Chapter 8

Application of BM3 mutants for on-line profiling of zearalenone metabolites

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Application of drug metabolizing mutants of cytochrome P450 BM3 as biocatalysts for the on-line profiling of estrogen receptor binding metabolites of the mycotoxin zearalenone

Submitted.
Abstract

The estrogenic mycotoxin zearalenone (ZEN) is known to undergo hepatic reductive metabolism to form the estrogenic α- and β-isomers of zearalenol (ZEL). Recently, it has been shown that ZEN can also undergo cytochrome P450 monooxygenase (P450) mediated oxidative metabolism to form monohydroxylated products but until now nothing is known about the estrogenic potency of these metabolites. In the present study, we aimed to investigate the metabolism of ZEN by different P450 isoforms and to determine the estrogen receptor α (ERα) affinities of the in vitro P450-generated ZEN metabolites in an on-line high resolution screening (HRS) setup. Human liver microsomes (HLM), recombinant P450s, and mutants of the bacterial P450 BM3 were used to investigate the oxidative metabolism of ZEN and produce large amounts of the metabolites. It was shown that P450 3A4 is mainly involved in the oxidative metabolism of ZEN and that mutants of the bacterial P450 BM3 produced not only the human relevant but also unique ZEN metabolites. Based on the ERα HRS affinity results it was concluded that P450 3A4-mediated hydroxylation of ZEN leads to the formation of products with a reduced estrogenic activity. The approach presented here can be used for the elucidation of the metabolism of other EDCs and xenobiotics in order to get clear pictures of the total effects of these compounds and their metabolites.
8.1 Introduction

Mycotoxins are low-molecular weight substances produced as secondary metabolites by mould fungi species [1], which grow in a wide range of climatic conditions and on a broad range of hosts (including barley, maize, millet, oat, rice, rye, and wheat) [2, 3]. Approximately 25% of the world’s crops are contaminated with mould or fungal growth and mycotoxins may be produced both before and after harvest during storage [4]. Mycotoxins can enter the human body directly by consumption of contaminated crops and their derived food products, or indirectly by “carry-over” in animal tissues, milk, and eggs after intake of contaminated feedstuff [5]. In both humans and animals, the presence of mycotoxins in foods, beverages [6], and feedstuff is acknowledged as a potential health threat since they can lead to mycotoxicoses, the possible symptoms of which are acute intoxication, losses in productivity, reduced weight gain, immunosuppression, and increased risk of cancer [4, 7].

![Chemical structures of zearalenone (ZEN), α-zearalenol (αZEL), and β-zearalenol (βZEL).](image)

The mycotoxin zearalenone (ZEN; Fig 1) is a macrocyclic resorcylic acid lactone that is produced as a secondary metabolite by several Fusarium species (Fusarium graminearum, F. culmorum, F. equiseti, and F. crookwellense) which are regular contaminants of cereal crops worldwide [8]. ZEN exhibits relatively low acute toxicity and carcinogenicity [9]. However, ZEN is harmful for health due to its strong estrogenic and anabolic properties, resulting in severe effects on the reproductive system of farm animals, particularly pigs [6, 10-12]. The structure is clearly flexible enough to allow ZEN to adopt a conformation able to bind to the estrogen receptor α (ERα), although with lower affinity than the natural estrogens estrone (E1), 17β-estradiol (E2), and estriol (E3) [13]. Accordingly, ZEN can be regarded as an endocrine disrupting chemical (EDC) that can alter function(s) of the endocrine system by mimicking or counteracting natural hormones [14]. It is known that endocrine disrupting effects of EDCs can be potentiated or increased after biotransformation occurs [15-20] which makes it very important to investigate the metabolism and estrogenic potency of the possibly formed metabolites of mycotoxins such as ZEN.

The metabolism of ZEN has been investigated in several animal species and in humans [5, 21-30] and it has been shown that three major biotransformation pathways exist for ZEN. These are: (1) formation of the α- (αZEL) and β-isomers (βZEL) of zearalenol (ZEL) through reduction of the carbonyl group at C-7 (Fig 1), assumed to be catalyzed by the hepatic enzymes 3α- and 3β-hydroxysteroid dehydrogenase [22, 25]; (2) conjugation of ZEN and its reduced metabolites with glucuronic acid, catalyzed by uridine diphosphate glucuronosyl transferases (UGTs) [27]; and (3) formation of monohydroxylated ZEN
metabolites, assumed to be catalyzed by the human cytochrome P450 (P450) isoforms 1A2 and 3A4 [29]. Previous studies have indicated that the estrogenic potencies of ZEN and its two major hepatic metabolites varies, with αZEL showing the highest binding affinity to ERα, followed by the parent ZEN. The lowest binding affinity to ERα has been found for βZEL [13, 31-33]. However, until now for the P450-generated monohydroxylated ZEN metabolites the estrogenic potencies are unknown.

In the present study, we have studied the estrogenic potencies of in vitro P450-generated metabolites of ZEN using human liver microsomes (HLM), recombinant human P450 isoforms, and bacterial P450 BM3 mutants. Bacterial P450 mutants were previously shown to bioactivate several drugs to higher amounts of metabolites and/or reactive intermediates than mammalian P450s, which facilitated their identification and structural elucidation [16, 34-37]. Experiments with recombinant human P450 enzymes were performed to identify the isoforms involved in the oxidative metabolism of ZEN. To individually assess the ERα affinity of ZEN and its metabolites, the on-line high resolution screening (HRS) ERα system (Fig 2) was used. This system consists of a HPLC system which is coupled on-line to a bioaffinity detection system [18]. In this system ERα ligand binding domain (LBD) is continuously mixed with coumestrol which shows fluorescence enhancement upon binding to the ERα LBD. Binding of affinity ligands to the ERα LBD will inhibit the coumestrol from binding to the receptor which will result in a fluorescent signal decrease. We report here that BM3 mutants have been successfully applied to biosynthesize two previously described [29] and several novel monohydroxylated ZEN products for which subsequently ERα affinity was determined in a HRS setup.

8.2 Materials and methods

8.2.1 Chemicals

All solvents used were of high purity for high-performance liquid chromatography (HPLC), and purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise. Ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), and β-nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Applichem (Lokeren, Belgium). Coumestrol was obtained from Fluka (Buchs, Germany). Dimethyl sulfoxide (DMSO) potassium dihydrogenphosphate (KH₂PO₄), and dipotassium hydrogenphosphate (K₂HPO₄) were obtained from Riedel de Haën (Seelze, Germany). Zearalenone (ZEN), α-zearalenol (αZEL), β-zearalenol (βZEL), 17β-estradiol (E2), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and polyethylene glycol 3350 (PEG 3350) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). All other chemicals were obtained from standard suppliers unless stated otherwise.

8.2.2 Enzymes and plasmids

Human liver microsomes (HLM), pooled from different individual donors were obtained from BD Gentest™ (San Jose, USA) and contained 20 mg/mL protein (Cat. No. 452161). Recombinant human P450 1A1, 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C18, 2C19, 2D6*1, 2E1, and 3A4 were also purchased from BD Gentest (Cat No. 456211, 456203, 456254, 456255, 456252, 456258, 456222, 456259, 456217, 456206, and 456202,
respectively). The bacterial P450 BM3 M02, M11, M11 V87A, MT35, and MT43 mutants were prepared according to the protocol described in Chapter 7. The LBD of the human ERα was expressed and purified using the protocol described by Eiler et al. [38], without E2 in the medium.

8.2.3 Enzymatic incubations with HLM and recombinant human P450s

All incubations had a final volume of 250 μL and consisted of 100 mM potassium phosphate buffer (pH 7.4), 200 μM substrate, 2 mg/mL protein, and final DMSO concentrations in the incubations were always below 1% (v/v). Reactions were initiated by addition of 50 μL of an NADPH regenerating system resulting in final concentrations of 0.5 mM NADPH, 0.38 mM glucose-6-phosphate, and 0.5 units/mL glucose-6-phosphate dehydrogenase. Reactions were allowed to proceed for 60 min at 37 °C and terminated by the addition of 250 μL ice-cold MeOH. Samples were subsequently centrifuged to remove precipitated protein (14,000 rpm for 15 min) and the supernatants were analyzed both in the on-line ERα bioaffinity assay and by LC-MS. HLM were used to metabolize the three substrates ZEN, αZEL, and βZEL. Recombinant human P450s were only used to perform metabolic incubations with ZEN.

8.2.4 Zearalenone (ZEN) incubations with BM3 mutants

Incubations of ZEN with the different BM3 mutants had a final volume of 500 μL and consisted of 500 nM enzyme and 250 μM ZEN in 100 mM potassium phosphate buffer (pH 7.4). The final DMSO concentration was always 5% and reactions were initiated by addition of 50 μL of an NADPH regenerating system resulting in final concentrations of 2 mM NADPH, 40 mM glucose-6-phosphate, and 2 units/mL glucose-6-phosphate dehydrogenase. Reactions were allowed to proceed for 60 min at 24 °C and terminated by the addition of 500 μL ice-cold methanol. Samples were subsequently centrifuged to remove precipitated protein (14,000 rpm for 15 min) and the supernatants were analyzed both in the on-line ERα bioaffinity assay and by LC-MS.

A large-scale incubation of ZEN by the M11 V87A mutant was performed to determine the bioaffinity of the formed metabolites in the ERα HRS setup. Briefly, 250 μM ZEN was incubated with 500 nM enzyme, 0.5 mM NADPH, 20 mM glucose-6-phosphate, and 0.5 units/mL glucose-6-phosphate dehydrogenase (final concentrations) in a total volume of 10 mL 100 mM potassium phosphate buffer (pH 7.4). Incubations were performed at 24 °C for 3 h and were subsequently terminated by applying the incubation mixture to a strata-X 33 μm Polymeric Sorbent 200 mg / 3 mL (Phenomenex, Amstelveen, The Netherlands) column for SPE sample preparation. Before sample application the column was conditioned with 2 mL MeOH and subsequently equilibrated with 2 mL H2O. The sample was washed with 3 mL 5% (v/v) of aqueous methanol and elution was performed using 3 mL 50% (v/v) of MeOH in ACN. After evaporation, the residue was dissolved in 2 mL 30% (v/v) of aqueous MeOH. A control incubation, which was applied to a strata-X 33 μM column for SPE sample directly at time zero, was also performed. Samples were subsequently analyzed both in the on-line ERα bioaffinity assay and by LC-MS.
8.2.5 Chromatography

All separations were performed on a Phenomenex (Amstelveen, The Netherlands) Luna C18(2) column (4.6 × 150 mm i.d., 5 μm particles) equipped with a 2.0 × 5.0 mm i.d. C18 guard column.

In the HRS setup, an injection volume of 100 μL was used and three different HPLC gradients were applied. For the HLM and recombinant P450 incubations, solvent A consisted of 125 mM ammonium acetate in deionized water, and solvent B consisted of 125 mM ammonium acetate in 99% MeOH. The HPLC column was eluted at a flow rate of 250 μL/min. A linear gradient was used, changing from 65% B to 95% B in 40 min. After 0.5 min of eluting the column with 95% B, the initial 65% B was reached in 0.5 min and kept for 15 min before the next injection. For the BM3 incubations on an analytical scale, solvent A consisted of 1% MeOH in deionized water, solvent B was 99% MeOH and the HPLC column was eluted at a flow rate of 250 μL/min. A linear solvent gradient was used, changing from 30% B to 95% B in 30 min. After 30 min of eluting the column with 95% B, the initial 30% B was reached in 0.5 min and kept for 15 min before the next injection. For the large-scale BM3 incubations, solvent A consisted of 1% MeOH in deionized water, solvent B was 99% MeOH and the HPLC column was eluted at a flow rate of 250 μL/min. A linear solvent gradient was used, changing from 75% B to 90% B in 30 min. After 10 min of eluting the column with 90% B, the initial 75% B was reached in 0.5 min and kept for 15 min before the next injection.

For identification of parent and metabolites by LC-MS, a Finnigan LCQ Deca mass spectrometer (ThermoQuest-Finnigan) was used with negative atmospheric pressure chemical ionization (APCI). An injection volume of 10 μL was always used and the gradients applied were identical to those used in the HRS setup, as described above. An UV detector (280 nm) was placed in series with the MS and by aligning UV spectra obtained with the bioassay setup and those obtained prior to MS, masses could be appointed to compounds, responsible to ERα-affinity signals. The Xcalibur solution software package from Finnigan was used to determine peak areas of the parent and the metabolites in the corresponding extracted ion chromatograms. Standard curves of the parent were linear between 0.5 and 500 μM.

8.2.6 On-line estrogen receptor alpha (ERα) bioaffinity assay

The setup used (Fig 2) was adapted from the homogeneous coumestrol-based ERα affinity detection system described by Kool et al. [18]. In the affinity detection system ERα LBD is continuously mixed with coumestrol which shows fluorescence enhancement upon binding to the ERα LBD. Binding of affinity ligands to the ERα LBD will inhibit the coumestrol from binding to the receptor which will result in a fluorescent signal decrease. The HRS system consists of a HPLC system which is coupled on-line to the bio-affinity detection system [18]. The eluent from the HPLC system is split and 90% is directed to the UV detector. The remaining 10% is mixed with a counteracting makeup gradient to maintain the percentage of organic solvent constant. The total flow is directly introduced into the coumestrol-based receptor affinity detection (RAD) system.

For operation of the on-line ERα assay, two Knauer K-500 HPLC pumps (Berlin, Germany) were used to control the 120 mL superloops, made in house, containing receptor (ERα) and tracer ligand (coumestrol), respectively, and one Knauer K-500 HPLC pump was used for delivery of the injected samples. The ERα and coumestrol solutions were
prepared in sodium phosphate buffer (10 mM; pH = 7.4) containing 150 mM NaCl and 0.4 mg/mL ELISA blocking reagent (Roche, Mannheim, Germany). Both superloops were kept on ice. Flow restrictors were inserted between the pumps and the superloops to ensure proper operation of the pumps at low flow rates. The flow restrictors consisted of natural peek tubing and resulted in back pressures of approximately 50 bar. The pressure limits of the pumps were set 20 bar higher than the working pressure and VICI Jour backpressure regulators (Schenkon, Switzerland) were inserted after the superloops to prevent damage due to possible clogging of the system. To maintain the HPLC column (25 °C) and the two reaction coils (37 °C) at a constant temperature, two Shimadzu CTO-10AC column ovens (Duisburg, Germany) were integrated in the system. Samples were injected using a Gilson 234 autoinjector (Villiers-le-Bel, France) equipped with a Rheodyne (Bensheim, Germany) 6-port injection valve (different injection loops). An Agilent 1100 (Waldbronn, Germany) series fluorescence detector ($\lambda_{\text{ex}}$ 340 nm; $\lambda_{\text{em}}$ 410 nm) was used for monitoring fluorescence of the on-line coumestrol-based ER$\alpha$ trace. The HPLC trace was monitored using UV (Agilent 1100 Series) detection at 280 nm.

![Figure 2](image-url)  
**Figure 2**  
Schematic overview of the ER$\alpha$ receptor affinity detection (RAD) system in high resolution screening (HRS) mode. Samples are injected with an autoinjector (A.I.) and are separated using a gradient reversed-phase high-performance liquid chromatography (HPLC) system controlled by P1 and P2, HPLC gradient pumps. After the column the flow is split by a flow splitter S (split ratio 1:9). HPLC elution is monitored by UV detection. A makeup gradient, controlled by make gradient pumps P3 and P4, is introduced and eluting compounds are mixed in the first reaction coil with ER$\alpha$ delivered by superloop-1 (SL-1). In the second reaction coil the probe ligand coumestrol is added to the mix by superloop-2 (SL-2). ER$\alpha$ affinity detection is performed using a fluorescence detector (FLD).

### 8.3 Results

#### 8.3.1 Biotransformation of ZEN, $\alpha$ZEL, and $\beta$ZEL by HLM

Human liver microsomes were incubated with ZEN and its two major metabolites $\alpha$ZEL and $\beta$ZEL in the presence of a NADPH-regenerating system and the incubations were subsequently analyzed in the HRS ER$\alpha$ setup and by LC-MS/MS. The resulting HRS UV and corresponding ER$\alpha$ bioaffinity traces are depicted in Figure 3A-F.
The UV trace of the HLM incubations with ZEN (Fig. 3A) shows the formation of four products which were not present in the control incubation at time zero. Two metabolites could be identified as αZEL (29.0 min) and βZEL (23.4 min) by co-chromatography with authentic reference compounds and mass spectra obtained by LC-MS/MS. The mass spectra obtained with APCI in the negative mode suggested that peak Z1 (20.1 min) was monohydroxylated ZEL (M-H ion at m/z 335) while peak Z2 (20.4 min) was identified as a monohydroxylated ZEN product (M-H ion at m/z 333). The ERα bioaffinity trace of ZEN (Fig. 3B) shows that only ZEN and αZEL produced a significant ERα affinity signal. The signal decrease in the bioaffinity trace caused by both compounds is similar while ZEN is present at a concentration which based on the UV data is 20 times higher (Fig. 3A). This indicates that the affinity of αZEL towards ERα is higher than that of ZEN.

Figure 3  
ERα bioaffinity traces of HLM incubations with ZEN, αZEL, and βZEL. 
(A) HPLC chromatogram of ZEN incubated for 60 minutes with human liver microsomes (HLM). Z1, Z2, βZEL, and αZEL are metabolites formed during the 60 min incubation. (B) The corresponding ERα bioaffinity trace. (C) HPLC chromatogram of αZEL incubated for 60 minutes with HLM. α1, α2, βZEL, and ZEN are metabolites formed during the 60 min incubation. (D) The corresponding ERα bioaffinity trace. (E) HPLC chromatogram of βZEL incubated for 60 minutes with HLM. β1, β2, and ZEN are metabolites formed during the 60 min incubation. (F) The corresponding ERα bioaffinity trace.

The UV trace of the HLM incubations with αZEL (Fig 3C) shows the formation of four products which were not present in the control incubation at time zero. Two metabolites could be identified as βZEL (23.4 min) and ZEN (30.9 min). The mass spectra suggested that peak α1 (19.2 min) and peak α2 (20.1 min) were both monohydroxylated ZEL products (M-H ion at m/z 335). Based on the identical retention times of Z1 and α2 and the observation that both MS/MS spectra showed a unique fragment at m/z 189, it was
concluded that these peaks were caused by one metabolite. The ERα bioaffinity trace of αZEL (Fig 3D) shows that only αZEL produced a significant ERα affinity signal. The concentration of ZEN present in the sample was probably too low to cause a significant decrease in the bioaffinity trace.

The UV trace of the HLM incubations with βZEL (Fig 3E) shows the formation of three products which were not present in the control incubation at time zero. One metabolite could be identified as ZEN (30.9 min). The mass spectra indicated that peak β1 (16.6 min) and β2 (19.8 min) were both monohydroxylated ZEL products (M-H ions at m/z 335). The ERα bioaffinity trace of βZEL (Fig 3D) shows that only βZEL and ZEN produced a significant ERα affinity signal. The signal decrease in the bioaffinity trace of both compounds is similar while ZEN is present at a much lower concentration (Fig 3E). This indicates that the affinity of βZEL towards ERα is lower than that of ZEN.

8.3.2 Activity of human P450 isoforms for the hydroxylation of ZEN

In order to get more information about the human P450 isoforms involved in the formation of the oxidative metabolites of ZEN, recombinant human P450s were incubated with ZEN in the presence of an NADPH regenerating system and the incubations were subsequently analyzed in the ERα HRS setup and by LC-MS/MS. The activities of eleven major isoforms i.e. 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, and 3A4 were tested in this experiment.

![Figure 4](image_url) Activities of recombinant human P450 isoforms for the hydroxylation of ZEN.
To obtain these results, the recombinant human P450 isoforms 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, and 3A4 were incubated with ZEN. Only 1A1 and 3A4 displayed formation of hydroxylated ZEN products. The activity of 3A4 has been set to 100% in this figure.

It was found that in all incubations minor amounts of αZEL and βZEL were formed. However, only the 1A1 and 3A4 isoforms also showed the formation of additional metabolites which were identified as being monohydroxylated ZEN products (M-H ion at m/z 333). The results of the experiment are shown in Fig 4. It can be seen from this figure that the activity of 3A4 is much higher than that of 1A1. Furthermore, 1A1 only produced one hydroxylated ZEN product which showed a unique fragment at m/z 191 while 3A4 produced three metabolites. One of these metabolites gave a unique fragment at m/z 191.
while another metabolite showed a unique fragment at \( m/z \) 187. For the remaining metabolite it was not possible to get more information about its identity based on the LC-MS/MS data.

All incubation mixtures were subsequently analyzed in the ER\( \alpha \) HRS setup and it was found that the levels of the formed monohydroxylated ZEN products were too low to detect any bioaffinity signals. However, we did observe significant bioaffinity signals for the minor amounts of \( \alpha \)ZEL that were formed.

### 8.3.3 Biotransformation of ZEN by P450 BM3 mutants

Analysis of the HLM and recombinant human P450 incubations did not show any ER\( \alpha \) affinity for the formed monohydroxyl metabolites which could be caused by the fact that the amounts of the formed metabolites were too low for detection. It was therefore decided to investigate if P450 BM3 mutants could be used for the generation of larger amounts of metabolites.

![Activities and metabolite distributions of ZEN incubations with BM3. All mutants tested formed the metabolites M1, M2, M3, M4, and M5.](image)

Five different BM3 mutants (M02, M11, M11 V87A, MT35, and MT43) were incubated with ZEN in the presence of a NADPH-regenerating system and the incubations were subsequently analyzed in the ER\( \alpha \) HRS setup and by LC-MS/MS. As can be seen from Fig 5, five products were formed that were not present in the control incubation at time zero. The LC-MS/MS data indicated that all these metabolites were monohydroxylated ZEN products (M-H ion at \( m/z \) 333). The ER\( \alpha \) bioaffinity traces showed that for all incubations, only the parent peak of ZEN caused a significant decrease in the affinity signal. Fig 5 shows that although all mutants formed the same five metabolites, the percentage of total conversion and the metabolite distribution varied between mutants. Mutant M18 V87A showed the highest amount of conversion (> 25%) and formed predominantly metabolite M1 in a significant larger amount. The other four mutants predominantly formed metabolite M3 while displaying a lower conversion. The conversion
of ZEN by HLM determined in the experiments described above was 25% where the α- and β-isomers of ZEL were the major products while the monohydroxylated products of ZEN were only minor metabolites.

Figure 6  ERα affinity traces of large-scale ZEN incubations with the M11 V87A mutant.
(A) HPLC UV chromatograms of ZEN incubated for 0 and 3 h with M11 V87A. M1, M2, M3, M4, M5, and M6 are metabolites formed during the 3 h incubation. (B) The corresponding ERα affinity traces.

Based on the BM3-mediated incubations on an analytical scale, mutant M11 V87A was selected to perform a large-scale incubation of ZEN to produce large amounts of the hydroxylated ZEN metabolites. The results of this large-scale incubation are depicted in Figure 6. The UV chromatogram (Fig 6A) shows that at least six metabolites were formed which were not present in the control incubation at time zero and ZEN was converted for more than 80%. The corresponding ERα bioaffinity trace (Fig 6B) clearly demonstrates that metabolite M1, M2, M4, M5 and M6 display significant affinity for the ERα. Metabolite M3 does not display any affinity and may exhibit a minor increase of
fluorescence. Based on the UV traces, it can be concluded that for M1-5 the ERα affinity is lower than that of ZEN. M6, however, seems to be more active than the other metabolites while due to its peak shape it is difficult to compare the activity to that of ZEN itself.

LC-MS/MS measurements were performed to identify the different metabolites and the daughter ion spectra of M1-5 are depicted in Fig 7. M1 and M2 were found to be monohydroxylated ZEN products (M-H ion at m/z 333). In both MS/MS spectra, the presence of the unique fragment at m/z 187 indicates that the aromatic ring of ZEN had not been hydroxylated and that therefore the hydroxylation had occurred elsewhere in the molecule [29]. The LC-MS/MS data indicated that the peak of M3 (Fig 6A) actually consisted of two monohydroxylated ZEN products. For both products, a unique fragment at
Peak M4 and M5 were identified to be monohydroxylated ZEN products where hydroxylation has also not occurred at the aromatic ring [29]. Peak M6, which exhibited the highest ERα affinity amongst the formed metabolites, could be detected by UV in the LC-MS/MS setup but it was very difficult to identify this metabolite since no clear mass signal was observed. However, the LC-MS data indicate that the mass of this metabolite was 332 since the corresponding extracted ion chromatogram (M-H ion at m/z 331) showed a peak with a similar shape as the UV peak and this peak was not present in the control incubation at time zero (data not shown).

8.4 Discussion

Recently, it has been shown that oxidative metabolites of ZEN were formed in vitro and in vivo through P450-mediated metabolism but for these metabolites, until now, no information about their estrogenic affinity was available. In the present study, we have employed HLM, recombinant human P450s, and mutants of the bacterial P450 BM3 to generate oxidative ZEN products and subsequently screen these metabolites for their ERα affinity in a HRS setup.

The HLM incubations showed that ZEN is metabolized into αZEL and βZEL. In addition, the formation of monohydroxylated products of ZEN and ZEL was observed. The major ZEN metabolites αZEL and βZEL were also incubated separately with HLM and the formation of ZEN and monohydroxylated products of ZEN and ZEL was observed. Recently, Pfeiffer et al. [29] have described that ZEN is metabolized by HLM into αZEL, βZEL, three different hydroxyl ZEN metabolites, which were identified as 6- or 8-OH-ZEN, 13-OH-ZEN, and 15-OH-ZEN, and three hydroxyl αZEL metabolites, which were identified as 6- or 8-OH-αZEL, 13-OH-αZEL, and 15-OH-αZEL. In our experiments the amounts of monohydroxylated products formed were very low which made it difficult to identify the structures of the products formed. However, based on the MS/MS spectra for some metabolites it was possible to get more information about the site of the hydroxylation. Z1, a hydroxylated product of ZEN, showed a unique fragment at m/z 191 which suggested that this hydroxylation had taken place on the aromatic ring and therefore this metabolite was identified as either 13- or 15-OH-ZEN [29]. A hydroxylated product of αZEL which was identified in the ZEN incubations as metabolite Z1 and in the αZEL incubations as metabolite α2, showed a unique fragment at m/z 189 (data not shown) which suggested that this metabolite is either 6- or 8-OH-αZEL [29]. The formed hydroxyl products did not display any ERα affinity, probably due to the fact that their concentrations were too low for detection. Based on these results it was decided to investigate if BM3 mutants could be used to increase the formation of the oxidative metabolites of ZEN and subsequently test these for their ERα affinity. Therefore no further efforts were made to improve the HLM-mediated ZEN metabolism or to further identify the metabolites formed in the in vitro HLM incubations described above.

To investigate which human P450 isoforms are responsible for the oxidative metabolism of ZEN, incubations were performed with recombinant human P450s. The results indicated that P450 1A1 and 3A4 are responsible for the oxidative metabolism since only in these incubations the presence of monohydroxylated ZEN products was observed. The MS/MS spectra suggested that the formed metabolites are either 6- or 8-OH-ZEN or 13- or 15-OH-ZEN. Pfeiffer et al. [29] also investigated the activity of human P450
isoforms for the hydroxylation of ZEN and they found that ZEN is mainly metabolized by 1A2 while 3A4 also displays low activity. We did not detect any oxidative metabolites in the experiments with recombinant P450 1A2. On the other hand, our finding that 3A4 is involved in the hydroxylation of ZEN is in agreement with the results of Pfeiffer et al. In our recombinant P450 incubations, the formation of minor amounts of the reductive ZEN products αZEL and βZEL was observed. The formation of these metabolites is assumed to be catalyzed by 3α- and 3β-hydroxysteroid dehydrogenase [22, 25] and was thus probably caused by other enzymes that were present in the recombinant P450 solutions.

Since the amounts of the monohydroxylated ZEN metabolites were too low for affinity detection in the HRS ERα setup, experiments were performed to investigate if bacterial P450 mutants could be used to generate these metabolites on a larger scale. Five different BM3 mutants were used which have been demonstrated to be active towards drug-like molecules in previous studies while showing different metabolite profiles [16, 34-36].

The initial BM3 experiments showed that these mutants produced five hydroxylated ZEN products in 90 min. The large-scale 3 h incubation showed the formation of two additional metabolites, one of which (M6 in Fig 6A) most likely is a secondary metabolite. Based on the LC-MS/MS data, we could establish that for two of the hydroxyl metabolites (M3A and M3B in Fig 7 which together form peak M3 in Fig 6A), hydroxylation had taken place on the aromatic ring and therefore these metabolites were identified to be the human relevant 13-OH-ZEN and 15-OH-ZEN. The ERα affinity trace indicates that hydroxylation at these positions in the aromatic ring results in a loss of ERα affinity which is in agreement with the observation by Shier et al. that blocking of the resorcinol OH groups results in a loss of estrogenicity [13]. Takemura et al. [31] have reported that ZEN is capable to occupy the active site of ERα in a strikingly similar manner as E2, such that the resorcinolic ring of ZEN occupies the same area as the A-ring of E2 [39] which suggests that introduction of substituents in the ZEN ring may influence binding. For the other four monohydroxylated metabolites (M1, M2, M4, and M5 in Fig 6A), we could only establish that hydroxylation had not occurred on the aromatic ring but elsewhere in the molecule. Based upon the ERα bioaffinity trace, we concluded that the estrogenic affinity of these four metabolites decreased. The metabolite which eluted just before ZEN (M6 in Fig 6A) with a molecular ion at m/z 331 may be a secondary metabolite of ZEN where one of the newly introduced hydroxyl groups was further oxidized into a ketone. The P450-mediated formation of a similar product originating from a monohydroxylated product of the mycotoxin 7,8′-dehydrozearalenol has been reported previously [40].

Based upon the experiments described above, we concluded that P450-mediated hydroxylation of ZEN does not lead to the formation of metabolites which display an increased ERα bioaffinity. However, two catechol metabolites are being formed (13- and 15-OH-ZEN) which may be a cause for concern since catechol metabolites of endogenous compounds, such as E2, and xenobiotics are often associated with toxic effects [41]. These include formation of DNA adducts after further oxidation to quinone or formation of reactive oxygen species through redox cycling [42]. In addition, the novel ZEN metabolite (at m/z 331) that displays a significant ERα affinity needs to be further investigated in order to establish the identity and the metabolic pathway of this product. It has been shown in the present study that the BM3 mutants may be very valuable for these studies in order to generate large amounts of the metabolites which facilitates structural elucidation and pharmacological and toxicological evaluation.
8.5 Conclusions

The aim of this study was to investigate the metabolism of ZEN by different P450s and to determine the estrogenic potencies of the \textit{in vitro} P450-generated metabolites of ZEN. It was shown that mutants of the bacterial P450 BM3 could be used to produce both human relevant and novel ZEN metabolites and that the HRS technology could be used to screen the metabolic mixtures for ER$\alpha$ affinity. In addition, it was shown that P450 3A4 is mainly involved in the oxidative metabolism of ZEN and that P450 3A4-mediated hydroxylation of ZEN leads to the formation of products with a reduced estrogenic activity.

The here presented experiments demonstrated to be useful to investigate the metabolism of the EDC ZEN and identify and characterize its metabolites. This general approach in the future can be used for the elucidation of the metabolism of other EDCs and xenobiotics in order to get clear pictures of the total effects of these compounds and their metabolites. In addition, the combination of BM3-generated biosynthesis and HRS screening can also be highly valuable in early drug discovery since it allows the rapid generation, optimization and more importantly toxicological and/or pharmacological evaluation of (novel) lead compounds and their metabolites.

Acknowledgements

The ER$\alpha$ LBD expressing \textit{Escherichia coli} cells were a kind gift of Dr. Marc Ruff and Dr. Dino Moras. The authors would like to acknowledge Prof. Dr. Wilfried M.A. Niessen for his assistance in elucidating the LC-MS data.
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