets deficient in (< 2 mg *RRR*- $\alpha$ -tocopheryl acetate/kg diet) or supplemented with vitamin E (60 mg *RRR*- $\alpha$ -tocopheryl acetate/kg diet; approximately twice the recommended  $\alpha$ -tocopherol content of diets for growing rats (24)), resulted in an almost complete depletion or a significant accumulation of  $\alpha$ -tocopherol in the plasma and liver, respectively (5). Despite these pronounced differences in the plasma and liver  $\alpha$ -tocopherol concentrations, no changes in gene expression of CYP enzymes were observed (Table II). In previous experiments, the employed rat model was successfully used to identify vitamin E-sensitive genes (5, 6).

Kluth *et al.* (17) fed mice for 3 months with diets containing 2, 20, or 200 mg *RRR*- $\alpha$ -tocopheryl acetate/kg. The hepatic levels of Cyp3a11 (the murine homolog of human CYP3A4 and rat CYP3A1 (25)) mRNA were about 2.5-fold higher in the 20- and 200-mg  $\alpha$ -tocopherol groups compared to the 2-mg group. Furthermore, after feeding

200 mg  $\alpha$ -tocopherol for 9 months, Cyp3a11 mRNA was 1.7-fold higher than after 3 months. The differences between our results and those of Kluth *et al.* (17) may be related to species-specific differences in the regulation of CYP gene expression. It has been reported that the induction of CYP3As by rifampicin is much more pronounced in humans than in rodents (26), and that rat CYP3A1 is not affected by rifampicin at all (27).

In conclusion, it was shown that vitamin E does not affect the CYP mRNA levels of the majority of CYPs in HepG2 cells or in rat liver *in vivo*. Moreover, no differences between natural and synthetic vitamin E on CYP gene expression were observed. Since the current study measured the effect of vitamin E on CYP gene expression at the level of mRNA, future experiments are warranted to study these effects at the level of CYP protein expression and enzyme activity.

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## **CHAPTER THREE**



## Effect of vitamin E and polyphenols on ochratoxin A-induced cytotoxicity in liver (HepG2) cells

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Oxidative stress;  
 $\alpha$ -Tocopherol

### Summary

It has been shown that oxidative damage contributes to the wide range of toxic effects of the mycotoxin ochratoxin A (OTA). Therefore, we examined the effects of  $\alpha$ -tocopherol ( $\alpha$ -TOC) and different polyphenols – catechin (CAT), daidzein (DAI), epicatechin (EC), epigallocatechin gallate (EGCG), genistein (GEN), and quercetin (QUE) – on OTA-induced cytotoxicity in HepG2 liver cells. Incubation of HepG2 cells with increasing concentrations of OTA resulted in a dose- and time-dependent cytotoxicity as measured by the neutral red assay. Half lethal concentrations ( $LC_{50}$ ) of OTA were 35 and 10  $\mu$ M after 48 and 72 h incubation, respectively. Incubation of HepG2 cells with  $\alpha$ -TOC as well as with different polyphenols (exhibiting different antioxidant potency as determined by the FRAP, TEAC and DPPH assays) did not counteract OTA-induced cytotoxicity. These findings indicate that OTA may exert its toxic effects by affecting other hepatic mechanisms than those directly modulated by  $\alpha$ -TOC and polyphenols.

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**Abbreviations:** CAT, catechin; DAI, daidzein; DPPH, diphenylpicrylhydrazyl; EC, epicatechin; EGCG, epigallocatechin gallate; EQU, equol; FRAP, ferric reducing antioxidant capacity of plasma; GEN, genistein; GSH, glutathione; OTA, ochratoxin A; PBS, phosphate buffered saline; QUE, quercetin; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute;  $\alpha$ -TOC,  $\alpha$ -tocopherol; TEAC, trolox equivalent antioxidant capacity

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## Introduction

Ochratoxin A (OTA), a mycotoxin mostly produced by *Aspergillus ochraceus* and *Penicillium verrucosum*, is a worldwide contaminant of food and feedstuff. Its occurrence has been reported in cereal grains, coffee beans, nuts, wine, pork, beer, cheese and several spices (Fazekas et al., 2002; Studer-Rohr et al., 1995; Van Egmond and Speijers, 1994). A number of studies have suggested a role for OTA in the development of several types of cancer (Reijula and Tuomi, 2003; Schwartz, 2002). In mice, oral administration of OTA led to an increase in hepatocellular tumours and renal-cell adenoma and carcinoma (Bendele et al., 1985). Besides, OTA toxicity is linked to an irreversible and fatal kidney disease referred to as Balkan Endemic Nephropathy (BEN; Stoev, 1998).

Chemically, OTA consists of a dihydroisocoumarin moiety linked via its carboxyl group to the amine of L-phenylalanine (Fig. 1). OTA toxicity has been attributed to the open lactone moiety which is structurally analogous to mitochondrial enzyme active sites and competitively binds enzyme substrates (Hussein and Brasel, 2001). In rat liver mitochondria, OTA competitively inhibits cytochrome C oxidase, succinate dehydrogenase and ATPase.

The mechanism by which OTA acts is not fully understood, but there is reason to assume that its toxicity may be the result of three major effects: (1) inhibition of ATP synthesis; (2) inhibition of protein synthesis; and (3) enhanced lipid peroxidation (Creppy et al., 1984; Rahimtula et al., 1988). Schaaf et al. (2002) attributed proximal tubular cell damage caused by OTA to the formation of reactive oxygen species (ROS), which in turn induce oxidative damage towards lipids, proteins and DNA.

Several natural components of the diet including  $\alpha$ -TOC and various polyphenols exhibit potent antioxidant activity and, thus, might partly counteract OTA toxicity (Baldi et al., 2004; Renzulli et al., 2004).

The present study aimed at investigating potential inhibitory effects of  $\alpha$ -TOC and the polyphenols

EGCG, QUE, CAT, EC, DAI, EQU and GEN on the OTA-induced cytotoxicity in human HepG2 liver cells.

## Materials and methods

### Cultivation and determination of OTA-cytotoxicity

Cells of the human hepatoblastoma derived cell line HepG2 were cultured in RPMI media supplemented with 10% foetal calf serum, 2 mM glutamine, 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin under standard conditions. Confluent cells were harvested and seeded at  $2 \times 10^5$  cells per well in 12-well plates. The medium was changed every 48 h.

In dose-response experiments, HepG2 cells were exposed to increasing concentrations of OTA ranging from 0 to 100  $\mu$ M for 24, 48, and 72 h. The cytotoxicity of OTA was measured by the neutral red (NR) assay (Valacchi et al., 2001). In this assay, the ability of viable cells to incorporate NR in cellular membranes is photometrically measured at 540 nm. Cell viability was expressed as percentage of solvent-exposed control (0.1% methanol) survival. Half lethal concentrations ( $LC_{50}$ ) for OTA were calculated. For each incubation period, two independent experiments were performed at least in triplicate.

### Antioxidant activity of test compounds

The antioxidant capacity of the test substances was determined with TEAC, FRAP and DPPH assays.

The TEAC assay, which is based on the reduction of the green coloured ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) radical cation by antioxidants, was performed with ethanolic solutions of the flavonoids (2.5  $\mu$ M). The increase in reduction was photometrically measured at  $\lambda = 734$  nm and compared to Trolox as internal standard. TEAC values give the  $\mu$ mol of Trolox with the same antioxidant capacity as 1.0  $\mu$ mol of the test substance. Two independent experiments were performed in triplicate.

The FRAP assay uses an oxidation/reduction reaction to measure the ability of a sample to reduce iron (III) to iron (II). Ascorbic acid was used as reference and FRAP values are given as ascorbic acid equivalents (AAE). The absorbance of the test compounds (3.3  $\mu$ M in ethanol) as compared to ascorbic acid was read at 593 nm 30 min after incubation at room temperature against a blank of FRAP reagent.

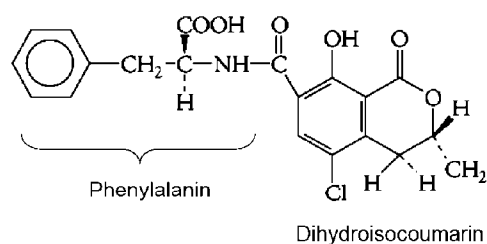


Figure 1. Chemical structure of OTA.

The colour of the DPPH radical (purple) in solution fades upon reduction by antioxidants and can be measured photometrically. DPPH values were determined 30 min after incubation with the test substances (5  $\mu$ M, EtOH) at  $\lambda = 515$  nm.

To study the possible preventative activity of vitamin E and flavonoids on OTA-induced cytotoxicity, cells were pre-treated for 48 h with flavonoids (50  $\mu$ M) or  $\alpha$ -TOC (100  $\mu$ M). Cells were washed twice with PBS and incubated for 48 h with OTA containing culture medium at a concentration of 20  $\mu$ M. Two independent replicates were performed in triplicate. The NR assay was performed as described above.

## Results

### Cytotoxic effects of ochratoxin A

Incubation of HepG2 cells with increasing concentrations of OTA resulted in a dose- and time-dependent cytotoxicity (Fig. 2). Half lethal concentrations of OTA were 35 and 10  $\mu$ M after 48 and 72 h incubation. An incubation period of 24 h only reduced the cell viability to 60%.

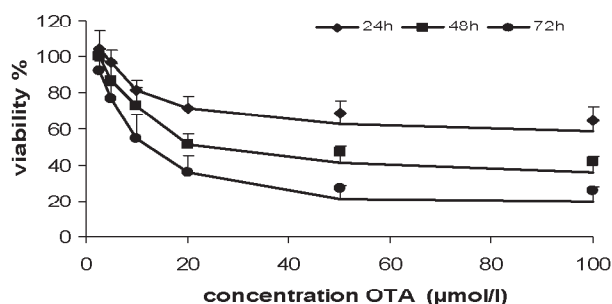


Figure 2. Viability of HepG2 after 24, 48, and 72 h of incubation with ochratoxin A at the given concentrations.

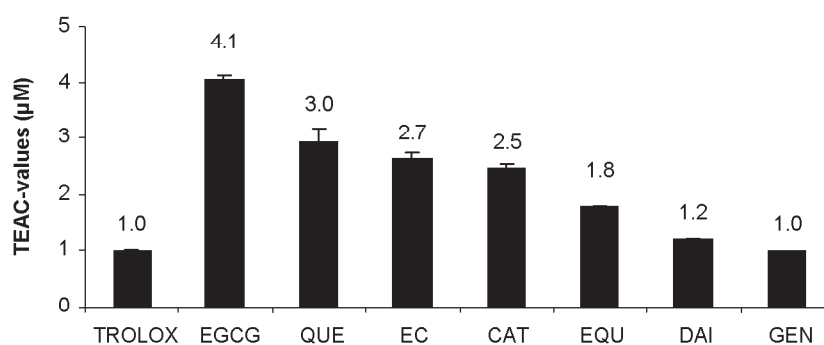


Figure 3. Antioxidant capacity of flavonoids as determined by the TEAC assay.

### Antioxidant activity of test compounds

Regarding the TEAC FRAP and DPPH assays EGCG exhibited the highest antioxidant activity, followed by QUE, EC and CAT. The isoflavones showed the lowest antioxidant capacity. However, EQU was more potent than GEN and DAI (Figs. 3–5).

### Effect of antioxidants on ochratoxin A cytotoxicity

Pre-incubation of HepG2 with  $\alpha$ -TOC as well as with flavonoids did not significantly counteract OTA-induced cytotoxicity (Fig. 6).

## Discussion

In the present study, the half lethal OTA concentration ( $LC_{50}$ ) in HepG2 cells was 35  $\mu$ M after 48 h incubation as measured by the NR assay. This value is similar to those reported by Baldi et al. (2004), who found in canine kidney cells (MDCK cells) a half lethal OTA concentration of 10–36  $\mu$ M.

Several studies propose the involvement of oxidative stress in the toxicity of OTA (Baudrimont et al., 1994; Belmadani et al., 1999; Kamp et al., 2005; Rahimtula et al., 1988). However, our data indicate that neither  $\alpha$ -TOC nor flavonoids, independent of their different antioxidant potency and solubility, had a protective effect on OTA-induced cell death.

In the study by Baldi et al. (2004), a small but significant protection (10%) of  $\alpha$ -TOC towards OTA-induced cell death was observed in bovine mammary epithelial cells (BME-UV1). Beside the differences in cell type it should be considered that BME-UV1 cells were incubated with  $\alpha$ -TOC for 3 h only. In contrast, we incubated the cells with  $\alpha$ -TOC for 48 h in order to ensure a complete cellular uptake and steady-state levels of  $\alpha$ -TOC in

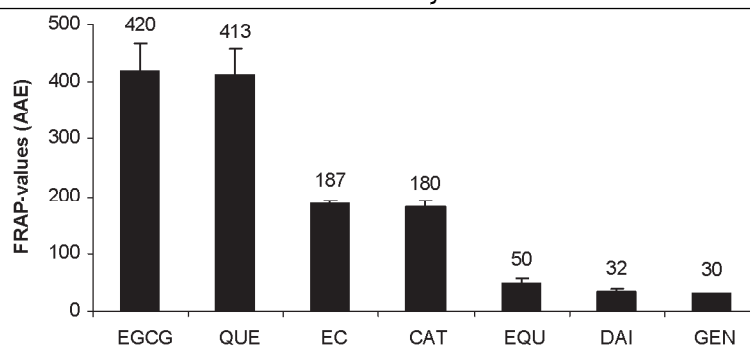


Figure 4. Antioxidant capacity of flavonoids as determined by the FRAP assay.

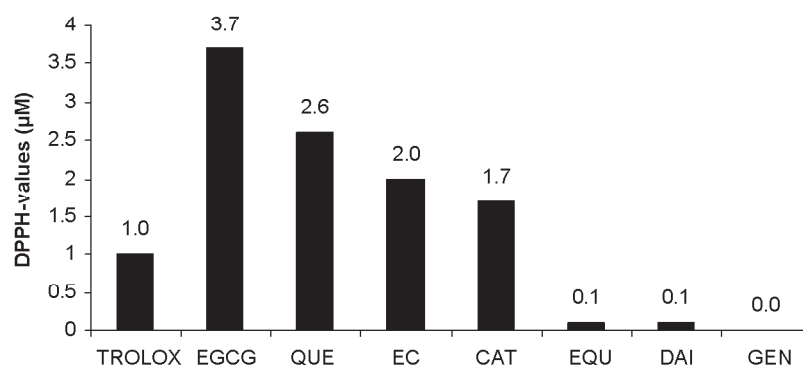


Figure 5. Antioxidant capacity of flavonoids as determined by the DPPH assay.

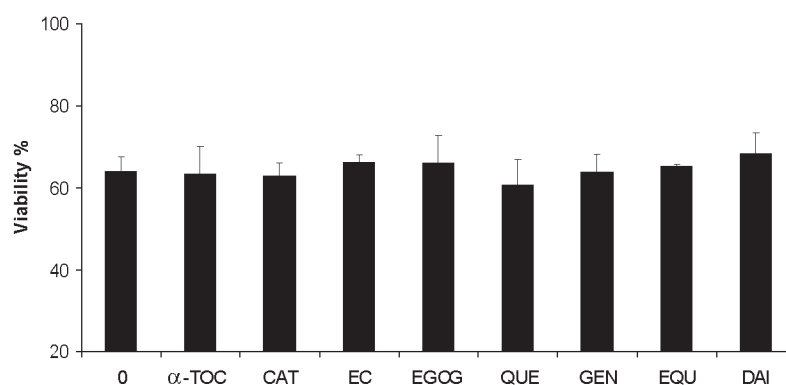


Figure 6. Viability of HepG2 cells after 48 h pre-incubation with  $\alpha$ -TOC (100  $\mu$ M) and flavonoids (50  $\mu$ M) followed by OTA treatment (20  $\mu$ M, 48 h). Results are expressed as mean  $\pm$  SEM. Statistical analysis of grouped data were performed as one way analysis of variance with post hoc test Scheffé. No significant differences ( $p < 0.05$ ) were obtained.

the cells. Furthermore, the cells were washed twice with PBS in order to avoid any chemical interaction between OTA and  $\alpha$ -TOC in the cell culture medium.

It needs to be taken into account that other mechanisms than oxidative stress are also likely to be involved in the cytotoxicity of OTA, including inhibition of protein synthesis (Creppy et al., 1984), inhibition of various enzymes such as ATPase (Siraj et al., 1981), succinate dehydrogenase (Wei et al.,

1985) and oxido-reductases (Singh et al., 1994), as well as the formation of DNA adducts (Faucet et al., 2004). Since OTA is a structural analogue of phenylalanine, there is competition with phenylalanine in the phenylalanine-tRNA aminoacylation reaction, which is a key reaction in protein synthesis. Aspartame (L-aspartyl-L-phenylalanine methyl ester), a structural analogue of both OTA and phenylalanine, was found to partially protect against the OTA-induced inhibition of protein

synthesis in monkey kidney cells (Vero cells) (Baudrimont et al., 1997).

It has been shown that in primary rat proximal tubulus (PT) cells and pig kidney (LLC-PK<sub>1</sub>) cells pre-incubation with *N*-acetyl-L-cysteine (NAC), a precursor of cellular glutathione (GSH), had a protective effect on OTA-induced cytotoxicity. In contrast  $\alpha$ -TOC decreased OTA-induced ROS production but not the cytotoxic effects of OTA (Schaaf et al., 2002) as observed in the current study. In a preliminary experiment, we found that incubation of HepG2 cells with buthionine-sulfoximine (BSO), known to deplete cellular GSH, significantly enhanced the cytotoxic effects of OTA. Hence, it appears plausible that covalent binding of OTA to GSH (Dai et al., 2002) may inactivate the mycotoxin and, thus, promote its cellular excretion.

Overall, present data demonstrate that vitamin E and polyphenols do not counteract OTA-induced cytotoxicity in liver HepG2 cells. These findings indicate that OTA may exert its toxic effects by affecting other hepatic mechanisms than those directly modulated by vitamin E and polyphenols.

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## **CHAPTER FOUR**

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## Ochratoxin A-induced cytotoxicity in liver (HepG2) cells: Impact of serum concentration, dietary antioxidants and glutathione-modulating compounds\*

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### Abbreviations

BSO, buthionine sulfoximine; CAT, catechin; DMSO, dimethyl sulfoxide; DTNB, dithio-bis-nitrobenzoic acid; EGCG, epigallocatechin gallate; FCS, foetal calf serum; GSH, glutathione; IARC, international agency for research on cancer; NAC, N-acetylcysteine; NO, nitric oxide; NR, neutral red; OATP, organic anion-transporting polypeptide; OTA, ochratoxin A; PBS, phosphate buffered saline; QUE, quercetin; ROS, reactive oxygen species; ROSAC, rosmarinic acid; RPMI, roswell park memorial institute;  $\alpha$ -TOC,  $\alpha$ -tocopherol;  $\alpha$ -TOC-P,  $\alpha$ -tocopherol phosphate

### Summary

Ochratoxin A (OTA) is a nephro- and hepatotoxic mycotoxin produced by various species of the genera *Aspergillus* and *Penicillium*. OTA is known to bind with high affinity to plasma proteins which may have a substantial impact on its bioavailability and, thus, on its toxicity. However, the underlying mechanisms of OTA-induced cellular toxicity have not yet been fully elucidated. It has been suggested that oxidative damage contributes to its cytotoxic effects. Dietary antioxidants such as vitamin E and polyphenols may therefore counteract OTA-induced cell death. Furthermore, compounds influencing the intracellular level of glutathione (L-gamma-glutamyl-L-cysteinylglycine, GSH), the most abundant thiol antioxidant in mammalian cells, may have an impact on OTA-induced cytotoxicity. In this study we investigated the effects of serum concentrations as well as different dietary antioxidants on the viability of OTA-exposed liver (HepG2) cells. Additionally, we determined the intracellular GSH-levels after incubation with OTA and N-acetylcysteine (NAC, a precursor of GSH) or buthionine sulfoximine (BSO, an inhibitor of gamma-glutamylcysteinyl synthetase). Incubation of human hepatoma cells (HepG2) for 24 h with increasing concentrations of OTA (0.25 - 50  $\mu$ mol/l) in the presence of 0, 2.5, 5, or 10% foetal calf serum (FCS) resulted in a dose-dependent decrease in cell viability. Decreasing the serum concentrations in the cell culture medium led to increased cell mortality. Pre-treatment for 24 h with RRR- $\alpha$ -tocopherol ( $\alpha$ -TOC), RRR- $\alpha$ -tocopherolphosphat ( $\alpha$ -TOC-P), epigallocatechin gallate (EGCG), quercetin (QUE), catechin (CAT), and rosmarinic acid (ROSAC) at concentrations of 25, 50 and 100  $\mu$ mol/l did not prevent OTA-induced toxicity.  $\alpha$ -TOC-P, EGCG, and QUE even amplified the cytotoxic effects of OTA in HepG2. Supplementation with NAC and BSO in the presence of OTA did not substantially change cell viability, although BSO treatment resulted in depletion of cellular GSH. OTA treatment increased GSH levels at concentrations of 50 and 100 nmol/l, but decreased cellular GSH at the higher concentrations (250, 500, and 1000 nmol/l). The decrease in cellular GSH concentrations was less pronounced than for BSO. Our results indicate that the cytotoxicity of OTA in HepG2

cells, which is strongly dependent on the protein concentration in the cell culture medium, can not be prevented by pre-incubation with dietary antioxidants. Although cellular GSH levels are influenced by OTA incubation, mechanisms other than oxidative stress are likely to be involved in OTA-induced cell death in HepG2 cells.

### Introduction

The mycotoxin ochratoxin A (OTA), mainly produced by *Aspergillus ochraceus* and *Penicillium verrucosum*, is the most toxic of the three known members of the ochratoxin family (ochratoxin A, B, and C). OTA was discovered and its chemical structure described in 1965 (VAN DER MERWE et al., 1965). Chemically, OTA consists of a dihydroisocoumarin moiety joined by a peptide bond to L-phenylalanine (Fig. 1). OTA can be found in many foods and beverages, e.g. cereal grains, coffee, and wine (FAZEKAS et al., 2002; STUDER-ROHR et al., 1995; VAN EGMONT et al., 1994). Due to its ubiquitous occurrence in foods, it is virtually impossible to completely avoid ingestion of OTA. Both in animals and humans, a wide range of toxic effects of OTA has been reported, including renal and hepatic toxicity (BENDELE et al., 1985), genotoxicity (CREPPY et al., 1985; BOSE and SINHA, 1994), neurotoxicity (MIKI et al., 1994; BRUNINK et al., 1998), and immunotoxicity (HARVEY et al., 1992; PETZINGER and ZIEGLER, 2000). Severe human renal diseases, such as Endemic Nephropathy (BEN), chronic interstitial nephritis and karyomegalic interstitial nephritis, may be a result of continued exposure to OTA (SIMON et al., 1996). Moreover, the International Agency for Research on Cancer has classified OTA as a class 2B carcinogen (possible human carcinogen) (IARC, 1991).

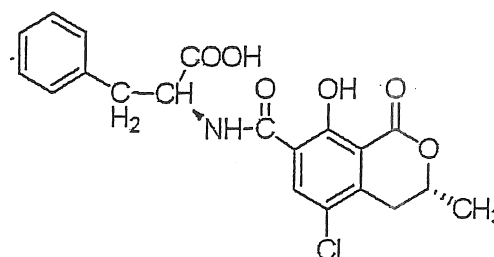


Fig. 1: Chemical structure of ochratoxin A

The toxicity of OTA is largely determined by its protein binding properties. In blood, 99% of OTA is bound to serum proteins (mainly albumin), which prolongs its systemic half-life, delays its elimination and may, therefore, amplify its toxicity (CHU, 1971; CHU, 1974). The exact mode of action of OTA toxicity is as yet unclear. The toxicity of OTA has been explained by three major mechanisms: 1.

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inhibition of protein synthesis via inhibition of phenylalanine-tRNA synthetases (CREPPY et al., 1979; CREPPY et al., 1998), 2. inhibition of mitochondrial function (MOORE and TRUELOVE, 1970; MEISNER, and CHAN, 1974; WEI et al., 1985), and 3. generation of reactive oxygen species (ROS) which may oxidise DNA, proteins, lipids, and other macromolecules (RAHMTULA et al., 1988; OMAR et al., 1990; SCHAAF et al., 2002; DOMJAN et al., 2005; KAMP et al., 2005). Therefore, antioxidants may counteract OTA-induced cytotoxicity. Recent studies have shown that supplementation with vitamin E or several polyphenols partly diminished the toxic effects of OTA (SCHAAF et al., 2002; BALDI et al., 2004; RENZULLI et al., 2004; GUERRA et al., 2005). Furthermore, an involvement of the glutathione (GSH) system in OTA cytotoxicity, has been suggested. GSH ( $\gamma$ -glutamylcysteinylglycine) functions as a free radical scavenger, and serves as a nucleophilic co-substrate for glutathione transferases in the detoxification of xenobiotics. It has been shown that pre-incubation with N-acetylcysteine (NAC), a precursor of GSH, completely prevented OTA-induced cell death in kidney (LLC-PK1) cells (SCHAAF et al., 2002).

The objective of the present study was to assess the impact of serum protein concentration in the cell culture medium and the effect of dietary antioxidants on OTA-induced cytotoxicity in HepG2 cells. Furthermore, we aimed at investigating whether modulation of cellular GSH levels affects OTA-induced cytotoxicity in human liver cells in culture.

## Materials and methods

### Cell culture and determination of OTA-cytotoxicity

The human hepatoblastoma derived cell line HepG2 was cultured in RPMI medium supplemented with 10% FCS, 2 mmol/l glutamine, 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin under standard conditions (37°C, 5% CO<sub>2</sub>). Confluent cells were harvested by trypsinisation and seeded at  $1 \times 10^5$  cells per well in 24-well plates. The medium was changed every 48 h.

In dose-response experiments, HepG2 cells were exposed to increasing concentrations of OTA ranging from 0.25 - 50  $\mu$ mol/l for 24 h. The FCS concentrations in the medium were 0, 2.5, 5 or 10%. In a separate experiment, HepG2 cells were incubated with OTA at concentrations of 10 - 1000 nmol/l to find out the highest non-toxic OTA concentration under serum-free conditions. The cytotoxicity of OTA was determined employing the neutral red (NR) assay (VALACCHI et al., 2001). This test is based on the ability of viable cells to incorporate NR in lysosomes which is photometrically measured at 540 nm. Cell viability was expressed as percentage of solvent-exposed control (0.1% methanol) survival. For each incubation period, two independent experiments were performed in triplicate (n = 6).

### Pre-incubation with test substances

To study the potential protective effects of vitamin E and polyphenols on OTA-induced cytotoxicity, cells were pre-treated with *RRR*- $\alpha$ -tocopherol ( $\alpha$ -TOC), *RRR*- $\alpha$ -tocopherolphosphate ( $\alpha$ -TOC-P), epigallocatechin gallate (EGCG), quercetin (QUE), catechin (CAT), and rosmarinic acid (ROSAC) (Fig. 2) for 24 h at concentrations of 25, 50, and 100  $\mu$ mol/l (non-toxic concentrations for HepG2 cells, data not shown). 100 mmol/l stock solutions were prepared in dimethyl sulfoxide (DMSO), ethanol or an acetic acid:ethanol mixture (1:3) for the polyphenols,  $\alpha$ -TOC and  $\alpha$ -TOC-P, respectively. Stock solutions were further diluted in culture medium containing 10% FCS with a maximum solvent concentration of 0.1%.

Cells were washed twice with PBS and incubated for another 24 h with 1, 5 or 15  $\mu$ mol/l OTA in serum-free RPMI. Additionally, HepG2 were pre-incubated for 24 h with 0, 0.2, 0.5, 1, 2, or 4 mmol/l NAC or 0, 5, 25, 50, 100, or 500  $\mu$ mol/l BSO. BSO and NAC were prepared as 100 mmol/l stock solutions in PBS. The pH of the NAC stock solution was adjusted to pH 7 with NaOH. Subsequently, cells were washed twice with PBS and incubated for 24 h with 1, 5 or 15  $\mu$ mol/l OTA in FCS-free medium. Two or three independent replicates were performed in triplicate.

### Determination of cellular glutathione levels

Total cellular GSH content was determined by the method of Griffith with minor modifications (GRIFFITH, 1980). HepG2 cells were grown at a density of  $9 \times 10^5$  cells per well in 6-well plates for 48 hours and incubated with OTA, BSO, or NAC at increasing concentrations. After 24 h, the incubation medium was aspirated; cells were washed with PBS and harvested by trypsinisation. Due to the limited cell material, cells of three wells were pooled together. Cell pellets were collected after centrifugation and stored immediately at -80°C. To measure glutathione concentrations, cell pellets were resuspended in lysis buffer (PBS, 0.05% Triton-X (w/v), 0.05 mmol/l EDTA) and homogenized. The cell suspension was treated with an equal volume of 10% sulfosalicylic acid (w/v) and separated by centrifugation (12,000  $\times$  g, 5 min, 4°C). 270  $\mu$ L of the supernatant was mixed with 20  $\mu$ L of a 150 mmol/l phosphate buffer (including 1% bovine serum albumin, pH 7.5) and 30  $\mu$ L triethanolamin (50%). Sample (75  $\mu$ L) was added to the reaction mixture containing 350  $\mu$ L of 0.28 mmol/l NADPH and 50  $\mu$ L of 6 mmol/l DTNB (5-5'-dithio-bis-2-nitro-benzoic acid). The reaction was started by addition of 50  $\mu$ L glutathione-reductase (10 U/ml) and the increase in absorption at 412 nm was followed for 180 min in 20 sec intervals. Each sample was measured in duplicate. Results were calculated by comparison of the difference in absorbance per minute with data from a standard curve generated with oxidised glutathione. The protein content of the cell suspension was determined with the commercially available BCA Protein Kit from Pierce. Three independent replicates were performed in duplicate.

### Statistical analysis

Data are presented as mean with standard deviation. The statistical program SPSS (version 13.0) was used to assess the effects of various treatments by analysis of variance (ANOVA) followed by the *post hoc* test Dunnett. In the case of inhomogeneous variances multiple t-tests were applied. Differences were considered significant if  $p < 0.05$ .

## Results

### Cytotoxicity of OTA

Incubation of HepG2 cells with concentrations of OTA ranging from 0.25 to 50  $\mu$ mol/l for 24 h resulted in a dose-dependent decline in cell viability (Fig. 3). Decreasing FCS concentrations were paralleled by a marked increase in OTA-induced cytotoxicity, with the strongest effects at OTA concentrations between 0.25 and 15  $\mu$ mol/l. In cells cultured in serum-free medium the strongest cytotoxicity was observed at OTA concentrations between 10 (100% viability) and 250 nmol/l (53% viability). However, in medium containing 5 or 10% serum, 24 h exposure to 1  $\mu$ mol/l OTA did not lead to cell death at all, while 0 or 2.5% serum decreased cell viability to 52 and 87%, respectively. The minimum level of cell viability (approximately 40%) was reached upon incubation with 15  $\mu$ mol/l OTA and did not

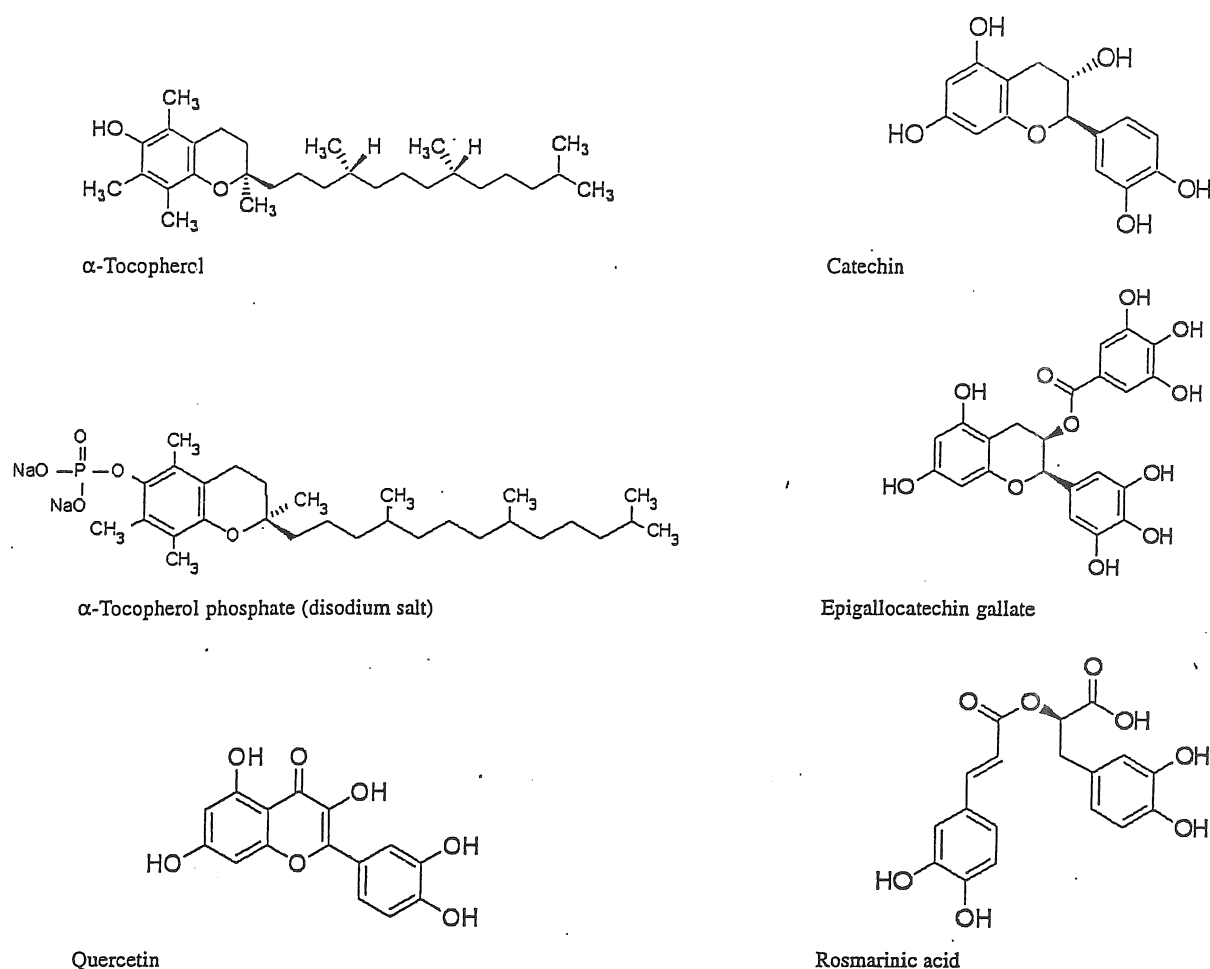


Fig. 2: Chemical structures of the antioxidants

decrease further even upon incubation with as much as 50  $\mu\text{mol/l}$  OTA in FCS-free medium (Fig. 3). The highest non-toxic OTA concentration was 10  $\text{nmol/l}$  (Fig. 4). Due to the higher bioavailability of OTA under these conditions, serum-free medium was chosen for further experiments.

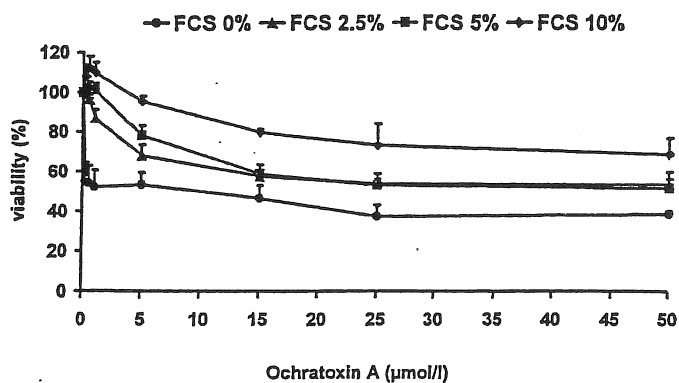


Fig. 3: Influence of foetal calf serum (FCS) concentration on the viability of HepG2 cells after 24 h incubation with ochratoxin A (0 - 50  $\mu\text{mol/l}$ ). Data are mean  $\pm$  SD from two experiments in triplicate ( $n = 6$ ).

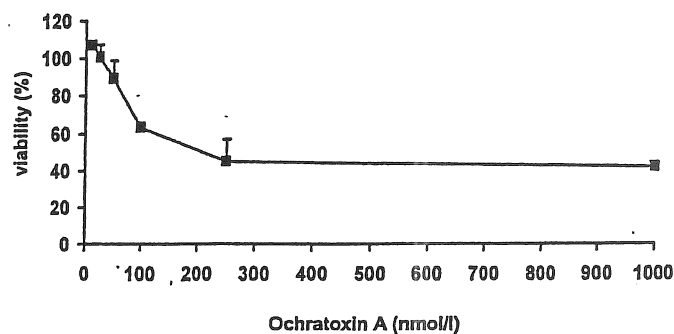


Fig. 4: Viability of HepG2 cells after 24 h incubation with ochratoxin A (0 - 1000  $\text{nmol/l}$ ). Data are mean  $\pm$  SD from two experiments in triplicate ( $n = 6$ ).

#### Pre-incubation experiments with antioxidants and glutathione-modulators

A 24 h pre-incubation of HepG2 cells with  $\alpha$ -TOC,  $\alpha$ -TOC-P, EGCG, QUE, CAT, and ROSAC at concentrations of 25, 50, or 100  $\mu\text{mol/l}$  and a subsequent incubation with 1, 5, or 15  $\mu\text{mol/l}$  OTA for 24 h did not prevent the cytotoxic effects of OTA (Fig. 5). Treatment with  $\alpha$ -TOC-P, EGCG, and QUE even enhanced OTA cytotoxicity dose-dependently. No changes in cell viability were observed upon pre-

incubation with  $\alpha$ -TOC, CAT and ROSAC. Cellular  $\alpha$ -TOC levels remained unchanged in response to OTA treatment (data not shown), cellular concentrations of EGCG, QUE, CAT, and ROSAC were not determined.

To further investigate the involvement of cellular glutathione levels in OTA-induced cytotoxicity, additional pre-incubations with the glutathione modulators BSO and NAC were carried out. Incubation with NAC did not protect OTA-treated cells from cytotoxicity (Fig. 6A). Pre-incubation with 500  $\mu$ mol/l BSO decreased cell viability by 10%, while lower concentrations of BSO were without effect (Fig. 6B).

#### Effect on cellular glutathione levels

As shown in Fig. 7A, incubation with BSO for 24 h resulted in a substantial and dose-dependent depletion of cellular glutathione levels

in HepG2 cells. Concentrations as low as 5  $\mu$ M decreased cellular GSH by about 40% compared to untreated controls. A complete GSH-depletion was observed at 100  $\mu$ mol/l BSO. Supplementation with NAC did not increase cellular GSH (data not shown).

Incubation with OTA decreased GSH levels at concentrations of  $\geq 0.25$   $\mu$ mol/l (Fig. 7B). 1  $\mu$ mol/l OTA led to an approximately 30% decrease in cellular GSH as compared to controls. In contrast, lower concentrations (0.05 or 0.1  $\mu$ mol/l) of OTA resulted in a moderate increase in GSH levels in HepG2 cells.

#### Discussion

In line with previous studies our data indicate that OTA induces cytotoxicity in HepG2 cells in a dose-dependent manner (BALDI et al., 2004; RENZULLI et al., 2004; HUNDHAUSEN et al., 2005). More-

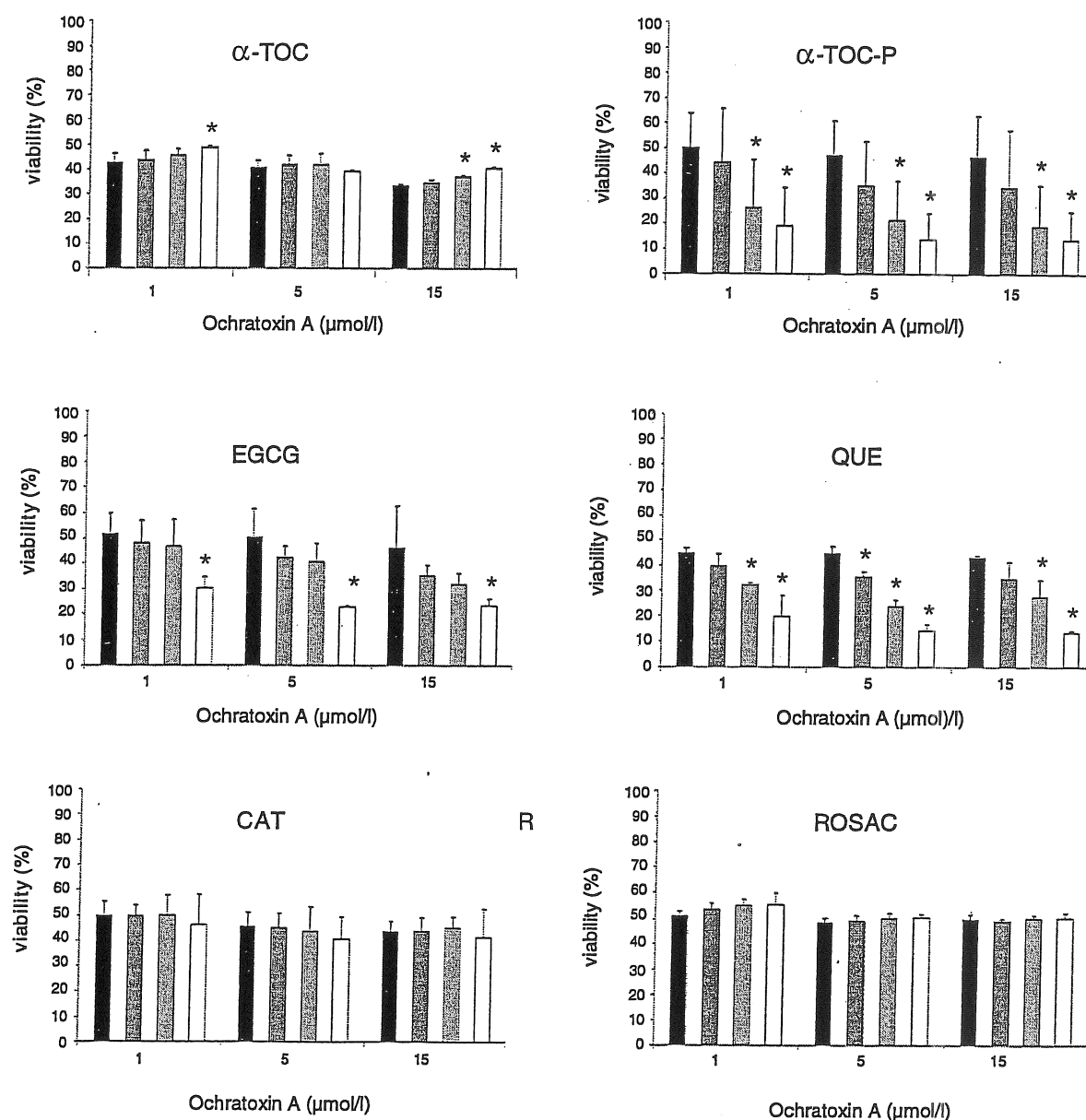


Fig. 5: Cytotoxicity of ochratoxin A (24 h, 1 - 15  $\mu$ mol/l) in HepG2 cells after pre-incubation with *RRR*- $\alpha$ -tocopherol ( $\alpha$ -TOC), *RRR*- $\alpha$ -tocopherolphosphate, epigallocatechin gallate (EGCG), quercetin (QUE), catechin (CAT), and rosmarinic acid (ROSAC) (24 h, 0  $\blacksquare$ , 25  $\square$ , 50  $\square$ , and 100  $\square$   $\mu$ mol/l). Data are mean  $\pm$  SD from two experiments in triplicate (n = 6). \*Indicates significant differences when compared to control (p < 0.05).

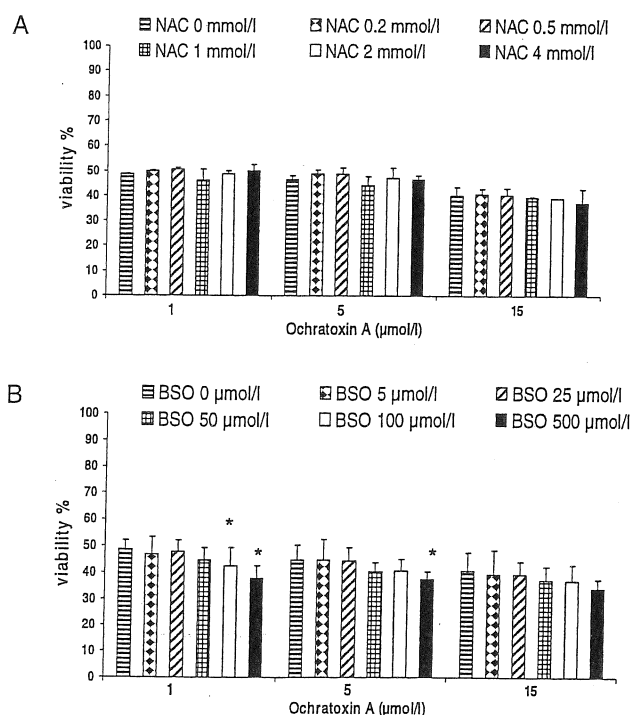


Fig. 6: Cytotoxicity of ochratoxin A (24 h, 1–15  $\mu\text{mol/l}$ ) on HepG2 cells after pre-incubation with N-acetylcysteine (NAC, A) (24 h, 0–4 mmol/l) or buthionine sulfoximine (BSO, B) (24 h, 0–500  $\mu\text{mol/l}$ ). Data are mean  $\pm$  SD from two experiments in triplicate ( $n = 6$ ). \*Indicates significant differences when compared to control (Dunnett;  $p < 0.05$ ).

over, we demonstrated a strong impact of serum protein concentration in the cell culture medium on the viability of OTA-exposed HepG2 cells. A decrease in serum concentration led to a dramatic increase in OTA-induced cell death in HepG2 cells. Importantly, under serum-free conditions even nanomolar OTA-concentrations resulted in a considerable loss of cell viability.

Concerning the protein binding properties of OTA, a high affinity of OTA for plasma proteins, both in animals and in humans, has been reported (RINGOT et al., 2006). In a wide range of species, including quail, mouse, rat and monkey, the fraction of free OTA in plasma was found to be less than 0.2% (HAGELBERG et al., 1989). Furthermore, it is well-known that OTA toxicity depends on its half-life in the body (O'BRIEN and DIETRICH, 2005), which in turn has been suggested to be influenced by binding of OTA to proteins (CHU, 1971; CHU, 1974). This is supported by findings that albumin-deficient rats had a 20- to 70-times faster OTA clearance from the systemic circulation than non-deficient rats (KUMAGAI, 1985).

However, studies investigating the dose-response relationship between the protein concentration in the cell culture medium and OTA toxicity are lacking. For this reason, a major focus of the present study was to determine the impact of different FCS levels in the culture medium on OTA-induced cell death in hepatocytes. We observed that a decrease of serum concentration in the medium was paralleled by a dramatic increase in OTA toxicity, which may indicate strong protein binding of OTA, possibly linked with inhibited cellular uptake and, thus, lower toxicity in HepG2 cells. Consistently, a previous study revealed decreased neurotoxic effects of OTA by protein binding to bovine serum albumin in embryonic chick brain and neural retina cell cultures (BRUININK and SIDLER, 1997). On the other hand, protein binding alone does not always decrease OTA

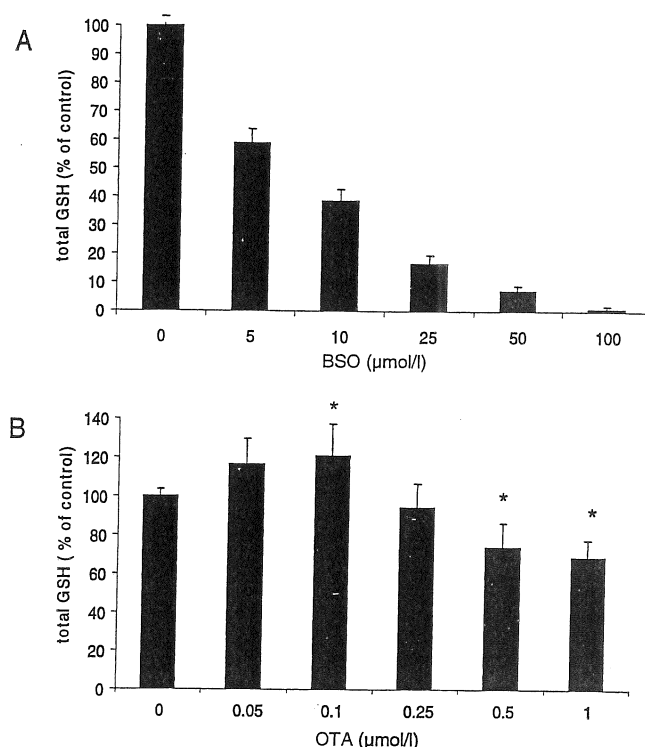


Fig. 7: Total glutathione (GSH, in % of control) after 24 h incubation with buthionine sulfoximine (BSO, A) or ochratoxin A (OTA, B) at the given concentrations. Data are mean  $\pm$  SD from two experiments in duplicate ( $n = 4$ , A) or triplicate ( $n = 6$ , B). \*Indicates significant differences when compared to control ( $p < 0.05$ ).

toxicity (STOJKOVIC et al., 1984). It has been suggested that binding of OTA to two small serum proteins (molecular mass 20,000 Da), being able to pass through the glomerular membrane, might explain the OTA-mediated nephrotoxic effect in mammals (STOJKOVIC et al., 1984).

Taken together, variations in the degree of protein binding as well as selective binding of OTA to distinct proteins may contribute to the often reported species-specific differences in OTA toxicity (DIETRICH et al., 2001; O'BRIEN et al., 2001; HEUSSNER et al., 2002).

In the present study, we investigated the potential cytotoxic effects of OTA at nanomolar concentrations. Previous studies have revealed that nanomolar OTA concentrations induced damage to renal and brain cells, due to an induction of apoptosis (GEKLE et al., 2000) and a loss of neuronal enzyme activity (MONNET-TSCHUDI et al., 1997). Our study, however, is the first to demonstrate that OTA causes cell death in hepatocytes at nanomolar concentrations, which is probably due to the absence of foetal calf serum (FCS) in the culture medium. Under serum-free conditions, incubation with 50, 100, or 250 nmol/l OTA for 24 hours resulted in an approximately 10, 40, or 60% decrease in viability of HepG2 cells, respectively. We observed toxicity with concentrations as low as 50 nmol/l OTA, which is within the range of OTA detected in human blood and serum. Between 6 and 26% of human blood and serum samples from the Balkan area contained OTA in the range of 1–35 ng/ml (2.5–88 nmol/l) and 1–40 ng/ml (2.5–100 nmol/l) (PETKOVA-BOCHAROVA et al., 1988), respectively, paralleled by an increased occurrence of the Balkan Endemic Nephropathy (BEN) (PETKOVA-BOCHAROVA et al., 1988). Considering the elimination half-life of OTA in humans of approximately 36 days (STUDER-ROHR et al., 2000), which is substantially

longer than that reported for other species – mice 40 h; rats 55–120 h (GALTIER *et al.*, 1979; HAGELBERG *et al.*, 1989); pigs 72–120 h (GALTIER *et al.*, 1981); monkeys 820 h (KUIPER-GOODMAN and SCOTT, 1989) – it is apparent that OTA constitutes a higher risk in humans.

Our study also demonstrated that at least 45% of the cells were still viable after incubation with 50  $\mu\text{mol/l}$  OTA for 24 h. In serum-free medium this maximum toxicity was reached at OTA concentrations as low as 0.25  $\mu\text{mol/l}$ . Incubation with 1–50  $\mu\text{mol/l}$  OTA did not further increase OTA-induced cell death in HepG2 cells. In accordance with our results it has been shown in LLC-PK1 cells that higher OTA-concentrations (high nmol/l to low  $\mu\text{mol/l}$ ) or longer times of exposure (96 h) did not further decrease cell viability (DREGER *et al.*, 2000). One hypothesis, which might explain the survival of a considerable number of HepG2 cells, suggests the existence of OTA carrier systems being saturated at low OTA-concentrations and, thus, not being able to increase intracellular OTA-transport at higher OTA-concentrations. Such OTA carriers, belonging to the family of human organic anion transporters, are well-known in proximal tubule cells from mice (JUNG *et al.*, 2001; BABU *et al.*, 2002). However, studies investigating the transport of OTA in liver cells are rare. It has been proposed, however, that the organic anion-transporting polypeptide (OATP) is involved in OTA-uptake in hepatocytes (KONTAXI *et al.*, 1996).

Neither the toxicokinetics of OTA, nor its precise toxicity mechanisms have been established to date. Nevertheless, the involvement of reactive oxygen species (ROS) in OTA toxicity seems to be likely (RAHIMTULA *et al.*, 1988; GAUTIER *et al.*, 2001; SCHAAF *et al.*, 2002). Therefore, one objective of our study was to counteract OTA toxicity by pre-incubation of HepG2 cells with various antioxidant test substances. We pre-incubated liver cells with 25, 50, or 100  $\mu\text{mol/l}$  of the respective antioxidants. Concerning  $\alpha$ -TOC these concentrations are similar to physiological plasma concentrations, which have been reported in the range of 25–40  $\mu\text{mol/l}$  (HENSLEY *et al.*, 2004), referring to flavonoid concentrations used in the cell culture experiments however exceeded physiological plasma concentrations (0.1–10  $\mu\text{mol/l}$ ) (MANACH *et al.*, 2004). To avoid interactions between OTA and the test compounds in the culture medium, cells were washed twice with PBS before incubation with OTA. None of the test compounds prevented HepG2 cells from OTA-induced cell death. This is in contrast to studies reporting protective effects of antioxidants towards OTA-induced cell damage. For example, 24 h of pre-treatment with 50  $\mu\text{mol/l}$  cyanidin-3-O-beta-glucopyranoside or 50  $\mu\text{mol/l}$  rosmarinic acid inhibited the cytotoxicity of 10  $\mu\text{mol/l}$  OTA in HepG2 cells by 25 and 35%, respectively (RENZULLI *et al.*, 2004; GUERRA *et al.*, 2005). Another experiment, also using OTA in serum-free medium, showed that three hours of pre-incubation with 1 nM  $\alpha$ -TOC decreased OTA-induced cytotoxicity in bovine mammary epithelium cells by 10% (BALDI *et al.*, 2004). The reasons for these conflicting results remain unclear, although cell-specific differences might be one plausible explanation.

We also found that EGCG, QUE, and  $\alpha$ -TOC-P enhanced the toxic effects of OTA.  $\alpha$ -TOC-P inhibits cell proliferation (OGRU *et al.*, 2004) and modulates membrane fluidity (REZK *et al.*, 2004). The latter might be the reason for a facilitated uptake of OTA into HepG2 cells, and thus, for the observed synergistic toxic effects of  $\alpha$ -TOC-P and OTA. Moreover, it has been demonstrated that both EGCG and QUE are able to exert not only antioxidant, but also pro-oxidant activity, partly due to the formation of hydrogen peroxide (METODIEWA *et al.*, 1999; ELBLING *et al.*, 2005; FERRARESI *et al.*, 2005), which might explain the increased cytotoxicity of OTA in HepG2 cells.

We further investigated the importance of the intracellular antioxidant glutathione for the prevention of OTA toxicity. Several studies report a depletion of glutathione by buthionine sulfoximine (BSO),

often paralleled by either loss of cell viability or increased susceptibility to compounds exerting oxidative stress (WRIGHT *et al.*, 1998; ANDERSON *et al.*, 1999; LONG *et al.*, 2000; ANDERSON and REYNOLDS, 2002; HONDA *et al.*, 2004). However, it has been shown that HepG2 cells are able to survive BSO treatment, which may be explained by an up-regulation of Bcl-2, a protein preventing apoptosis (D'ALESSIO, 2004). In the present study we confirmed the resistance of HepG2 cells to BSO treatment and showed, additionally, that there were no synergistic effects of OTA and BSO on cytotoxicity in HepG2 cells. Although GSH was dose-dependently depleted by both BSO and OTA (Fig. 7), pre-treatment with BSO did not enhance cell death (Fig. 6). Furthermore, NAC did not increase cellular GSH levels (data not shown), and was not able to prevent HepG2 cells against the toxic effects of OTA (Fig. 6). Consistent with these findings are the results of a study with cultured rat embryonic cells demonstrating a decrease of GSH induced by OTA, but no protection due to exogenous GSH supplementation (HONG *et al.*, 2000). On the other hand, it has been shown that NAC completely protected LLC-PK1 from OTA-induced cell death (SCHAAF *et al.*, 2002).

Interestingly, we observed a biphasic effect of OTA on intracellular GSH levels in HepG2 cells. 50 and 100 nmol/l OTA increased cellular GSH to 116 and 121% compared to controls, respectively, while OTA at concentrations between 0.1 and 1  $\mu\text{mol/l}$  dose-dependently decreased cellular GSH levels to approximately 70%. This effect may indicate that GSH is involved in the detoxification of OTA, possibly by directly binding to OTA as proposed by DAI and co-workers (2002). On the other hand, it has been shown for brain cells that OTA-exposure leads to an increased generation of nitric oxide (NO) (ZURICH *et al.*, 2005), which in turn is detoxified through conjugation with GSH.

In conclusion, the presented data indicate a dose-dependent effect of foetal calf serum concentrations in the cell culture medium on OTA-induced cytotoxicity in HepG2 cells. Supplementation with dietary antioxidants did not counteract cytotoxic effects of OTA. Although cellular GSH levels are modulated by OTA, mechanisms other than oxidative stress are likely to be involved in OTA-induced cell death in HepG2 cells.

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## **CHAPTER FIVE**

## ORIGINAL ARTICLE

# Ochratoxin A impairs Nrf2-dependent gene expression in porcine kidney tubulus cells

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## Keywords

ochratoxin A, Nrf2-dependent gene expression, kidney, pig

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## Summary

The mycotoxin, ochratoxin A (OTA), which is produced by *Aspergillus* and *Penicillium* subspecies, is frequently present in feedstuffs. Ochratoxin A exhibits a wide range of toxic activities including nephrotoxicity. However, the underlying molecular mechanisms of OTA-induced cellular nephrotoxicity have yet not been fully elucidated. Nrf2 is a basic leucine zipper transcriptional activator essential for the coordinated transcriptional induction of antioxidant and xenobiotic metabolizing enzymes in the kidney. Therefore, in the present study, the effects of OTA on the nuclear translocation and transactivation of the transcription factor Nrf2 as well as mRNA levels of Nrf2 target genes including glutathione-S-transferase and  $\gamma$ -glutamylcysteinyl synthetase have been studied in cultured porcine kidney tubulus cells (LLC-PK1). Nrf2 was induced by sulforaphane, a well-known activator of this transcription factor. Ochratoxin A significantly decreased  $\gamma$ -glutamylcysteinyl synthetase and glutathione-S-transferase mRNA levels in LLC-PK1 cells. Decreased mRNA levels of  $\gamma$ -glutamylcysteinyl synthetase and glutathione-S-transferase were accompanied by a lowered nuclear translocation and transactivation of Nrf2. Furthermore, OTA also lowered Nrf2 mRNA levels. Current data indicate that OTA nephrotoxicity may be, at least partly, mediated by an Nrf2-dependent signal transduction pathway.

## Introduction

Ochratoxin A (OTA) is an ubiquitous mycotoxin mostly produced by *Aspergillus* and *Penicillium* subspecies and present in feedstuffs (Bayman et al., 2002). Ochratoxin A exerts various toxic effects including nephro-, hepato-, terato-, immuno- and neurotoxicity (Kuiper-Goodman and Scott, 1989; Petzinger and Ziegler, 2000). The kidney is the main target tissue of OTA toxicity (Castegnaro et al., 1998; Luehe et al., 2003) and the chronic dietary exposure to OTA may be linked to the pathogenesis of nephropathy, a chronic tubulointerstitial kidney disease. Furthermore, OTA is considered as a key factor of porcine nephropathy, which is endemic in Denmark (Elling

and Moller, 1973; Krogh, 1987, 1992). Currently, the underlying mechanisms of OTA toxicity have not yet been fully elucidated. Several mechanisms have been proposed to explain OTA toxicity including inhibition of protein synthesis (Dirheimer and Creppy, 1991), mitochondrial activity alteration (Meisner and Chan, 1974) and oxidative stress (Petrik et al., 2003). Recently, it has been proposed that OTA may affect Nrf2 signal transduction pathways (Marin-Kuan et al., 2006; Cavin et al., 2007). However, so far, systematic studies in cell culture models also relevant to animal nutrition are missing.

Under basal conditions, the transcription factor Nrf2 is bound to the cytosolic Kelch-like ECH-associated protein 1 (Keap1), a cysteine-rich

actin-associated repressor protein that keeps Nrf2 complexed in the cytosol (Dinkova-Kostova et al., 2002; Itoh et al., 2003; Kang et al., 2004). Several groups demonstrated the rapid degradation of Nrf2 by the ubiquitin-proteasome pathway and the stabilization of Nrf2 by phase II inducers like sulforaphane (SFN) (Alam et al., 2003; Itoh et al., 2003; McMahon et al., 2003; Nguyen et al., 2003). In the presence of electrophilic substances, the Nrf2-Keap1 complex is disrupted, Nrf2 translocates into the nucleus, binds to the antioxidant response element (ARE) (Dinkova-Kostova et al., 2002; Wakabayashi et al., 2004) and controls the expression of phase II enzymes including  $\gamma$ -glutamylcysteinyl synthetase ( $\gamma$ GCS) and glutathione-S-transferase (GST) (McMahon et al., 2001; Chanas et al., 2002). The aim of this study was to further characterize the effect of OTA on the nuclear translocation and transactivation of the transcription factor Nrf2 as well as mRNA levels of Nrf2 and its target genes including GST and  $\gamma$ GCS in cultured kidney tubulus cells derived from pigs.

## Materials and methods

### Chemicals

Ochratoxin A and SFN were obtained from Sigma (Deisenhofen, Germany). An OTA stock solution of 25 mmol/l was prepared in methanol and stored at  $-20^{\circ}\text{C}$ . Sulforaphane was dissolved in dimethylsulfoxide (5 mmol/l stock solution) and stored at  $-20^{\circ}\text{C}$  until use. Cell culture medium and supplements were obtained from PAA (Coelbe, Germany). Western blotting reagents and materials were purchased from Bio-Rad (Muenchen, Germany). All other chemicals and reagents were acquired from Sigma, unless specified otherwise.

### Cell culture and treatments

Porcine renal epithelial proximal tubulus cells (LLC-PK1) purchased from the Institute for Applied Cell Culture (IAZ, Munich, Germany) were cultured in Dulbecco's modified Eagle's medium with high glucose content (4.5 g/l). Medium was supplemented with 10% foetal bovine serum (FBS), 100 IU/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. Cells were maintained in 75  $\text{cm}^2$  flasks kept at  $37^{\circ}\text{C}$  under standard conditions. A maximum of 15 in-house passages were used for the experiments. Cells were seeded at an initial density of  $0.15 \times 10^6$  cells/ $\text{cm}^2$ , grown for 48 h and exposed to SFN, OTA and OTA plus SFN at varying concentrations for different time-points (6, 12 and 24 h). After treatment, cyto-

toxicity was measured via neutral red assay or cells were harvested for Nrf2-dependent gene expression and measurement of Nrf2-transactivation via reporter gene assay and western blot analysis.

### Determination of cell viability

The cytotoxicity of SFN, OTA and SFN/OTA in LLC-PK1 was evaluated by the neutral red assay (Borenfreund and Puerner, 1985). Cells seeded in 24-well plates were treated with SFN (0–50  $\mu\text{mol}/\text{l}$ ), OTA (0–50  $\mu\text{mol}/\text{l}$ ) and 5  $\mu\text{mol}/\text{l}$  SFN/ 0–50  $\mu\text{mol}/\text{l}$  OTA. The medium was replaced after 24 h by medium containing neutral red (50  $\mu\text{g}/\text{ml}$ , Carl Roth, Karlsruhe, Germany) and incubated for 3 h. Neutral red medium was aspirated and cells were washed with phosphate buffered saline (PBS). The incorporated neutral red dye was extracted using a bleaching solution (50% ethanol, 1% acetic acid, 49%  $\text{H}_2\text{O}$ ) and the absorbance read at 540 nm using a plate reader (Labsystems, Helsinki, Finland). Cell viability was expressed as percent viability compared to non-treated control cells.

### Western blot analysis

LLC-PK1 cells were incubated with SFN and SFN plus OTA supplemented medium for 6, 12 and 24 h. Cells were harvested in PBS and centrifuged at 800  $g$  ( $4^{\circ}\text{C}$ , 3 min). The remaining cell pellet was resuspended in 10 mmol/l *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.9 (10 mmol/l KCl, 1.5 mmol/l  $\text{MgCl}_2$ , 0.5 mmol/l dithiothreitol, 0.1% Nonidet-P40 and protease inhibitors) and incubated on ice for 15 min and centrifuged (10 min,  $4^{\circ}\text{C}$ , 4000  $g$ ). The cell pellets were resuspended in ice-cold 40 mmol/l HEPES, pH 7.9 (400 mmol/l KCl, 1 mmol/l dithiothreitol, 5 mol/l NaCl, 10% glycerol and protease inhibitors), left on ice for 30 min and centrifuged at 18 000  $g$  at  $4^{\circ}\text{C}$  for 30 min. Supernatants (nuclear extracts) were removed and stored at  $-80^{\circ}\text{C}$  until further analysis. Protein concentrations of the samples were determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Thirty microgram protein was mixed with loading buffer [0.5 mol/l Tris buffer, pH 6.8, 87% glycerol, 10% sodium dodecyl sulfate (SDS), 0.5% bromophenol blue,  $\text{H}_2\text{O}$ , 0.05%  $\beta$ -mercaptoethanol] and denatured at  $95^{\circ}\text{C}$  for 5 min. Subsequently, the samples were separated on a 10% SDS-polyacrylamide gel (Roth, Karlsruhe, Germany) in Tris/Glycine/SDS buffer and blotted onto an immunoblot polyvinylidene difluoride membrane. The membrane was blocked with 3% non-fat dried

milk in Tris-buffered saline, pH 7.4, in 0.05% Tween-20 for 2 h and probed with polyclonal rabbit anti-human Nrf2 primary antibody (Santa Cruz, Heidelberg, Germany) in a 1:500 dilution at 4 °C overnight. Then, the membrane was incubated with a goat anti-rabbit IgG secondary antibody (1:4000) conjugated with horseradish peroxidase for 45 min. Specific bands were visualized by enhanced chemiluminescence (ECL) reagent on a ChemiDoc system (both Bio-Rad). The predicted size for Nrf2 is 68 kDa, which corresponds to the lower band in the western blots. The upper band (100 kDa) refers to the Nrf2/actin complex, which is formed upon activation and nuclear translocation of Nrf2 (Kang et al., 2002).

#### Transient transfection and luciferase reporter gene assay

To investigate the effects of OTA on Nrf2-transactivation and transcription of Nrf2 target genes, a pGL3 plasmid containing the ARE-motif from the rat NQO1-promoter linked to a *luc* sequence was used. The plasmids pARE\_pGL3 of rat NQO1 (kindly provided by C. Wruck, University of Kiel, Germany), pGL3, phRLTK (Promega, Mannheim, Germany) were amplified in *Escherichia coli* BL 21 (Invitrogen, Karlsruhe, Germany) grown in *Luria Bertoni* medium supplemented with 100 µg/ml ampicillin at 37 °C overnight. Bacteria were harvested by centrifugation and plasmid DNA was extracted by QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's description. LLC-PK1 cells were seeded into 24-well plates, cultured for 24 h and transfected with phRLTK (endogenous control) and either pARE\_pGL3 or pGL3 (control) using FuGene HD transfection reagent (Roche, Mannheim, Germany) for another 24 h. Following transfection, cells were incubated with SFN (5 µmol/l), OTA (2.5, 10, 25 µmol/l) and SFN plus OTA (using concentrations mentioned above) for another 24 h. Cell lysis and determination of luciferase and renilla activity were performed by a commercially available Dual luciferase kit (Promega) according to the manufacturer's instructions. Firefly and renilla luciferase activities were measured in a luminometer (Tecan

GmbH, Crailsheim, Germany). Relative luciferase activity was calculated by the firefly luciferase to renilla luciferase ratio. The empty pGL3 plasmid served as control. Luciferase activity of pARE\_pGL3 plasmid without SFN treatment served as basis (100% control) for OTA-treated cells, and SFN-induced luciferase activity was used as basis (100% control) for SFN/OTA-coapplied cells.

#### RNA isolation and real time PCR

To determine mRNA levels of Nrf2 and Nrf2 target genes  $\gamma$ GCS (heavy chain) and GST, LLC-PK1 cells were stimulated for 12 h with the highest non-toxic concentration of OTA (25 µmol/l), SFN (5 µmol/l) and SFN plus OTA. After 12 h treatment, the cells were washed with PBS and lysed with TRIsure (Bioline, Luckenwalde, Germany). RNA was isolated according to the manufacturer's instructions. Total RNA was quantified photometrically at 260 nm and stored at -80 °C until analysis. Quality of RNA was determined by agarose gel electrophoresis. Primers for human  $\gamma$ GCS (as porcine sequence is unknown) and porcine GST (no isoform-specified information available) were designed by standard software tools (Primer3, NCBI BLAST) and synthesized by MWG (Ebersberg, Germany). Primer sequence information is given below (Table 1). Real time PCR was performed as one-step procedure with QuantiTect Sybr-Green Kit from Qiagen (Hilden, Germany) on the Rotorgene RG-3000 machine. Serial dilution of control cell RNA was used to generate standard curves for calculation of sample mRNA level. Gene expression was normalized to the housekeeping gene 18SRNA and the results were calculated as per cent of medium-treated control cells. PCR product size was controlled by gel electrophoresis.

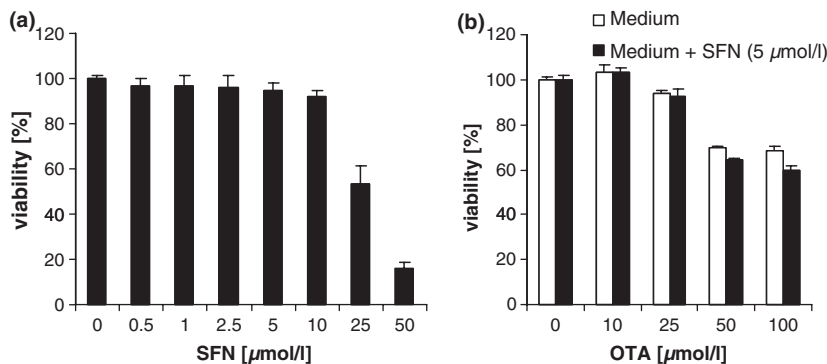
#### Statistical analysis

Results are expressed as mean  $\pm$  SD. Statistical analysis was carried out with SPSS Version 15.0 (SPSS GmbH Software, Munich, Germany). Normally distributed data were compared via *t*-test; otherwise, the non-parametric Mann-Whitney *U*-test was used.

**Table 1** Primer sequences and conditions for real time PCR experiments

Gene	Forward primer	Reverse primer	Annealing temperature
18SRNA	5'-AGTCGGCATCGTTTATGGTC-3'	5'-CCGCAGCTAGGAATAATGGA-3'	57 °C
$\gamma$ GCS	5'-GGCGATGAGGTGGAATACAT-3'	5'-CCTGGTGTCCCTTCAATCAT-3'	58 °C
GST	5'-CCTCCTATGGATGCGAAAAA-3'	5'-ATGATATTGCGTGCGAACAA-3'	58 °C

GCS,  $\gamma$ -glutamylcysteinyl synthetase; GST, glutathione-S-transferase.



**Fig. 1** (a) Sulforaphane (SFN)-, (b) ochratoxin A (OTA)- and SFN/OTA- induced cytotoxicity in LLC-PK1 cells. Cells were treated with increasing concentrations of SFN and OTA and SFN/OTA for 24 h. Cytotoxicity was determined with neutral red assay and viability is expressed as percentage of control cells. Data represent mean + SD of three independent experiments performed in triplicate.

## Results

### Cytotoxic effects of OTA and SFN on cell viability

To assure that SFN does not affect cell viability in the following experiments, kidney cells were incubated with increasing concentrations of SFN (1–50 µmol/l) for 24 h. As shown in Fig. 1a, SFN did not influence cell viability up to 10 µmol/l. However, 25 µmol/l led to a 50% reduction of viability in LLC-PK1 cells and 50 µmol/l decreased viability approximately more than 80% compared with control. Based on the cytotoxicity data, a SFN concentration of 5 µmol/l was chosen for all further experiments.

To determine the toxicity of OTA, cell viability was analysed following treatment of cells with increasing concentrations of OTA ranging from 0 to 100 µmol/l in control and SFN-supplemented medium. Ochratoxin A and a co-treatment of OTA and SFN did not affect cell viability up to 25 µmol/l. However, 50 and 100 µmol/l OTA reduced cell viability by 30% and 40% alone and in combination with SFN as shown in Fig. 1b. Based on these findings, the highest non-cytotoxic concentration of 25 µmol/l OTA was chosen for all further experiments.

cells were transiently transfected with ARE plasmid constructs and transactivation was measured by a luciferase reporter gene assay. As shown in Fig. 3, SFN induced a threefold transactivation of Nrf2 whereas OTA lowered luciferase activity both in the absence and presence of SFN.

### OTA and SFN induced changes in gene expression

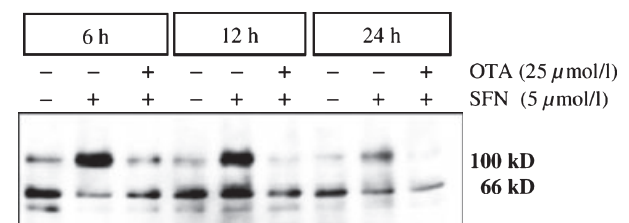
To study the effects of SFN, OTA and SFN/OTA on the mRNA levels of Nrf2 and its target genes including GST and γGCS, LLC-PK1 cells were stimulated for 12 h with non-cytotoxic concentrations of OTA and SFN. The effects of OTA, SFN and OTA plus SFN on the transcriptional levels of GST, γGCS and Nrf2 are summarized in Fig. 4. Treatment of cells with SFN significantly increased the mRNA level of GST up to 1.5-fold, whereas OTA- and SFN/OTA administration significantly decreased GST transcription (Fig. 4a). Similar results were obtained in the case of γGCS mRNA (Fig. 4b). Interestingly, OTA lowered also Nrf2 mRNA levels (Fig. 4c).

### Effects of OTA and SFN on Nrf2 nuclear translocation

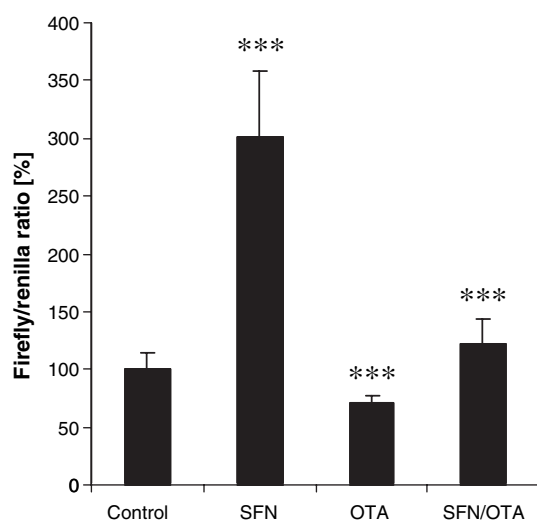
To study the translocation of the Nrf2 protein into the nucleus after stimulation of LLC-PK1 with SFN in the presence and absence of OTA, western blot analyses were performed with nuclear extracts of porcine kidney cells. As shown in Fig. 2, Nrf2 nuclear translocation was significantly induced by SFN and decreased because of OTA treatment compared with LLC-PK1 cells treated with SFN only.

### Effects of OTA and SFN on Nrf2 transactivation

To investigate the effects of OTA, SFN and SFN/OTA on ARE-dependent transactivation by Nrf2, kidney



**Fig. 2** Western blot analyses of Nrf2 protein in nuclear extracts of LLC-PK1 cells. Nuclear extracts were prepared from LLC-PK1 cells treated with sulforaphane and SFN ochratoxin A for 6, 12 and 24 h. Nrf2 translocates into the nucleus and binds to the antioxidant response element motif in response to SFN exposure in a time-dependent manner. In the presence of OTA, Nrf2 translocation decreases significantly. The immunoblot shown is one representative out of three independent experiments.



**Fig. 3** Effect of ochratoxin A (OTA) on the antioxidant response element (ARE)-dependent transactivation by Nrf2 in LLC-PK1 cells. Cells were transfected with a pGL3 plasmid containing the ARE-motif (from rat NQO1 promoter). Twenty-four hours after transfection, cells were incubated with control medium or medium containing sulforaphane (SFN, 5  $\mu\text{mol/l}$ ) supplemented with OTA (2.5, 10 and 25  $\mu\text{mol/l}$ ) for 24 h. Relative luciferase activity was determined by calculating the firefly luciferase to renilla luciferase ratio. The transfected empty luciferase plasmid (pGL3) served as a control. Values are means  $\pm$  SD of three experiments measured in quadruplicate. \*\*\*Significantly different compared with control ( $p < 0.001$ ; Mann-Whitney U-test).

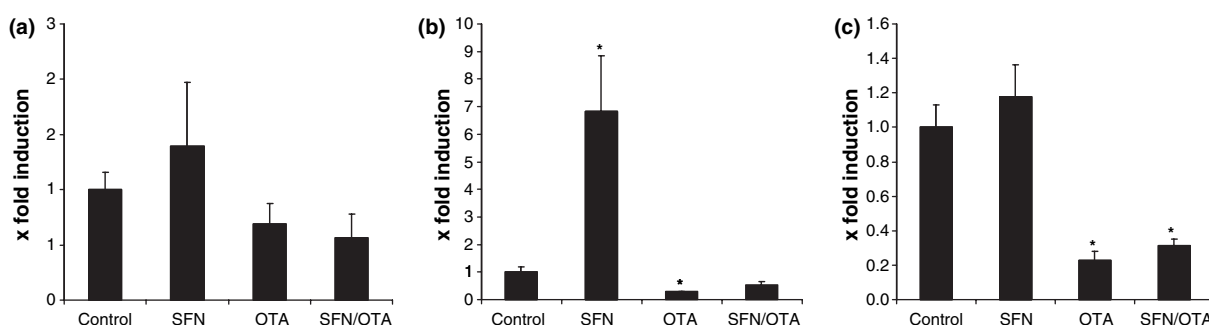
## Discussion

The mycotoxin OTA is a nephrotoxin and carcinogen in rodents, whereas its potential cancer promoting activity in humans is still under scientific debate (Pfohl-Leszkowicz *et al.*, 2007; Stefanović *et al.*, 2007). Although the nephrotoxic potential of OTA has been recognized for many years, the mechanisms underlying its nephrotoxicity have not yet

been fully elucidated. It has been suggested that the transcription factor Nrf2 may be implicated in OTA toxicity (Marin-Kuan *et al.*, 2006; Cavin *et al.*, 2007). Therefore, the present study aimed to further characterize the impact of OTA on Nrf2 activation in kidney cells.

## OTA cytotoxicity

As the kidney is the main target organ of OTA, LLC-PK1 cells, a porcine kidney cell culture model was used. Several studies revealed relatively high OTA concentrations in kidney cells following OTA exposure probably as a result of the reabsorption of OTA at all nephron segments (Gekle *et al.*, 2005) and an active transport of OTA via the organic anion transporter (Jung *et al.*, 2001). Concerning the susceptibility of cells towards OTA, there are great differences between various cell types. However, all cells have in common that the cytotoxic effects of OTA are time- and dose-dependent (Schaaf *et al.*, 2002; Baldi *et al.*, 2004; Guerra *et al.*, 2005; Hundhausen *et al.*, 2005). In addition, incubation conditions substantially influence OTA toxicity in cellular systems because the binding of OTA to serum proteins from FBS supplementation prevents its cellular uptake. However, many studies reported in the literature were performed under serum-free conditions (Schaaf *et al.*, 2002; Baldi *et al.*, 2004; Guerra *et al.*, 2005) leading to a cellular toxicity of OTA which is approximately 10-fold higher compared with experiments using serum (Schwerdt *et al.*, 1999; Boesch-Saadatmandi *et al.*, 2006). For the present investigation, cells were grown in medium supplemented with 10% of FBS to provide optimal growth conditions. Under these experimental conditions, OTA did not exhibit any cytotoxic effects



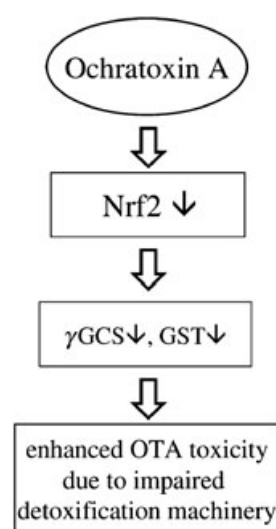
**Fig. 4** Effects of ochratoxin A (OTA), sulforaphane (SFN) and SFN/OTA on glutathione-S-transferase (a),  $\gamma$ -glutamylcysteinyl synthetase (b) and Nrf2 (c) mRNA levels in LLC-PK1 cells. Cells were incubated for 12 h with either 5  $\mu\text{mol/l}$  SFN or 25  $\mu\text{mol/l}$  OTA or co-incubated with 5  $\mu\text{mol/l}$  SFN and 25  $\mu\text{mol/l}$  OTA. Data represent mean  $\pm$  SD of three experiments performed in duplicate. \*Significantly different compared with control ( $p < 0.05$ ; Mann-Whitney U-test).

in LLC-PK1 cells up to 25  $\mu\text{mol/l}$  after 24 h incubation. However, it needs to be taken into account that this OTA concentration is manifold higher than those concentrations detected in animal and human plasma (Studer-Rohr et al., 2000).

#### Modulation of Nrf2 nuclear translocation, transactivation and Nrf2-dependent gene expression by OTA

Sulforaphane significantly induced Nrf2 nuclear translocation at any time-point studied. Nuclear translocation is following disruption of Nrf2/Keap1 complex caused by SFN (Dinkova-Kostova et al., 2002; Wakabayashi et al., 2004), which enhances the level of Nrf2 as well as its stability (Jeong et al., 2005). The Nrf2 protein seems to be stabilized and protected from degradation by ubiquitin-proteasome pathway as Nrf2 levels in the cytoplasm were not changed (data not shown), whereas Nrf2 levels in the nucleus were increased in response to SFN treatment. The effect of OTA on nuclear translocation of Nrf2 protein was investigated only in the presence of SFN because protein activation is only evident after stimulation. Interestingly, OTA decreased Nrf2 activity also on the mRNA level. The lowered Nrf2 mRNA levels in the kidney after OTA treatment are possibly caused by inhibition of its translocation into the nucleus, which could be explained by the fact that Nrf2 regulates its own gene expression (Kwak et al., 2002).

It has been recently shown that SFN induces a number of Nrf2-regulated genes (Thimmulappa et al., 2002). Thus in this study, mRNA levels of Nrf2-regulated enzymes including GST and  $\gamma\text{GCS}$  were analysed in response to OTA treatment. Glutathione-S-transferase, a multigen family of enzymes, detoxify endobiotic and xenobiotic compounds by covalent linkage of glutathione to a hydrophobic substrate forming less reactive and more polar glutathione-S-conjugates, which are then eliminated via urine (Eaton and Bammler, 1999). Six GST classes have been described yet: GST-A, -M, -P, -T, -Z, -O (Hayes et al., 2005), which exhibit a tissue- and species-specific expression pattern. In pigs, however, information regarding different subclasses of GST is very limited.  $\gamma$ -glutamylcysteine synthetase (also known as glutamate cysteine ligase) is a key enzyme in the glutathione biosynthesis pathway catalyzing the synthesis of  $\gamma\text{GCS}$  from L-glutamate and cysteine. This reaction represents the rate limiting step in the synthesis of glutathione, which is the most important cytosolic antioxidant in the kidney (Lu, 1999; Blokhina et al., 2003). In previous studies, the cellu-



**Fig. 5** Potential signal transduction pathway by which ochratoxin A (OTA) may impair its own detoxification. GCS,  $\gamma$ -glutamylcysteinyl synthetase; GST, glutathione-S-transferase.

lar content of glutathione in LLC-PK1 cells was significantly decreased following OTA treatment, either indicating an inhibition of  $\gamma\text{GCS}$  expression by OTA or an increased conjugation of OTA with glutathione (Schaaf et al., 2002). The down-regulation of  $\gamma\text{GCS}$  by OTA is likely to result in chronic elevation of oxidative stress in the kidney as reported previously (Costa et al., 2007). One important finding of this study is that OTA significantly decreased GST and  $\gamma\text{GCS}$  mRNA levels most likely via an Nrf2 signal transduction pathway. As GST and  $\gamma\text{GCS}$  are centrally involved in OTA detoxification, our data indicate that OTA may inhibit its own detoxification machinery (Fig. 5).

Future *in vivo* studies are necessary to test the hypothesis whether the finding in cultured porcine kidney cells regarding the effect of OTA on Nrf2, GST and  $\gamma\text{GCS}$  have also relevance in pigs.

#### Acknowledgements

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## CHAPTER SIX

# **Ochratoxin A lowers mRNA levels of genes encoding for key proteins of liver cell metabolism**

**Running title: ochratoxin A and gene expression in hepatocytes**

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**Keywords:** ochratoxin A, gene expression, liver, HepG2

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## Abstract

Ochratoxin A (OTA) is a nephro- and hepatotoxic mycotoxin that frequently contaminates food and feedstuffs. Although recent studies indicate that OTA modulates renal gene expression, little is known regarding its impact on differential gene expression in the liver. We therefore performed a microarray study of the HepG2 liver cell transcriptome in response to OTA exposure (0; 0.25; 2.5  $\mu\text{mol/l}$  for 24 h) using Affymetrix GeneChip technology. Selected microarray results were verified by real-time PCR and Western blotting as independent methods. Out of 14500 genes present on the microarray, 13 and 250 genes were down regulated by 0.25 and 2.5  $\mu\text{mol/l}$  OTA, respectively. Decreased mRNA levels of calcineurin A beta (PPP3CB) which regulates inflammatory signalling pathways in immune cells, and of the uncoupling protein 2 (UCP2) that has been suggested to control the production of reactive oxygen species (ROS), were observed in response to 0.25  $\mu\text{mol/l}$  OTA. A particularly strong down regulation due to 2.5  $\mu\text{mol/l}$  OTA was evident for the mRNA levels of the insulin-like growth factor binding protein 1 (IGFBP1) and tubulin beta 1 (TUBB1) which have been demonstrated to function as a pro-survival factor in hepatocytes and as an important cytoskeletal component, respectively. In addition, many genes involved in energy and xenobiotics metabolism, including phosphoglycerate kinase 1 (PGK1), stearoyl-Coenzyme A desaturase 1 (SCD), and glutathione S-transferase omega 1 (GSTO1) were down regulated by OTA. Furthermore, OTA significantly inhibited the capacitative calcium entry in HepG2 cells, indicating an alteration of calcium homeostasis. Overall, the present data indicate that OTA dose-dependently affects multiple genes encoding for key proteins of liver cell metabolism.

## Introduction

OTA is produced by several *Aspergillus* and *Penicillium* species and was firstly isolated from *Aspergillus ochraceus* [1]. Chemically, OTA consists of a dihydroisocoumarin moiety linked to phenylalanine (Figure 1). Over the last years, ochratoxin A (OTA) which is frequently present in cereals [2], has received increasing attention because it has also been detected in relatively large amounts in non cereal plant products including coffee, nuts, spices, olives, grapes, and beans [3, 4]. Moreover, OTA has also been found in meat [5, 6] and in human blood, urine and milk [7-10]. Numerous animal studies demonstrated predominantly nephrotoxic, but also hepatotoxic, teratogenic, immunosuppressive, and recently also genotoxic effects of OTA [11-14]. The International Agency for Research on Cancer (IARC) classified OTA as a putative human carcinogen [15].

The toxicity mechanisms of OTA are incompletely understood. Although it has been shown that OTA damages the mitochondria [16], inhibits protein biosynthesis [17] and causes oxidative stress [18, 19], little is known about the mode of action of OTA on the molecular level. Particularly, the impact of OTA on hepatic gene expression has remained elusive.

A recent microarray study in primary rat kidney cells and a continuous kidney cell line revealed an influence of OTA on the transcription of genes involved in DNA repair, antioxidative defence, and inflammation [20]. Additionally, the study demonstrated that OTA modulates the same set of genes *in vitro* and *in vivo*. Moreover, recent data suggest an impairment of the “nuclear factor (erythroid-derived 2)-like 2” (Nrf2) dependent gene expression [21, 22].

Only a few hepatic genes have been reported to be affected by OTA including genes involved in the detoxification of reactive oxygen species (e.g. superoxide dismutase) and in tumour development (e.g. connexin) [23]. Since the molecular targets of OTA are largely unknown we performed a large-scale gene expression profile to study the impact of OTA on the liver transcriptome using gene chip technology.

## Materials and Methods

### *Cell culture, RNA isolation and microarray production*

HepG2 cells were seeded at a density of  $2.5 \times 10^5$  cells/ml in six well plates (3 ml/well) and cultivated under standard conditions (RPML-1640 medium with 10% foetal calf serum, 37°C, 5% CO<sub>2</sub>). At confluence two wells were treated identically with 0, 0.25 and 2.5  $\mu\text{mol/l}$  OTA (Sigma, Deisenhofen, Germany) for 24 h. After washing twice with phosphate-buffered saline (PBS), cells of the identically treated wells were pooled and total RNA was extracted by use of the RNeasy mini kit (Qiagen, Hilden, Germany). Treatment was repeated once in an independent experiment, resulting in a total of six RNA samples, two for each experimental condition (0, 0.25, and 2.5  $\mu\text{mol/l}$  OTA) (n=2). Agarose gel electrophoresis was performed to check the RNA quality. Purity and concentration of RNA were determined spectrophotometrically at 260 nm (Beckman Coulter, Fullerton, USA). The six RNA samples were further processed for microarray hybridization as described in the “Affymetrix GeneChip Expression Analysis Technical Manual” by a regional German Affymetrix Service Provider, “KFB - Center of Excellence for Fluorescent Bioanalytics” (Regensburg,

Germany), resulting in six microarrays of the "Human Genome U133A 2.0" type that contains more than 22000 probe sets representing 14500 well-characterized human genes.

#### *Real-time polymerase chain reaction (PCR; SybrGreen®) experiments*

For real-time PCR verification cell culture experiments were performed as described in the previous section with the exception that cells from identically treated cells were not pooled but RNA was isolated from cells of each single well of the cell culture plate. The procedure was repeated twice in independent experiments resulting in a total of 18 RNA samples, six for each experimental condition (0, 0.25, and 2.5  $\mu\text{mol/l}$  OTA) ( $n=6$ ). Primers were designed for glutathione S-transferase omega 1 (GSTO1), phosphoglycerate kinase 1 (PGK1), stearoyl-Coenzyme A desaturase 1 (SCD), tubulin beta 1 (TUBB1), insulin-like growth factor binding protein 1 (IGFBP1), and the uncoupling protein 2 (UCP2) with standard software tools (Spidey, Primer3, NCBI BLAST) and synthesized by MWG (Ebersberg, Germany). Primer sequence information is given in Table 1.

Real time PCR was performed as one-step procedure with the QuantiTect SybrGreen Kit (Qiagen) on the Rotorgene RG-3000 thermo cycler (Corbett Life Science, Sidney, Australia). Gene expression was normalized to the housekeeping gene 18SRNA (Qiagen) and results were calculated in percent of control cells.

#### *Microarray data analysis*

The raw data files (Cel files) were processed and normalized by dCHIP algorithm [24]. The gene expression values (arbitrary units, a.u.) were calculated with the "Perfect Match (PM) / Mismatch (MM) difference model". In the following "Compare samples" analysis the two control arrays were firstly compared with the two "low dose" and secondly with the "high dose" arrays. For each gene the ratio of the mean expression values of control and experiment were calculated, resulting in the so-called "fold change" (FC) value. From the list of genes obtained we discriminated between genes with detectable ("present") and undetectable ("absent") expression values. Genes were selected on the basis of the following criteria: 1. "present" signal on all arrays, 2. "present" signal on both control arrays and "absent" signal on both experiment arrays, 3. "absent" signal on both control arrays and "present" signal on both experiment arrays. Furthermore, we selected for genes that showed a fold change  $\geq + 2.0$ , or  $\leq - 2.0$  with  $P < 0.05$ . In order to exclude noise signals, genes with a mean expression level lower than 27 a.u. representing 5% of the mean control expression level, were not considered. Finally, we selected genes on the basis of reproducibility by considering only those genes that revealed similar expression values within the two control or the two experiment arrays (coefficient of variation of expression value  $\leq 25\%$ ).

#### *Western blotting*

HepG2 cells were incubated with 0, 0.25 and 2.5  $\mu\text{mol/l}$  OTA for 48 h, harvested in ice cold PBS and centrifuged. The remaining cell pellets were resuspended in RIPA buffer (25 mmol/l Tris/HCl pH 7.6, 150 mmol/l NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitor (Protease Inhibitor Cocktail, Sigma), and incubated on ice for 30 min. After centrifugation, supernatants were removed and stored at  $-80^\circ\text{C}$  until further analysis. Protein concentrations of the samples were determined by BCA protein assay (Pierce, Rockford, Illinois, USA). 40  $\mu\text{g}$  of protein was mixed with loading buffer (0.5 mol/l Tris buffer, pH 6.8, 87% glycerol, 10% SDS, 0.5% bromophenol blue, 0.05%  $\beta$ -mercaptoethanol) and denaturated at  $95^\circ\text{C}$  for 5 min. Subsequently, the samples were separated on a 10% SDS/polyacrylamide gel (Roth, Karlsruhe, Germany) in Tris/Glycine/SDS buffer and blotted onto immuno-blot PVDF membranes. The membranes were blocked with 3% non-fat dried milk in Tris-buffered saline/Tween-20 (TBS/T) for 2 h and probed with the primary antibodies for IGFBP1 and GSTO1 (Santa Cruz, Heidelberg, Germany) at  $4^\circ\text{C}$  overnight (1:200 dilution). In the following, the membranes were incubated with goat anti-mouse IgG secondary antibodies (1:4000 dilution) (Santa Cruz, Heidelberg, Germany) conjugated with horseradish peroxidase for 45 min. Specific bands were visualized by ECL reagent on a ChemiDoc system (Bio-Rad).

#### *Measurement of intracellular $\text{Ca}^{2+}$ ("Calcium imaging")*

The impact of OTA on the intracellular calcium concentration of HepG2 cells was determined by fluorescence imaging. The acetomethyl ester of the fluorescent dye fura-2 (fura-2/AM) is an uncharged molecule that can easily permeate into the cell, where unspecific esterases cleave the lipophilic groups. This results in the charged form of the dye (referred to as fura-2) which binds to the target ion  $\text{Ca}^{2+}$ . Upon calcium binding the fluorescent excitation maximum of fura-2 undergoes a blue shift from 380 nm ( $\text{Ca}^{2+}$ -free) to 340 nm ( $\text{Ca}^{2+}$ -saturated). Therefore, the ratio of the emissions at those wavelengths is directly correlated to the amount of intracellular calcium.

Fluorescence measurements were carried out with an inverted phase-contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany). Cells were excited alternatively at 340 or 380 nm and the light was deflected by a dichroic mirror into either the objective (Fluar 40 $\times$ /1.30 oil, Zeiss, Oberkochen, Germany) or a camera (Proxitronic, Bensheim, Germany). Emitted fluorescence intensity was recorded at 505 nm and data acquisition was accomplished by using specialized computer software (Metafluor, Universal Imaging,

Downingtown, USA). As a measure for the increase of cytosolic  $\text{Ca}^{2+}$  activity, the peak of the change in the 340/380 nm ratio was calculated for each experiment.

HepG2 cells were pre-treated with 2.5  $\mu\text{mol/l}$  OTA for 24 h at 37°C or left untreated and subsequently loaded with 3  $\mu\text{mol/l}$  fura-2/AM for 30 min at 37°C. After running a baseline in the presence of extracellular  $\text{Ca}^{2+}$ , changes in cytosolic  $\text{Ca}^{2+}$  were monitored upon depletion of the intracellular  $\text{Ca}^{2+}$  stores. Experiments were carried out prior to and during exposure to  $\text{Ca}^{2+}$ -free solution. In the absence of  $\text{Ca}^{2+}$  the intracellular  $\text{Ca}^{2+}$  stores were depleted by inhibition of the sarco/endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) by thapsigargin (1  $\mu\text{mol/l}$ ). Store-depletion subsequently activates plasma membrane calcium channels, allowing an influx of calcium into the cytosol when calcium was re-added to the bath.

Experiments were performed with Ringer solution containing: 125 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l  $\text{MgSO}_4$ , 2 mmol/l  $\text{CaCl}_2$ , 2 mmol/l  $\text{Na}_2\text{HPO}_4$ , 32 mmol/l Hepes, 5 mmol/l glucose, pH 7.4. Nominally  $\text{Ca}^{2+}$ -free solutions contained: 125 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l  $\text{MgSO}_4$ , 2 mmol/l  $\text{Na}_2\text{HPO}_4$ , 32 mmol/l Hepes, 0.5 mmol/l EGTA, 5 mmol/l glucose, pH 7.4. For calibration purposes ionomycin (10  $\mu\text{mol/l}$ , Sigma, Germany) was applied at the end of each experiment.

## Results

### *Microarray*

Out of 14500 genes represented on the array, 13 and 250 genes were at least twofold down regulated in response to 24 h treatment with 0.25 and 2.5  $\mu\text{mol/l}$  OTA, respectively.

Those 13 genes that were down regulated by 0.25  $\mu\text{mol/l}$  OTA encode for proteins which are important in transcription, cell growth, cytoskeletal organization, immune response and energy metabolism (Table 2). The strongest down regulation (-3.9) due to 0.25  $\mu\text{mol/l}$  OTA was observed for the insulin-like growth factor 2 (IGF2). Furthermore, the calcineurin A beta (PPP3CB) gene and the “uncoupling protein 2” (UCP2) gene were both twofold down regulated.

The fold changes of the 250 genes down regulated by 2.5  $\mu\text{mol/l}$  OTA ranged from -2.0 up to -12.0. A classification of the 250 genes by fold change revealed as largest group (70%) the twofold down regulated genes (Figure 2). 10 genes (4%) were more than fivefold down regulated (Table 3). The highest fold change (-12.4) showed a gene called “death-inducer obliterator 1” (DIDO1). Glutathione S-transferase omega 1 (GSTO1), one isozyme of the GST family, revealed a twofold down regulation of its mRNA levels, and the insulin-like growth factor binding protein 1 (IGFBP1) exhibited a fold change of -4.0 in response to OTA treatment.

In addition, 33 genes that encode for proteins which are involved in the metabolism of carbohydrates and lipids were more than twofold down regulated, for example phosphoglycerate kinase 1 (PGK1) and stearoyl-CoA desaturase (SCD) (Tables 4, 5a+b). Moreover, 2.5  $\mu\text{mol/l}$  OTA decreased the mRNA levels of 16 genes predominantly involved in the cell cycle and the cell proliferation (Table 6). Out of the 28 down regulated mitochondrial genes the majority of genes encode for enzymes, mainly oxidoreductases such as isocitrate dehydrogenase 3 (NAD<sup>+</sup>) beta (IDH3B, Figure 3). 19 down regulated genes encode for proteins of the cytoskeleton including tubulin beta 1 (TUBB1, Figure 4). Furthermore, OTA decreased mRNA levels of three genes involved in calcium homeostasis, namely “chemokine (C-C motif) ligand 14” (CCL14), “CD24 molecule” (CD24) and “ATPase, Ca<sup>++</sup> transporting, cardiac muscle, slow twitch 2” (ATP2A2) (Table 7). An overview of gene classification by function and localization is given in Figures 5 and 6, respectively.

### *Verification of microarray data by real-time PCR and Western Blots*

Lowered mRNA levels due to OTA could be verified for selected genes in independent experiments by real-time PCR (TUBB1, IGFBP1, PGK1, SCD, UCP2 and GSTO1). However, despite of the same tendency of gene regulation, the remarkably strong down regulation (FC > -100) of TUBB1 and IGFBP1 mRNA levels resulted from PCR experiments was not observed in the microarray study. Furthermore, Western blots for IGFBP1 and GSTO1 indicate also changes on the protein level (Figures 7+8) in response to OTA treatment. A particularly strong down regulation by OTA on both the transcriptional and protein level was observed for IGFBP1.

### *Intracellular $\text{Ca}^{2+}$ imaging*

Figure 9 A shows that the basal fura-2 ratio (first 3 minutes in calcium containing bath solution) and, thus, the intracellular  $\text{Ca}^{2+}$  level was not significantly different between control and OTA treated cells. Removal of calcium from the bath led to a decrease in the intracellular  $\text{Ca}^{2+}$  concentration to similarly low levels in control and OTA treated cells. Addition of 1  $\mu\text{mol/l}$  thapsigargin to the calcium free bath solution resulted in a slight increase in the fura-2 ratio (peak 1) with no difference in the peak amplitude between control and OTA treated cells. Re-addition of extracellular  $\text{Ca}^{2+}$  in the continuous presence of thapsigargin led to a strong

increase in the fura-2 ratio (peak 2). The peak amplitude of this second peak was significantly smaller in OTA treated cells.

## Discussion

Our microarray study demonstrated that OTA lowered mRNA levels of genes centrally involved in liver cell metabolism. The decrease of mRNA levels could have been either due to a decreased mRNA stability or a decreased gene transcription following the OTA treatment. Importantly, the applied OTA concentrations (0.25  $\mu\text{mol/l}$  and 2.5  $\mu\text{mol/l}$ ) did not impair cell viability over 24 h treatment as shown previously [25]. Thus, the present data are indicative for the gene regulating activity of OTA in viable cells.

### *Dose dependent effects of OTA*

A 24 h treatment of HepG2 cells with 0.25 or 2.5  $\mu\text{mol/l}$  OTA resulted in a twofold or stronger down regulation of 13 or 250 genes, respectively. This demonstrates that OTA at concentrations that have already been detected in serum of pigs (0.62  $\mu\text{mol/l}$ ) [26] and humans (4.5  $\mu\text{mol/l}$ ) [27] affects the transcriptome of liver cells in culture.

The immune-relevant calcineurin A beta gene (PPP3CB, previous symbol CALNB) was dose-dependently down regulated in response to 0.25 and 2.5  $\mu\text{mol/l}$  OTA (FC = -2.0 and -7.6, respectively). PPP3CB is one of three isoforms (alpha, beta, gamma) of calcineurin A, the catalytic subunit of the  $\text{Ca}^{2+}$ /calmodulin dependent phosphatase calcineurin, which is essential for T cell activation and survival in mice [28]. On the protein level inhibition of calcineurin has been reported to be nephrotoxic [29]. The OTA induced inhibition of PPP3CB transcription suggests that calcineurin is a molecular target of OTA contributing to its nephrotoxic, and possibly also hepatotoxic effects.

### *Protein metabolism*

The 250 genes down regulated in response to 2.5  $\mu\text{mol/l}$  OTA were classified into clusters according to the biological function and localization of the encoded proteins (Figures 5 + 6). Whereas Creppy and coworkers related OTA induced inhibition of protein biosynthesis mainly to the inhibition of a single group of enzymes (aminoacyl-tRNA synthetases) involved in the translational process [30], our data show that OTA affects a large number of genes involved in protein metabolism. Clustering resulted in 66 down regulated genes which are involved in protein biosynthesis, proteolysis or posttranslational protein modification and mainly encode for protein phosphatases, ligases, transferases, and hydrolases. Overall, our results are in accordance with previous studies demonstrating an inhibitory effect of OTA on protein biosynthesis [17, 31, 32].

### *Carbohydrate metabolism*

Remarkably, all 12 genes of the cluster "carbohydrate metabolism" were down regulated in response to a 24 h treatment with 2.5  $\mu\text{mol/l}$  OTA (Table 4). These genes predominantly encode for proteins which are important in glycolysis, tricarboxylic acid cycle, and gluconeogenesis. For example, two genes encoding for key glycolytic enzymes, namely phosphofructokinase (PFK) and phosphoglycerate kinase 1 (PGK1), which catalyze the conversion of D-fructose 6-phosphate to D-fructose 1,6-bisphosphate and 1,3-diphosphoglycerate to 3-phosphoglycerate, respectively, were both twofold down regulated.

Interestingly, PGK1 may also act as a cofactor for polymerase alpha and, thus, probably influences DNA synthesis and cell cycle progression [33]. Because of these two important functions of PGK1 we aimed to confirm the microarray data for this gene by an independent method. Real-time PCR data for PGK1 also indicate a substantial down regulation due to OTA (Figure 7). In addition, the genes encoding for isocitrate dehydrogenase 3 (NAD<sup>+</sup>) beta (IDH3B) and isocitrate dehydrogenase 3 (NAD<sup>+</sup>) gamma (IDH3G), revealed fold changes of -2.0 and -2.5, respectively. IDH3B and IDH3G belong to the NAD (+)-dependent isocitrate dehydrogenases which catalyze the allosterically regulated rate-limiting step of the tricarboxylic acid cycle.

Also, our study demonstrated a substantial down regulation of the mRNA level of phosphoenolpyruvate carboxykinase 2, a well-known key enzyme of gluconeogenesis in the liver. This is in line with the down regulation of the C/EBP beta transcription factor (FC = -2.4) that has been reported to regulate gluconeogenesis and phosphoenolpyruvate carboxykinase gene transcription [34].

Taken together, our data suggest that OTA massively inhibits the carbohydrate metabolism on the cytosolic and mitochondrial level in HepG2 cells, and, thus, strongly impairs the cellular energy supply.

### *Lipid metabolism*

As shown in Tables 5a + b OTA treatment of HepG2 liver cells resulted in a substantial change of mRNA levels of several genes involved in lipid metabolism. In this study we report that a gene encoding for a key regulator in cellular fatty acid composition, the stearoyl-CoA desaturase (SCD), was down regulated in response to OTA. This finding which derived from the gene chip analysis could be verified by real-time PCR as an independent method (Figure 7).

SCD converts saturated fatty acids (SFAs) into monounsaturated fatty acids (MUFAs). Alterations in SCD mRNA levels could change the phospholipid composition and, thus, the fluidity of cell membranes which, in

turn, possibly damages the function of membrane-attached proteins. Interestingly, for OTA it has been shown in different tissues of rats (brain, pancreas, and kidney) that the activity of many membrane-bound enzymes, for example alanine aminopeptidase and gamma-glutamyl transferase, is impaired [35, 36]. In addition, hepatic SCD down regulation has been demonstrated to correlate with an increase of pro-inflammatory cytokines [37] and with anorexic effects in mice [38]. Similarly, also OTA has been reported to cause inflammation and anorexia in several animal species [39, 40]. In conclusion, we propose that the OTA induced SCD gene down regulation, as observed in this study, may be involved in several well-known effects of OTA such as inhibition of membrane-bound enzymes, inflammation and anorexia.

#### *Cell cycle and cell proliferation*

We observed that OTA decreased the mRNA levels of several genes involved in cell cycle and cell proliferation (Tables 2 + 6), among them the insulin-like growth factor 2 (IGF2), the insulin-like growth factor binding protein 1 (IGFBP1) and tubulin beta 1 (TUBB1). IGF2 and IGFBP1 are part of a complex system (often referred to as the IGF "axis") consisting of two cell-surface receptors (IGF1R and IGF2R), two ligands (IGF1 and IGF2), a family of six high-affinity IGF binding proteins (IGFBP 1-6), as well as associated IGFBP degrading enzymes. The IGF "axis" has been shown to play an important role in the promotion of cell proliferation and the inhibition of cell death. Disruption of the IGF2 gene leads to growth deficiency in mice [41]. Similar effects have been observed for OTA: the fetuses of pregnant Wistar rats fed with different doses of OTA revealed dose dependent skeletal anomalies like incomplete closure of the skull and fused ribs [42]. As we demonstrated an almost fourfold down regulation of IGF2 this may contribute to the well-known OTA induced skeletal malformations in animals.

IGFBPs mainly serve as carrier proteins for IGF1 and IGF2 which show high sequence similarity to insulin. IGFBPs modulate IGF action in complex ways that involve both inhibiting IGF action by preventing binding to the IGF1 receptor as well as promoting IGF action possibly through delivering it to the receptor and increasing its half-life. Recently, however, it has been documented that IGFBP1 exhibits an important intracellular function that is independent of the interactions with the IGFs: Leu et al. found in HepG2 cells that IGFBP1 is a pro-survival factor that binds to the "BCL2-antagonist/killer (BAK) and antagonizes the pro-apoptotic actions of the nuclear transcription factor p53 in mitochondria [43]. In our study we show that OTA significantly inhibited IGFBP1, both on the transcriptional and protein level (Figure 8 A). Thus, OTA possibly induces mitochondrial damage partly by preventing the anti-apoptotic effects of IGFBP1.

TUBB1 is one of six isoforms of beta tubulin. Polymers of beta and alpha tubulin dimers form the microtubules that are one of the components of the cytoskeleton and part of the mitotic spindle. Several studies demonstrated that disruption of the cytoskeleton or impairment of the mitotic process leads to cell death [44]. Our microarray and PCR data reveal that OTA lowers TUBB1 mRNA levels. This finding is in line with a previous study demonstrating defects in spindle formation caused by OTA [45].

#### *Mitochondrial function*

Although it is known for more than 30 years that OTA impairs mitochondrial function, the underlying molecular mechanisms, especially on the transcriptional level, are not fully understood. It has been shown that OTA dose-dependently inhibits the respiratory chain of isolated rat liver mitochondria [46]. Also, abnormally shaped mitochondria have been detected in kidneys of chicks fed with OTA [47]. These results are in line with our observation that key genes of mitochondrial function are affected by OTA (Figure 3). In particular, genes encoding for oxidoreductases such as IDH3B and IDH3G (we discussed these proteins in the section "carbohydrates"), were significantly down regulated by OTA. Furthermore, also mRNA levels of the uncoupling protein 2 (UCP2) gene, that has been shown to limit the generation of reactive oxygen species (ROS) in mice [48], were down regulated by OTA (verified by real-time PCR) indicating that an impairment of this gene might be a factor of the often reported OTA induced increase of oxidative stress [18, 19].

#### *Impact of OTA on calcium homeostasis*

Over the past two decades several studies demonstrated an effect of OTA on cellular calcium homeostasis in different cell types [49-52]. Whereas some studies report an increase of cellular calcium induced by OTA [49, 53], others showed an inhibition of cellular calcium uptake [50, 51]. However, no data were available elucidating the impact of OTA on calcium homeostasis in HepG2 cells. In this study we demonstrated that OTA (2.5  $\mu\text{mol/l}$ , 24 h) substantially decreased the calcium influx in HepG2 cells induced by depletion of intracellular calcium stores (Figure 9), which points to an inhibitory effect of OTA on store-operated  $\text{Ca}^{2+}$  channels (SOCs). This result is in line with previous *in vitro* studies showing a concentration-dependent inhibition of calcium uptake by OTA in liver and kidney microsomes [50, 51]. As blocking of  $\text{Ca}^{2+}$  entry has been shown to limit proliferation of cancer cells *in vitro* [54], the inhibition of calcium influx by OTA could well contribute to the OTA induced reduction of cell numbers that has been observed in many cell lines [55]. Furthermore, we identified four genes involved in calcium homeostasis and down regulated by OTA, namely CCL14, CD24, ATP2A2 and PPP3CB (Table 7). CCL14, for example, represents one of several CC cytokine genes and has been shown to raise intracellular  $\text{Ca}^{2+}$  in human monocytes and to enhance the proliferation

of human bone marrow cells [56]. Down regulation of this cytokine gene by OTA is consistent with our observed inhibition of calcium influx and the previously described immunosuppressive effects of OTA [57, 58]. Cellular  $\text{Ca}^{2+}$  homeostasis may further be affected by down regulation of the ATP dependent ATP2A2 gene, encoding for an intracellular calcium pump of the SERCA type.

## Conclusion

Overall, our results indicate that OTA affects multiple genes encoding for key proteins of liver cell metabolism in cultured HepG2 hepatocytes. We have decided to perform our studies in HepG2 cells since they are of human origin, well characterized, widely used for toxicity studies [59], and sensitive towards OTA treatment [25, 60]. Furthermore, HepG2 cells are an excellent model to investigate mitochondrial toxicity because of their high content of organelles and mitochondrial DNA [61]. However, a limitation of the HepG2 model is that these cells do not express some phase II enzymes to the same extend like primary hepatocytes [62]. Finally studies in laboratory animals are warranted to test the effect of OTA on liver cell metabolism *in vivo*.

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**Table 1 Primer sequences and conditions for real time PCR experiments**

<b>Gene</b>	<b>Forward primer</b>	<b>Reverse Primer</b>	<b>Annealing Temperature</b>
<b>TUBB1</b>	CAAAAGCATGACAGGCAGAA	GGACAATTTACGCATCCTT	57°C
<b>IGFBP1</b>	CTGCCAAACTGCAACAAGAA	TATCTGGCAGTTGGGGTCTC	57°C
<b>PGK1</b>	CTGTGGGGGTATTTGAATGG	CTTCCAGGAGCTCCAACTG	57°C
<b>SCD</b>	CGAGCCGGAGTTTACAGAAG	TATTCCTCAGCCCCCTTTT	57°C
<b>UCP2</b>	TCTTTCCCCACCTCTTCCTT	AGGACGAAGATTCTGGCTGA	57°C
<b>GSTO1</b>	AGGACGCGTCTAGTCCTGAA	TTCCCTGGGTATGCTTCATC	57°C

**Table 2 Genes down regulated by 0.25  $\mu$ mol/l OTA**

Affy_ID	Gene title	Abbreviation	Gene function	fold change at
				24 h OTA 0.25 $\mu$ mol/l
202410_x_at	insulin-like growth factor 2 (somatomedin a)	IGF2	growth-promoting activity	-3.9
210175_at	chromosome 2 open reading frame 3	C2orf3	represses transcription	-2.7
207401_at	prospero-related homeobox 1	PROX1	may play a fundamental role in early development of CNS	-2.6
208998_at	uncoupling protein 2 (mitochondrial, proton carrier)	UCP2	mitochondrial transporter protein that creates proton leaks across the inner mitochondrial membrane	-2.5
215918_s_at	spectrin, beta, non-erythrocytic 1	SPTBN1	tetrameric cytoskeletal protein essential for determination of cell shape, positioning of transmembrane proteins, and organization of organelles and molecular traffic	-2.5
212877_at	kinesin 2	KNS2	microtubule-associated force-producing protein that may play a role in organelle transport	-2.4
208194_s_at	signal transducing adaptor molecule (sh3 domain and itam motif) 2	STAM2	involved in intracellular signal transduction mediated by cytokines and growth factors.	-2.4
215629_s_at	deleted in lymphocytic leukemia, 2	DLEU2	may act as a tumor suppressor	-2.3
204713_s_at	coagulation factor v (proaccelerin, labile factor)	F5	encodes coagulation factor V which is an essential factor of the blood coagulation cascade	-2.2
222120_at	hypothetical protein mgc13138	MGC13138	unknown	-2.1
206348_s_at	pyruvate dehydrogenase kinase, isozyme 3	PDK3	inhibits the mitochondrial pyruvate dehydrogenase, thus contributing to the regulation of glucose metabolism	-2.1
209817_at	protein phosphatase 3 (formerly 2b), catalytic subunit, beta isoform (calcineurin a beta)	PPP3CB	belongs to the PPP phosphatase family, plays an important role in immune response, inhibitors of calcineurin can cause nephrotoxicity	-2.0
205594_at	zinc finger protein 652	ZNF652	transcription / cell division and chromosome partitioning	-2.0

**Table 3 Genes with FC  $\geq$  -5 in response to 2.5  $\mu$ mol/l OTA**

Affy_ID	Gene title	Abbreviation	Gene function	fold change at 24 h
				OTA 2.5 $\mu$ mol/l
218325_s_at	death inducer-oblierator 1	DIDO1	is thought to be involved in apoptosis and regulation of chromatin-based processes	-12.4
217861_s_at	prolactin regulatory element binding	PREB	binds to the prolactin promotor, may act as a transcriptional regulator	-7.8
209817_at	protein phosphatase 3 (formerly 2b), catalytic subunit, beta isoform (calcineurin a beta)	PPP3CB	subunit of calcineurin, regulation of immune response, inhibitors of calcineurin induce nephrotoxicity	-7.6
53076_at	xylosylprotein beta 1,4-galactosyltransferase, polypeptide 7 (galactosyltransferase i)	B4GALT7	belongs to type II membrane-bound glycoproteins that appear to have exclusive specificity for the donor substrate UDP-galactose	-5.9
202492_at, 203192_at	atp-binding cassette, sub-family b (mdr/tap), member 6	ABCB6	encodes for a membrane-associated protein, member of the superfamily of ATP-binding cassette (ABC) transporters, plays a role in mitochondrial function	-5.9
203123_s_at	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	SLC11A2	encodes for a divalent metal transporter (DMT1), which carries iron, manganese, cobalt, nickel, cadmium, lead, copper, and zinc	-5.7
222369_at	hypothetical protein flj13848	—	unknown	-5.5
202257_s_at	cd2 antigen (cytoplasmic tail) binding protein 2	CD2BP2	binds the cytoplasmic domain of CD2 through the GYF domain	-5.5
218556_at	orm1-like 2 (s. cerevisiae)	ORMDL2	belongs to the ORM family, encodes for a membrane protein, widely expressed	-5.3
218439_s_at	comm domain containing 10	COMMD10	unknown	-5.1

**Table 4 Genes of carbohydrate metabolism down regulated in response to OTA treatment (24 h, 2.5  $\mu\text{mol/l}$ ) in HepG2 cells**

Affy_ID	Gene title	Abbreviation	Gene function	fold change at 24 h
				OTA 2.5 $\mu\text{mol/l}$
53076_at	xylosylprotein beta 1,4-galactosyltransferase, polypeptide 7 (galactosyltransferase i)	B4GALT7	one of seven beta-1,4-galactosyltransferase genes, potential targets for the control of cell growth and apoptosis, inflammation and infections	-5.9
219015_s_at	glycosyltransferase 28 domain containing 1	ALG13	involved in N-linked glycosylation, important for glycoprotein folding and stability	-4.9
211885_x_at	fucosyltransferase 6 (alpha (1,3) fucosyltransferase)	FUT6	essential for peripheral fucosylation of acute phase proteins	-3.4
202847_at	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	PCK2	mitochondrial enzyme that catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, may be involved in gluconeogenesis in liver	-2.6
209208_at	mannose-p-dolichol utilization defect 1	MPDU1	functions in synthesis of lipid-linked oligosaccharides (LLOs), important for protein folding and membrane anchoring	-2.6
202471_s_at	isocitrate dehydrogenase 3 (NAD+) gamma	IDH3G	catalyzes rate-limiting step of the tricarboxylic acid cycle	-2.5
204053_x_at	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	PTEN	functions as a tumor suppressor by negatively regulating AKT/PKB signaling pathway	-2.4
201102_s_at, 211065_x_at	phosphofructokinase, liver	PFKL	catalyzes a key step in glycolysis, namely the conversion of D-fructose 6-phosphate to D-fructose 1,6-bisphosphate	-2.3
210820_x_at	coenzyme q7 homolog, ubiquinone (yeast)	COQ7	involved in ubiquinone biosynthesis	-2.2
201509_at, 210418_s_at, 210014_x_at	isocitrate dehydrogenase 3 (NAD+) beta	IDH3B	catalyzes rate-limiting step of the tricarboxylic acid cycle	-2.1
202944_at	n-acetylgalactosaminidase, alpha-	NAGA	encodes the lysosomal enzyme alpha-N-acetylgalactosaminidase, which cleaves alpha-N-acetylgalactosaminyl moieties from glycoconjugates	-2.0
200738_s_at	phosphoglycerate kinase 1	PGK1	glycolytic enzyme that catalyzes the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate	-2.0

**Table 5a Genes of lipid metabolism down regulated in response to OTA treatment (24 h, 2.5 µmol/l) in HepG2 cells**

Affy_ID	Gene title	Abbreviation	Gene function	fold change at 24 h
				OTA 2.5 µmol/l
219015_s_at	glycosyltransferase 28 domain containing 1	ALG13	involved in N-linked glycosylation, important for glycoprotein folding and stability	-4.9
208964_s_at	fatty acid desaturase 1	FADS1	member of the fatty acid desaturase (FADS) gene family. Desaturase enzymes regulate unsaturation of fatty acids through the introduction of double bonds between defined carbons of the fatty acyl chain	-4.0
220041_at	phosphatidylinositol glycan, class z	PIGZ	this gene encodes a protein that is localized to the endoplasmic reticulum, and is involved in GPI anchor biosynthesis	-3.5
221142_s_at	peroxisomal trans-2-enoyl-coa reductase	PECR	key enzyme for a proposed peroxisomal chain elongation of fatty acids	-3.5
202883_s_at, 202884_s_at	protein phosphatase 2 (formerly 2a), regulatory subunit a (pr 65), beta isoform	PPP2R1B	protein phosphatase 2 is one of the four major Ser/Thr phosphatases, and it is implicated in the negative control of cell growth and division	-3.4
209160_at	aldo-keto reductase family 1, member c3 (3-alpha hydroxysteroid dehydrogenase, type ii)	AKR1C3	member of the aldo/keto reductase superfamily, these enzymes catalyze the conversion of aldehydes and ketones to their corresponding alcohols by utilizing NADH and/or NADPH as cofactors	-3.4
209546_s_at	apolipoprotein I, 1	APOL1	apolipoprotein A-I is a relatively abundant plasma protein and is the major apoprotein of HDL. It is involved in the formation of most cholesteryl esters in plasma and also promotes efflux of cholesterol from cells	-3.4
204151_x_at, 216594_x_at	aldo-keto reductase family 1, member c1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	AKR1C1	member of the aldo/keto reductase superfamily, these enzymes catalyze the conversion of aldehydes and ketones to their corresponding alcohols by utilizing NADH and/or NADPH as cofactors	-2.7
211423_s_at	sterol-c5-desaturase (erg3 delta-5-desaturase homolog, fungal)-like	SC5DL	this gene encodes an enzyme of cholesterol biosynthesis. The encoded protein catalyzes the conversion of lathosterol into 7-dehydrocholesterol	-2.7
202025_x_at	acetyl-coenzyme a acyltransferase 1 (peroxisomal 3-oxoacyl-coenzyme a thiolase)	ACAA1	acetyl-Coenzyme A acyltransferase (ACAA1) is an enzyme operative in the beta-oxidation system of the peroxisomes	-2.6
219238_at, 51146_at	phosphatidylinositol glycan, class v, official full name: phosphatidylinositol glycan anchor biosynthesis, class V	PIGV	glycosylphosphatidylinositol (GPI) is a complex glycolipid that anchors many proteins to the cell surface. PIGV adds the second mannose to the GPI core	-2.6

**Table 5b Genes of lipid metabolism down regulated in response to OTA treatment (24 h, 2.5 µmol/l) in HepG2 cells**

Affy_ID	Gene title	Abbreviation	Gene function	fold change at 24 h
				OTA 2.5 µmol/l
209699_x_at	aldo-keto reductase family 1, member c2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type iii)	AKR1C2	member of the aldo/keto reductase superfamily, These enzymes catalyze the conversion of aldehydes and ketones to their corresponding alcohols by utilizing NADH and/or NADPH as cofactors	-2.5
209759_s_at	dodecenoyl-coenzyme a delta isomerase (3,2 trans-enoyl-coenzyme a isomerase)	DCI	this gene encodes a member of the hydratase/isomerase superfamily. The protein encoded is a key mitochondrial enzyme involved in beta-oxidation of unsaturated fatty acids	-2.4
201885_s_at	cytochrome b5 reductase 3	CYB5R3	functions in desaturation and elongation of fatty acids, in cholesterol biosynthesis, and in drug metabolism	-2.4
209509_s_at	dolichyl-phosphate (udp-n-acetylglucosamine) n-acetylglucosaminophosphotransferase 1 (glcnac-1-p transferase)	DPAGT1	the protein encoded by this gene is an enzyme that catalyzes the first step in the dolichol-linked oligosaccharide pathway for glycoprotein biosynthesis.	-2.4
204053_x_at	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	PTEN	functions as a tumor suppressor by negatively regulating AKT/PKB signaling pathway	-2.4
201790_s_at	7-dehydrocholesterol reductase	DHCR7	the DHCR7 gene encodes delta-7-sterol reductase, the enzyme of mammalian sterol biosynthesis that converts 7-dehydrocholesterol (7-DHC) to cholesterol	-2.3
212393_at, 39835_at	set binding factor 1	SBF1 (MTMR5)	cellular function of MTMR5 is unknown, but male mice deficient for MTMR5 are infertile	-2.2
209122_at	adipose differentiation-related protein	ADFP	adipocyte differentiation-related protein is associated with the globule surface membrane material. This protein is a major constituent of the globule surface	-2.2
211708_s_at, 200831_s_at	stearoyl-coa desaturase (delta-9-desaturase)	SCD	converts saturated fatty acids (SFAs) into monounsaturated fatty acids (MUFAs)	-2.1

**Table 6 Genes of cell cycle and cell proliferation down regulated in response to OTA treatment (24 h, OTA 2.5 µmol/l) in HepG2 cells**

Affy_ID	Gene title	Abbreviation	Gene function	fold change at 24 h OTA 2.5 µmol/l
205302_at	insulin-like growth factor binding protein 1	IGFBP1	binds both insulin-like growth factors (IGFs) I and II, pro-survival function in liver	-4.2
202883_s_at, 202884_s_at	protein phosphatase 2 (formerly 2a), regulatory subunit a (pr 65), beta isoform	PPP2R1B	subunit of protein phosphatase 2, implicated in the negative control of cell growth and division	-3.4
208601_s_at	tubulin, beta 1	TUBB1	one of six isotypes of beta tubulin that forms as heterodimer with alpha tubulin the microtubule in mammals	-3.4
203558_at	cullin 7	CUL7	inhibitor of Myc-induced apoptosis	-3.3
205436_s_at	h2a histone family, member x	H2AFX	belongs to the family of histones, that are basic nuclear proteins responsible for the nucleosome structure of the chromosomal fiber in eukaryotes	-2.8
200712_s_at, 200713_s_at	microtubule-associated protein, rp/eb family, member 1	MAPRE1	during mitosis, the protein is associated with the centrosomes and spindle microtubules	-2.8
201469_s_at	shc (src homology 2 domain containing) transforming protein 1	SHC1	longevity-associated gene, might be involved in regulating mitochondrial metabolism	-2.8
202642_s_at	transformation/transcription domain-associated protein	TRRAP	involved in chromatin based processes	-2.6
220386_s_at	echinoderm microtubule associated protein like 4	EML4	essential for microtubule formation	-2.5
204053_x_at	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	PTEN	functions as a tumor suppressor by negatively regulating AKT/PKB signaling pathway.	-2.4
216870_x_at	deleted in lymphocytic leukemia, 2	DLEU2	may act as a tumor suppressor	-2.3
208796_s_at	cyclin g1	CCND1	functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition	-2.2
212099_at	ras homolog gene family, member b	RHOB	regulates endosome transport by promoting actin assembly on endosomal membranes through Dia1	-2.1
217301_x_at	retinoblastoma binding protein 4	RBBP4	involved in histone acetylation and chromatin assembly	-2.1
209817_at	protein phosphatase 3 (formerly 2b), catalytic subunit, beta isoform (calcineurin a beta)	PPP3CB	belongs to the PPP phosphatase family, plays an important role in immune response, inhibitors of calcineurin can cause nephrotoxicity	-2.0
200020_at	tar dna binding protein	TARDBP	transcriptional repressor	-2.0

**Table 7 Genes involved in calcium homeostasis down regulated in response to OTA treatment (24 h, OTA 2.5  $\mu$ mol/l) in HepG2 cells**

Affy_ID	Gene title	Abbreviation	Gene function	fold change at
				24 h OTA 2.5 $\mu$ mol/l
210390_s_at	chemokine (c-c motif) ligand 14	CCL14	one of several CC cytokine genes, induces changes in intracellular calcium concentration	-2.6
209771_x_at, 216379_x_at, 266_s_at, 209772_s_at	cd24 antigen (small cell lung carcinoma cluster 4 antigen)	CD24	cell surface glycoprotein, involved in cell adhesion and signalling	-2.6
209186_at	atpase, ca++ transporting, cardiac muscle, slow twitch 2	ATP2A2	encodes one of the SERCA Ca(2+)-ATPases, which are intracellular Ca (2+) pumps located in the sarcoplasmic or endoplasmic reticula	-2.3

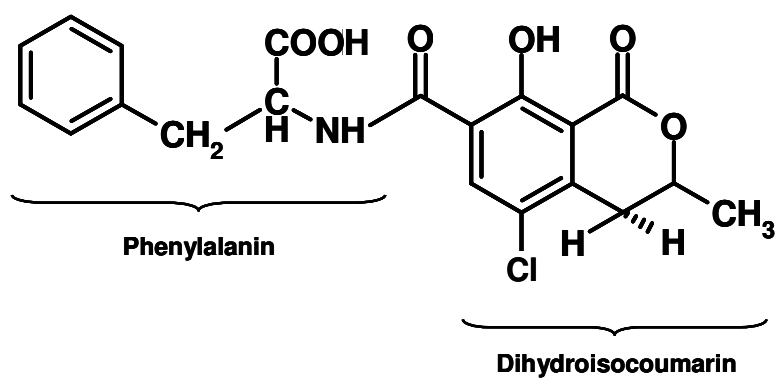


Figure 1. Chemical structure of ochratoxin A.

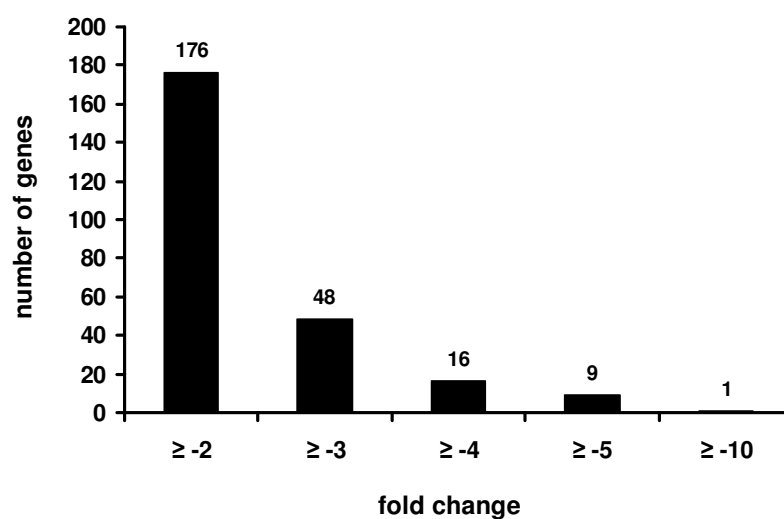
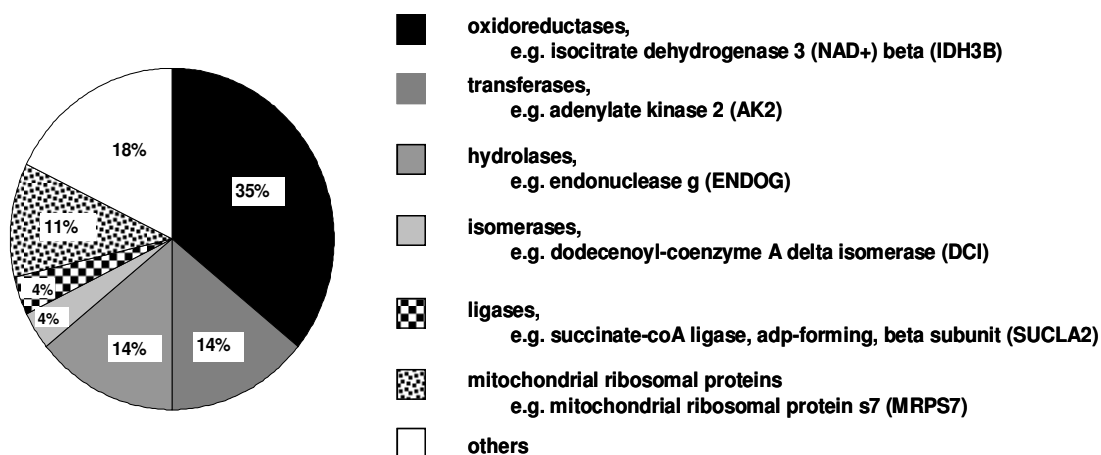
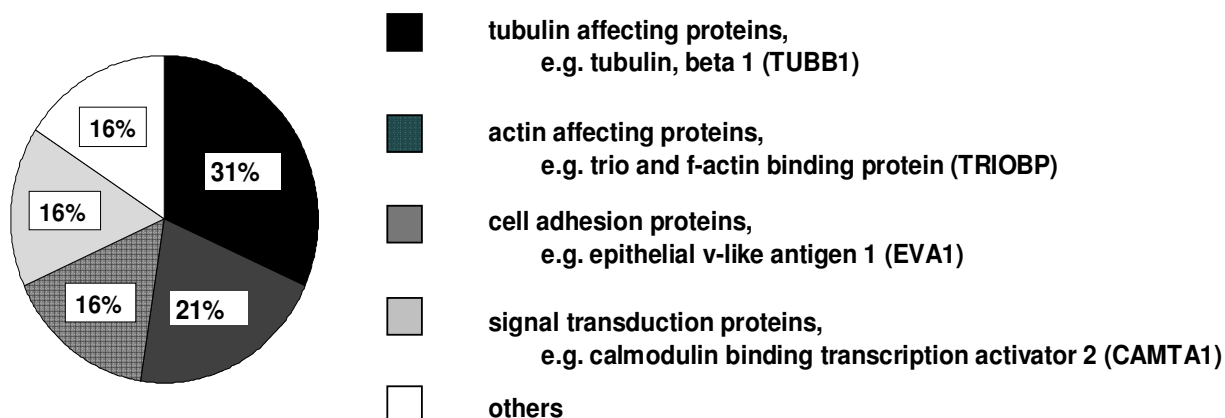


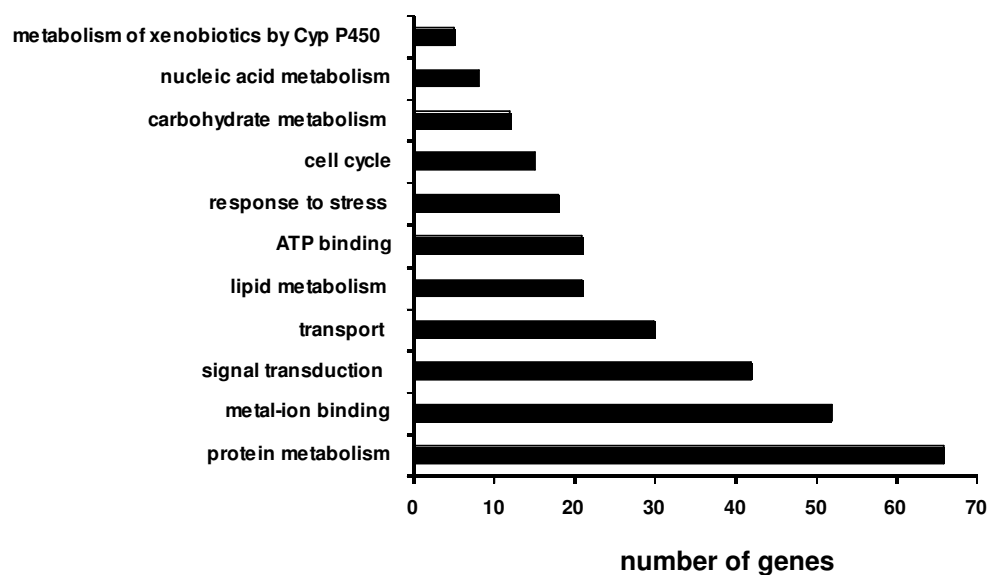
Figure 2. Classification by fold change of down regulated HepG2 genes after 24 h treatment with 2.5  $\mu\text{mol/l}$  OTA (dchip,  $p \leq 0.05$ ).



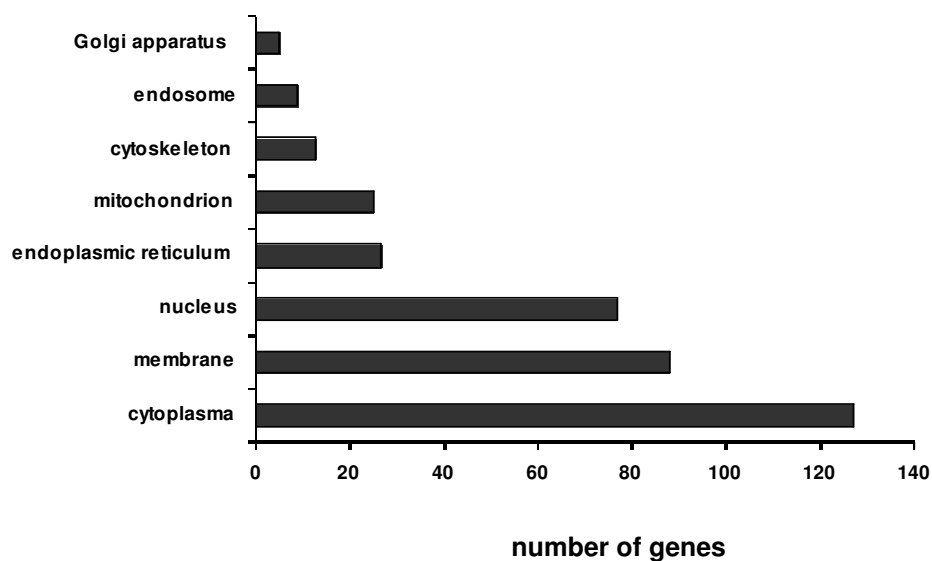
**Figure 3.** Classification of mitochondrial proteins encoded by down regulated genes in HepG2 cells after 24 h treatment with 2.5 µmol/l OTA (dchip,  $p \leq 0.05$ , DAVID functional clustering).



**Figure 4** Classification of cytoskeleton proteins encoded by down regulated genes in HepG2 cells after 24 h treatment with 2.5 µmol/l OTA (dchip,  $p \leq 0.05$ , DAVID functional clustering).



**Figure 5.** Classification by function of at least twofold down regulated genes in HepG2 cells after 24 h treatment with 2.5  $\mu\text{mol/l}$  OTA (dchip,  $p \leq 0.05$ , DAVID functional clustering).



**Figure 6.** Classification by localization of at least twofold down regulated genes in HepG2 cells after 24 h treatment with 2.5  $\mu\text{mol/l}$  OTA (dchip,  $p \leq 0.05$ , DAVID functional clustering).

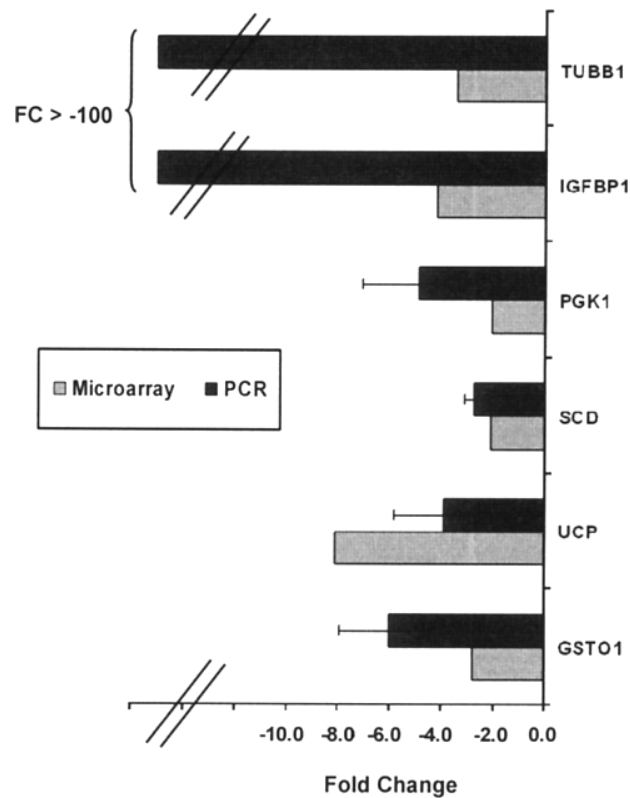


Figure 7. Verification of gene expression changes in HepG2 cells following ochratoxin A treatment ( $2.5 \mu\text{mol/l}$ , 24h) as indicated by microarray data. Expression of selected target genes was determined via quantitative real-time PCR as an independent method. Data of real-time PCR are mean values with SEM.

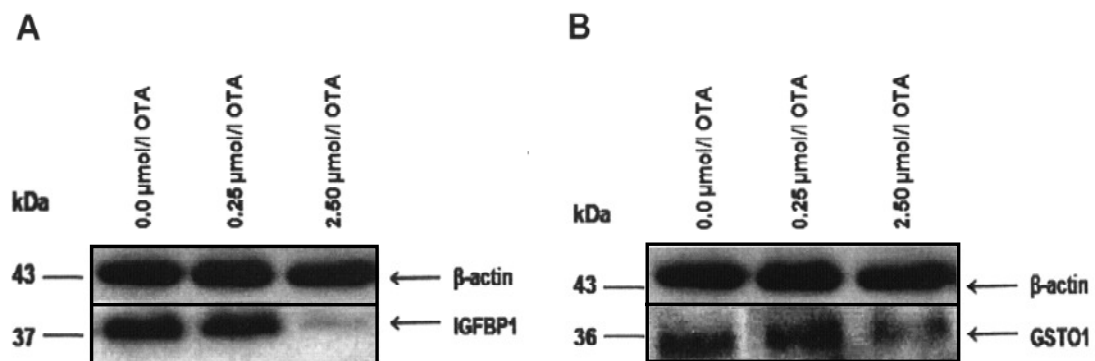


Figure 8. Western Blot analysis: Down regulation of IGFBP1 (A) and GSTO1 (B) by 48 h treatment of HepG2 cells with  $2.5 \mu\text{mol/l}$  OTA. One representative out of three experiments is shown.

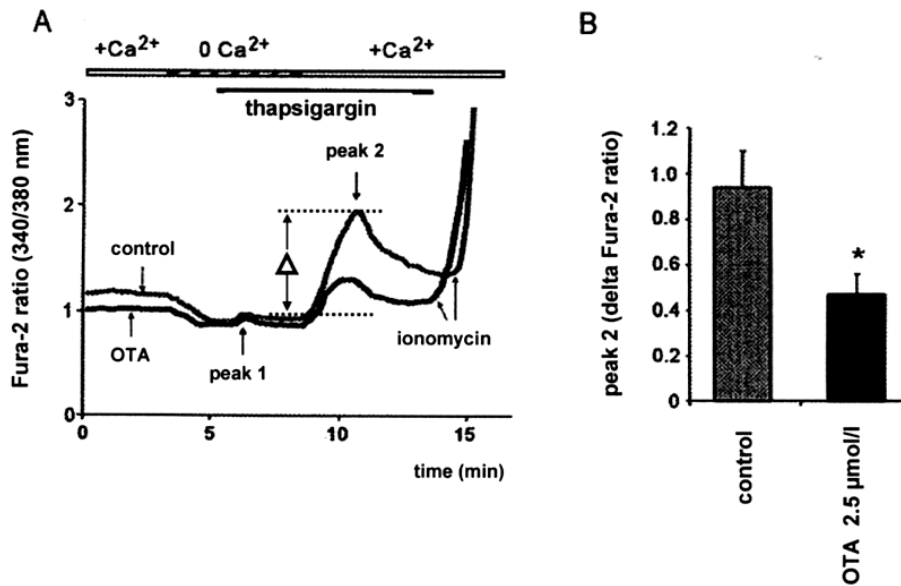


Figure 9. (A) Representative original tracings showing the Fura-2 fluorescence ratios (340/380 nm) - indicating intracellular  $\text{Ca}^{2+}$  concentration - in control (upper line) and OTA-treated (lower line, 2.5  $\mu\text{mol/l}$  for 24 h) HepG2 cells.

Where indicated, thapsigargin (1  $\mu\text{mol/l}$ ) was added to  $\text{Ca}^{2+}$ -free bath solution, resulting in a release of  $\text{Ca}^{2+}$  from intracellular stores (peak 1). Readdition of extracellular  $\text{Ca}^{2+}$  in the presence of thapsigargin led to an influx of extracellular  $\text{Ca}^{2+}$  (peak 2).

(B) refers to peak 2: Changes in Fura-2 fluorescence ratios (340/380 nm) for control (n=7) and OTA-treated (n=9) HepG2 cells in the presence of thapsigargin. Data are mean values with SEM ( $p \leq 0.05$ ).

## CHAPTER SEVEN

## General Discussion

The mycotoxin ochratoxin A (OTA) has been shown to exhibit a wide range of toxic effects including nephro- and hepatotoxicity in animals [1]. Furthermore, OTA has been classified as a possible human carcinogen by the International Agency for Research on Cancer (IARC) [2]. The toxicity mechanisms of OTA are still incompletely understood. Numerous *in vitro* and *in vivo* studies indicate that the generation of reactive oxygen species (ROS) may play a pivotal role in OTA toxicity. In fact, OTA has been shown to enhance NADPH and ascorbate-mediated lipid peroxidation in rat liver microsomes [3]. In monkey kidney cells (Vero cells), a concentration dependent induction in the formation of malondialdehyde (MDA), a biomarker of lipid peroxidation, was observed in response to OTA treatment [4]. In Wistar rats, OTA injection enhanced ethane exhalation, which is again indicative of an increase in lipid peroxidation [3]. Furthermore, there is increasing experimental evidence of OTA induced DNA damage [5] and protein oxidation [6].

Several studies aimed to counteract OTA induced oxidative stress by inducing antioxidant enzymes such as superoxide dismutase (SOD) and catalase [3, 7]. A broad range of antioxidants, including lipophilic synthetic antioxidants like butylated hydroxytoluene (BHT) [3], natural fat-soluble antioxidants such as vitamin E [8-10], hydrophilic antioxidants like vitamin C [8, 11], and also the hormone melatonin [12], have been tested for their potential to diminish OTA mediated oxidative damage in cultured cells and in rodents. As far as polyphenols are concerned, one study used berry and leaf juice of the common grape *Vitis vinifera* to counteract OTA toxicity in mice [13].

Although the above mentioned studies indicate a potential for the tested antioxidants to counteract OTA toxicity, their efficacy may depend on various factors including: duration of OTA treatment, concentrations of the tested antioxidants, molar ratio between OTA and the test compound, concentration of serum in the cell culture medium, and cell type, to name a few. Furthermore, the way the antioxidant is administered may be an important determinant of its efficacy to counteract OTA toxicity. BHT, for example, abolished the OTA enhanced

lipid peroxidation in rat liver microsomes while SOD and catalase had no effect [3]. In contrast, when injected subcutaneously in rats, SOD and catalase prevented most of the nephrotoxic effects induced by OTA [7]. Similarly,  $\alpha$ -tocopherol counteracted the OTA induced loss of cell viability in bovine mammary epithelia (BME-UV1) cells by about 10% [9], but did not protect LLC-PK1 kidney cells from OTA induced cell death [10], although a significant decrease in ROS levels was observed. Vitamin C exhibited no effect on MDA production in chickens fed an OTA containing diet [8], but significantly minimized the incidence of abnormalities in mitotic and meiotic chromosomes observed in mice when administered OTA orally for 45 days [11]. The application of juice of the grape *Vitis vinifera* partly protected from OTA induced hepatorenal carcinoma [13], but the active principle accounting for this protection was not identified.

Summarising the evidence from the above cited studies, it remained unclear, which antioxidants might be most potent in counteracting OTA induced cytotoxicity and biomarkers of oxidative stress. Therefore, we studied the potency of selected antioxidants to counteract OTA induced cell death. As cell death in general represents the endpoint of toxicity, a decrease in OTA induced cell death by antioxidants would be an indication of their effectiveness in counteracting OTA toxicity. Since the liver is one of the main targets for OTA and studies concerning the effects of antioxidants in OTA treated liver cells are rare, we performed our experiments in the human hepatoma derived cell line HepG2, which represents a frequently used human model for biotransformation and cytotoxicity [14, 15]. Furthermore, we regarded HepG2 cells as especially suitable for our studies, because they are rich in mitochondria [16] and thus relatively potent producers of ROS. We studied the antioxidant vitamin  $\alpha$ -tocopherol and the dietary flavonoids EGCG, CAT, EC, GEN, DAI, EQU, and QUE for their abilities to protect liver cells from OTA induced cytotoxicity, because so far little information is available regarding the role of flavonoids in counteracting OTA toxicity.

At first we determined the *in vitro* antioxidative capacities of the flavonoids using the FRAP, DPPH, and TEAC assays. While the DPPH and TEAC assays are based on the ability of a compound to scavenge coloured radicals converting them into colourless products [17], the FRAP assay basically measures the ferric reducing ability of an antioxidant [18]. Remarkably, all three assays revealed similar antioxidative capacities for the flavonoids, in the order EGCG > QUE > CAT/EC > GEN/DAI/EQU. However, neither pre-treatment with  $\alpha$ -tocopherol (48 h, 100  $\mu$ mol/l) nor flavonoids (48 h, 50  $\mu$ mol/l) increased the viability of OTA treated (48 h, 20  $\mu$ mol/l) HepG2 cells which was approximately 65% compared to untreated cells as determined by the neutral red assay [19]. Furthermore, we observed that pre-incubation with  $\alpha$ -tocopheryl phosphate ( $\alpha$ -TOC-P) and quercetin (QUE) even enhanced OTA toxicity in a dose dependent manner.

There may be several reasons why antioxidants do not protect from OTA toxicity in HepG2 cells: 1. Oxidative stress might not play a pivotal role in OTA induced cytotoxicity in HepG2 cells, 2. The uptake of the antioxidants into HepG2 cells might not be sufficient, 3. The antioxidants might be rapidly metabolised in HepG2 cells, 4. The uptake into the cytosol might be sufficient but the antioxidants might not reach the relevant sites of free radical generation such as the inner mitochondrial membrane.

It has been previously shown that an overnight incubation with 1-30  $\mu$ mol/l  $\alpha$ -tocopherol attenuated lipid peroxidation in HepG2 cells [20]. However, we found that a 48 h pre-treatment with 100  $\mu$ mol/l  $\alpha$ -tocopherol, which is a concentration more than twice the upper range in human plasma (15- 40  $\mu$ mol/l) [21], did not prevent from OTA induced cell death in HepG2 cells. Similarly, Schaaf and co-workers observed a significant decrease of ROS production in LLC-PK1 cells incubated with  $\alpha$ -tocopherol, but no protection against OTA induced cytotoxicity. The authors concluded that lipid peroxidation might not be centrally involved in OTA toxicity in LLC-PK1 cells. Furthermore, as it has also been demonstrated that OTA inhibits site I and II mitochondrial respiration in proximal tubular cells [22], Schaaf

and co-workers hypothesized that mitochondrial dysfunction might be the main cause for OTA induced cytotoxicity in LLC-PK1 cells. The lack of protection of  $\alpha$ -tocopherol against OTA cytotoxicity and the massive down regulation of mitochondrial genes observed in our studies in HepG2 cells (Chapter six) support the hypothesis that an impairment of mitochondrial function is more likely to cause OTA toxicity in cultured hepatocytes than lipid peroxidation.

Our finding that  $\alpha$ -TOC-P and QUE even increased OTA toxicity in a dose dependent manner may possibly be due to their effects on glutathione-S-transferases (GSTs). GSTs are a family of enzymes that catalyze the conjugation of glutathione (GSH) to electrophiles forming more polar glutathione-S-conjugates which are then eliminated via urine [23]. Thus, GSTs play a crucial role in detoxifying endobiotic and xenobiotic compounds; they also have been linked to OTA detoxification [24, 25]. Six GST classes have been described to date: GST-A, -M, -P, -T, -Z, and -O [26]. GSTO with its members GSTO1 and GSTO2 represents a relatively new class of GSTs which were characterized by Board and co-workers in 2000 [27]. Unlike other GSTs GSTO has thiol transferase and dehydroascorbate reductase activity and is therefore associated with the cellular response to oxidative stress [27]. Recently it has been demonstrated that  $\alpha$ -TOC-P inhibits the activity of GSTO1 in a concentration dependent manner [28]. Similarly, it has been reported that QUE decreases GST activity [29]. Since our results demonstrated that OTA decreased GSTO1 mRNA and protein levels in HepG2 cells (Chapter five), we suggest that the synergistic toxic effects of  $\alpha$ -TOC-P / OTA and CAT / OTA, respectively, are due to a simultaneous inhibition of GSTs by both OTA as well as  $\alpha$ -TOC-P and CAT, respectively.

In addition to investigating the possible protective role of antioxidants in OTA induced cytotoxicity, we tested the impact of OTA on gene expression in liver cells. To this end, we performed a large-scale gene expression profile of OTA treated HepG2 cells using Affymetrix GeneChip<sup>®</sup> technology. Out of 14500 genes represented on the microarray, 13 and 250

genes were down regulated in HepG2 cells incubated with 0.25 and 2.5  $\mu\text{mol/l}$  OTA, respectively. Decreased mRNA levels of calcineurin A beta (PPP3CB) which regulates inflammatory signalling pathways in immune cells [30, 31] and of the uncoupling protein 2 (UCP2), which has been suggested to control the production of reactive oxygen species (ROS) [32], were observed in response to 0.25  $\mu\text{mol/l}$  OTA. In addition, numerous genes encoding for proteins involved in energy and xenobiotics metabolism showed an at least twofold down regulation due to incubation with 2.5  $\mu\text{mol/l}$  OTA. For example, two genes encoding for key enzymes in glycolysis, namely phosphofructokinase (PFK) and phosphoglycerate kinase 1 (PGK1), which catalyze the conversion of D-fructose 6-phosphate to D-fructose 1,6-bisphosphate and 1,3-diphosphoglycerate to 3-phosphoglycerate, respectively, were both twofold down regulated. mRNA levels of critical genes for mitochondrial function, mainly encoding for oxidoreductases such as isocitrate dehydrogenase 3 (NAD<sup>+</sup>) beta (IDH3B), which belongs to the NAD (+)-dependent isocitrate dehydrogenases which catalyze the allosterically regulated rate-limiting step of the tricarboxylic acid cycle, were significantly lowered by OTA. Overall, our results indicate that OTA might lead to ATP depletion in HepG2 cells via down regulation of important genes involved in carbohydrate metabolism and mitochondrial function.

We further observed that OTA decreased the insulin-like growth factor binding protein 1 (IGFBP1), both at the mRNA and protein level. IGFBP1 is one of six members of the family of IGFBPs (IGFBP1-6) which belong to the so-called “IGF axis”, a complex system consisting of two cell-surface receptors (IGF1R and IGF2R), two ligands (IGF1 and IGF2), six high-affinity IGF binding proteins (IGFBPs), as well as associated IGFBP degrading enzymes. The IGF “axis” has been shown to play an important role in the promotion of cell proliferation and the inhibition of cell death [33]. Interestingly, IGFBP1 has recently been demonstrated to exhibit an important intracellular function that is independent of the interactions with the IGFs: Leu and co-workers found in HepG2 cells that IGFBP1 is a pro-survival factor that binds to the “BCL2-antagonist/killer” (BAK) and antagonizes the pro-apoptotic actions of the

nuclear transcription factor p53 in mitochondria [34]. Moreover, IGFBP1 production in HepG2 cells has been reported to be influenced by ROS [35]. Therefore our finding that OTA lowers both mRNA and protein levels of IGFBP1 in HepG2 cells indicates that IGFBP1 might be an important factor in OTA induced liver cytotoxicity with mitochondria being a major target of OTA.

Taken together, the results of our studies show that down regulation of key genes of the cellular hepatic metabolism, including PGK1, UCP2, IDH3B, IGFBP1 and GSTO1, seem to play a critical role in OTA induced liver toxicity. Our hypothesis that the inhibition of mitochondrial function is the pivotal event in OTA toxicity is underlined by our finding that all analyzed mitochondrial genes in HepG2 cells were strongly down regulated. Our observation that antioxidants were not able to protect HepG2 cells from OTA-induced cytotoxicity does not fully exclude an involvement of ROS in OTA toxicity, which may also be due - to a certain extent - to insufficient concentrations of the antioxidants at the relevant sites of cellular ROS generation, such as mitochondria. Systematic studies on the distribution of OTA in specific subcellular compartments may contribute to a better understanding of OTA mediated toxicity. Our data suggest that one approach to counteract OTA cytotoxicity may be the use of mitochondria-targeted antioxidants. Finally, studies in rodents are warranted to test if and to what extent changes in gene expression observed in HepG2 cells apply to the situation *in vivo*.

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## APPENDIX

**Table A1 Cytotoxicity of OTA in HepG2 liver cells after 24, 48 and 72 h incubation with 2.5 – 100 µmol/l OTA**

OTA (µmol/l)	Viability %				MW	STABW	coefficient of variation (%)
	V1	V2	V3	V4			
<b>2,5</b>	96,3	104,6	117,4	99,0	<b>104,3</b>	9,4	9,0
<b>5</b>	92,7	96,0	106,4	91,5	<b>96,7</b>	6,8	7,0
<b>10</b>	73,3	83,9	83,8	83,9	<b>81,2</b>	5,3	6,5
<b>20</b>	<b>23,5</b>	72,9	63,4	77,3	<b>71,2</b>	7,1	10,0
<b>50</b>	67,9	51,4	54,2	77,9	<b>62,9</b>	12,4	19,7
<b>100</b>	63,1	48,4	47,3	75,2	<b>58,5</b>	13,3	22,7

**48 h:**

OTA (µmol/l)	Viability %				MW	STABW	coefficient of variation (%)
	V1	V2	V3	V4			
<b>2,5</b>	99,8	99,1	102,3	99,5	<b>100,2</b>	1,4	1,4
<b>5</b>	88,3	77,9	98,9	82,1	<b>86,8</b>	9,1	10,5
<b>10</b>	79,2	67,0	82,9	60,9	<b>72,5</b>	10,3	14,2
<b>20</b>	56,5	54,9	51,3	42,5	<b>51,3</b>	6,3	12,2
<b>50</b>	53,0	43,6	37,5	30,1	<b>41,1</b>	9,7	23,6
<b>100</b>	46,0	39,8	32,5	25,3	<b>35,9</b>	9,0	25,0

**72 h:**

OTA (µmol/l)	Viability %				MW	STABW	coefficient of variation (%)
	V3	V4	V5	V5			
<b>2,5</b>	98,7	93,2	87,5	89,3	<b>92,2</b>	5,0	5,4
<b>5</b>	86,9	76,4	70,8	73,8	<b>77,0</b>	7,0	9,1
<b>10</b>	74,0	44,3	49,1	50,4	<b>54,5</b>	13,3	24,4
<b>20</b>	45,0	22,7	37,4	38,9	<b>36,0</b>	9,5	26,3
<b>50</b>	19,4	12,4	25,5	29,2	<b>21,6</b>	7,4	34,0
<b>100</b>	16,3	11,2	26,1	27,2	<b>20,2</b>	7,7	38,3

**Table A2 FRAP (ferric reducing ability of plasma) test of selected flavonoids**

Flavonoid	c( µmol/l)	Absorption 593nm			m	STABW	VK (%)	FRAP-values (µmol/l aae**)
		1	2	3				
Quercetin	200	0,852	0,926	0,999	<b>0,926</b>	0,074	7,90	<b>619,0</b>
	100	0,475	0,471	0,485	<b>0,477</b>	0,007	1,47	<b>319,7</b>
EGCG	200	0,843	0,922	0,903	<b>0,889</b>	0,041	4,61	<b>594,3</b>
	100	0,593	0,633	0,636	<b>0,621</b>	0,024	3,86	<b>415,7</b>
Catechin	200	0,342	0,337	0,386	<b>0,355</b>	0,027	7,61	<b>259,0</b>
	100	0,170	0,187	0,167	<b>0,175</b>	0,011	6,29	<b>118,3</b>
Epicatechin	200	0,340	0,348	0,386	<b>0,358</b>	0,025	6,98	<b>240,3</b>
	100	0,182	0,190	0,177	<b>0,183</b>	0,007	3,83	<b>123,7</b>
Genistein	200	0,024	0,012	0,018	<b>0,018</b>	0,006	33,30	<b>13,7</b>
	100	0,005	0,004	0,007	<b>0,005</b>	0,002	40,00	<b>5,0</b>
Equol	200	0,031	0,026	0,026	<b>0,028</b>	0,003	10,71	<b>20,3</b>
	100	0,024	0,021	0,021	<b>0,022</b>	0,002	9,09	<b>16,3</b>

\*\* aae= ascorbic acid equivalents

**Table A3 TEAC (trolox equivalent antioxidant capacity) test of selected flavonoids**

**A**

Flavonoid	Absorption (734 nm)				STABW	VK (%)	Reduction of Absorption %	TEAC-values
	1	2	3	m				
BW (ABTS-Rad.Lsg.)	0,724	0,728	0,724	<b>0,725</b>	0,002	0,318		
Trolox (2,5 µM)	0,639	0,629	0,632	<b>0,633</b>	0,005	0,810	12,7	<b>1,0</b>
Quercetin (2,5 µM)	0,469	0,471	0,465	<b>0,468</b>	0,003	0,652	35,4	<b>2,8</b>
EGCG (2,5 µM)	0,351	0,367	0,357	<b>0,358</b>	0,008	2,256	50,6	<b>4,0</b>
Catechin (2,5 µM)	0,498	0,508	0,506	<b>0,504</b>	0,005	1,050	30,5	<b>2,4</b>
Epicatechin (2,5 µM)	0,495	0,468	0,492	<b>0,485</b>	0,015	3,051	33,1	<b>2,6</b>
Genistein (2,5 µM)	0,607	0,629	0,613	<b>0,616</b>	0,011	1,845	15,0	<b>1,2</b>
Equol (2,5 µM)	0,561	0,553	0,558	<b>0,557</b>	0,004	0,725	23,2	<b>1,8</b>

**B**

Flavonoid	Absorption (734 nm)				STABW	VK (%)	Reduction of Absorption %	TEAC-values
	1	2	3	m				
BW (ABTS-Rad.Lsg.)	0,730	0,716	0,727	<b>0,724</b>	0,007	1,018		
Trolox (2,5 µM)	0,635	0,626	0,639	<b>0,633</b>	0,007	1,051	12,6	<b>1,0</b>
Quercetin (2,5 µM)	0,345	0,358	0,348	<b>0,350</b>	0,007	1,943	38,8	<b>3,1</b>
EGCG (2,5 µM)	0,470	0,427	0,433	<b>0,443</b>	0,023	5,253	51,7	<b>4,1</b>
Catechin (2,5 µM)	0,500	0,470	0,515	<b>0,495</b>	0,023	4,629	31,6	<b>2,5</b>
Epicatechin (2,5 µM)	0,486	0,466	0,488	<b>0,480</b>	0,012	2,534	33,7	<b>2,7</b>
Genistein (2,5 µM)	0,649	0,648	0,665	<b>0,654</b>	0,010	1,459	9,7	<b>0,8</b>
Equol (2,5 µM)	0,557	0,545	0,557	<b>0,553</b>	0,007	1,253	23,6	<b>1,8</b>

**Table A4 Viability of HepG2 cells after preincubation with Vitamin E (VE) or flavonoids followed by OTA treatment (20  $\mu$ M, 48 h)**

test compound	Viability %				STABW
	1	2	3	m	
control 1	62,1	65,7	64,7	<b>64,2</b>	1,9
control 2	67,2	73,2	70	<b>70,1</b>	3,0
200 $\mu$ M (48h)	67	67,3	66,2	<b>66,8</b>	0,6
VE 100 $\mu$ M (48h)	58,8	62,9	54,4	<b>58,7</b>	4,3
VE 200 $\mu$ M (6h)	65,3	73,7	73,9	<b>71,0</b>	4,9
VE 100 $\mu$ M (6h)	76,9	73,2	76,7	<b>75,6</b>	2,1
EGCG 100 $\mu$ M (48h)	66,4	69	68,3	<b>67,9</b>	1,3
EGCG 50 $\mu$ M(48h)	72,5	68,9	75,1	<b>72,2</b>	3,1
Quercetin 100 $\mu$ M (48h)	69,2	64,8	69,2	<b>67,7</b>	2,5
Quercetin 50 $\mu$ M (48h)	65,4	64,3	65,2	<b>65,0</b>	0,6
Genistein 50 $\mu$ M (48h)	66,7	66,3	67,6	<b>66,9</b>	0,7

**Table A5 Influence of foetal calf serum concentration (FCS) on the viability of HepG2 cells after 24 h incubation with Ochratoxin A (OTA)**

**1. FCS 10 %**

OTA (µM)	viability (%)			MW	STABW	VC (%)
	m1	m2	m3			
0,0	100,0	100,0	100,0	<b>100,0</b>	0,0	0,0
0,25	101,7	104,4	104,9	<b>103,7</b>	1,7	1,7
0,5	108,8	103,6	111,6	<b>108,0</b>	4,1	3,8
1	108,6	102,3	109,5	<b>106,8</b>	3,9	3,7
5	93,0	89,2	97,2	<b>93,1</b>	4,0	4,3
15	78,1	78,2	80,3	<b>78,9</b>	1,2	1,6
25	65,0	62,0	69,3	<b>65,4</b>	3,7	5,6
50	59,3	65,3	63,4	<b>62,7</b>	3,1	4,9

**2. FCS 5%**

OTA (µM)	viability (%)			MW	STABW	VC (%)
	m1	m2	m3			
0,0	100,0	100,0	100,0	<b>100,0</b>	0,0	0,0
0,25	96,4	96,9	101,7	<b>98,3</b>	2,9	3,0
0,5	104,3	95	102,2	<b>100,5</b>	4,9	4,9
1	98,0	105,1	100,0	<b>101,0</b>	3,7	3,6
5	79,4	72,3	82,0	<b>77,9</b>	5,0	6,4
15	61,3	53,3	61,6	<b>58,7</b>	4,7	8,0
25	54,2	46,8	58,1	<b>53,0</b>	5,7	10,8
50	55,9	52,0	46,9	<b>51,6</b>	4,5	8,7

**3. FCS 2,5%**

OTA (µM)	viability (%)			MW	STABW	VC (%)
	m1	m2	m3			
0,0	100,0	100,0	100,0	<b>100,0</b>	0,0	0,0
0,25	107,1	108,6	114,2	<b>110,0</b>	3,7	3,4
0,5	97,5	93,1	99,4	<b>96,7</b>	3,2	3,3
1	86,9	93,1	89,5	<b>89,8</b>	3,1	3,5
5	64,9	60,3	68,9	<b>64,7</b>	4,3	6,7
15	52,8	54,1	53,3	<b>53,4</b>	0,7	1,2
25	53,7	49,2	52,8	<b>51,9</b>	2,4	4,6
50	46,4	49,3	52,6	<b>49,4</b>	3,1	6,3

**4. FCS 0%**

OTA (µM)	viability (%)			MW	STABW	VC (%)
	m1	m2	m3			
0,0	100,0	100,0	100,0	<b>100,0</b>	0,0	0,0
0,25	50,4	50,2	45,9	<b>48,8</b>	2,5	5,2
0,5	50,6	45,8	48	<b>48,1</b>	2,4	5,0
1	46,3	47,2	45,9	<b>46,5</b>	0,7	1,4
5	51,5	48,4	47,9	<b>49,3</b>	2,0	4,0
15	40,5	44,1	41,8	<b>42,1</b>	1,8	4,3
25	37,6	43,8	43,1	<b>41,5</b>	3,4	8,2
50	41,3	36,7	35,1	<b>37,7</b>	3,2	8,5

**Table A6 Cytotoxicity of ochratoxin A (24 h, 1 – 15 µmol/l) on HepG2 cells after pre-incubation with N-acetylcysteine (NAC, 24 h, 0 – 4 mmol/l)**

**OTA 1 µM**

NAC (mM)	viability (%)			m	stabw	vc (%)	mortality (%)			m	stabw	vc (%)
0	46,2	47,8	49,6				53,8	52,2	50,4			
	46,3	51,4	50,7	48,7	2,2	4,6	53,7	48,6	49,3	51,3	2,2	4,3
0,2	47,5	50,1	51,0	49,5	1,8	3,7	52,5	49,9	49,0	50,5	1,8	3,6
0,5	49,2	50,9	52,6	50,9	1,7	3,3	50,8	49,1	47,4	49,1	1,7	3,5
1	48,5	50,6	48,2	49,1	1,3	2,7	51,5	49,4	51,8	50,9	1,3	2,6
2	48,1	48,7	51,4	49,4	1,8	3,6	51,9	51,3	48,6	50,6	1,8	3,5
4	53	49,6	52	51,5	1,7	3,4	47	50,4	48	48,5	1,7	3,6

**OTA 5 µM**

NAC (mM)	viability (%)			m	stabw	vc (%)	mortality (%)			m	stabw	vc (%)
0	44,7	46,4	43,1				55,3	53,6	56,9			
	45,2	47,2	44,2	45,1	1,5	3,3	54,8	52,8	55,8	54,9	1,5	2,7
0,2	48,0	48,2	46,4	47,5	1,0	2,1	52,0	51,8	53,6	52,5	1,0	1,9
0,5	44,8	47,4	47,6	46,6	1,6	3,4	55,2	52,6	52,4	53,4	1,6	2,9
1	35,4	46,5	43,6	41,8	5,8	13,8	64,6	53,5	56,4	58,2	5,8	9,9
2	44,2	46,6	41,5	44,1	2,6	5,8	55,8	53,4	58,5	55,9	2,6	4,6
4	43,9	44,5	46,5	45,0	1,4	3,0	56,1	55,5	53,5	55,0	1,4	2,5

**OTA 15 µM**

NAC (mM)	viability (%)			m	stabw	vc (%)	mortality (%)			m	stabw	vc (%)
0	35,8	37,5	35,2				64,2	62,5	64,8			
	39,0	37,7	38,2	37,2	1,5	3,9	61,0	62,3	61,8	62,8	1,5	2,3
0,2	39,3	36,2	41,2	38,9	2,5	6,5	60,7	63,8	58,8	61,1	2,5	4,1
0,5	38,5	37,0	39,2	38,2	1,1	2,9	61,5	63,0	60,8	61,8	1,1	1,8
1	42,0	37,9	38,4	39,4	2,2	5,7	58,0	62,1	61,6	60,6	2,2	3,7
2	41,7	37,6	37,5	38,9	2,4	6,2	58,3	62,4	62,5	61,1	2,4	3,9
4	41,9	41,6	39,7	41,1	1,2	2,9	58,1	58,4	60,3	58,9	1,2	2,0

**Table A7 Cytotoxicity of ochratoxin A (24 h, 1 – 15 µmol/l) on HepG2 cells after pre-incubation with buthionine-sulfoximine (BSO, 24 h, 0 – 500 µmol/l)**

**OTA 1 µM**

BSO (µM)	viability (%)			m	stabw	vc (%)	mortality (%)			m	stabw	vc (%)
0	49,9	49,7	53,7	51,3	1,8	3,5	50,1	50,3	46,3	48,7	1,8	3,6
	50,1	51,1	53,3				49,9	48,9	46,7			
5	48,9	51,3	54,3	51,5	2,7	5,3	51,1	48,7	45,7	48,5	2,7	5,6
25	51,1	49,7	52,2	51,0	1,3	2,5	48,9	50,3	47,8	49,0	1,3	2,6
50	47,0	49,1	48,0	48,0	1,1	2,2	53,0	50,9	52,0	52,0	1,1	2,0
100	43,8	49	49,6	47,5	3,2	6,7	56,2	51	50,4	52,5	3,2	6,1
500	43,3	40,4	39,3	41,0	2,1	5,0	56,7	59,6	60,7	59,0	2,1	3,5

**OTA 5 µM**

BSO (µM)	viability (%)			m	stabw	vc (%)	mortality (%)			m	stabw	vc (%)
0	48,7	48,9	48,0	48,6	1,8	3,6	51,3	51,1	52,0	51,4	1,8	3,4
	45,6	49,7	50,8				54,4	50,3	49,2			
5	49,3	51,5	49,9	50,2	1,1	2,3	50,7	48,5	50,1	49,8	1,1	2,3
25	47,0	47,4	49,4	47,9	1,3	2,7	53,0	52,6	50,6	52,1	1,3	2,5
50	40,9	41,5	45,5	42,6	2,5	5,9	59,1	58,5	54,5	57,4	2,5	4,4
100	41,2	44,8	45,3	43,8	2,2	5,1	58,8	55,2	54,7	56,2	2,2	4,0
500	41,2	40,0	36,9	39,4	2,2	5,6	58,8	60	63,1	60,6	2,2	3,7

**OTA 15 µM**

BSO (µM)	viability (%)			m	stabw	vc (%)	mortality (%)			m	stabw	vc (%)
0	43,7	45,1	46,0	45,4	2,2	4,9	56,3	54,9	54,0	54,6	2,2	4,0
	43,0	49,3	45,5				57,0	50,7	54,5			
5	44,8	45,1	47,7	45,9	1,6	3,5	55,2	54,9	52,3	54,1	1,6	2,9
25	41,7	42,4	43,6	42,6	1,0	2,3	58,3	57,6	56,4	57,4	1,0	1,7
50	39,6	41,1	41,7	40,8	1,1	2,7	60,4	58,9	58,3	59,2	1,1	1,8
100	39,9	39,7	43,4	41,0	2,1	5,1	60,1	60,3	56,6	59,0	2,1	3,5
500	38	37,9	33,4	36,4	2,6	7,2	62	62,1	66,6	63,6	2,6	4,1