



Zearalenone and its metabolite exposure directs oestrogen metabolism towards potentially carcinogenic metabolites in human breast cancer MCF-7 cells

Faezeh Malekinejad^{1,2} · Johanna Fink-Gremmels³ · Hassan Malekinejad^{1,4}

Received: 23 March 2022 / Revised: 20 November 2022 / Accepted: 22 November 2022 / Published online: 15 December 2022

© The Author(s) under exclusive licence to Society for Mycotoxin (Research Gesellschaft für Mykotoxinforschung e.V.) and Springer-Verlag GmbH Germany, part of Springer Nature 2022

Abstract

Zearalenone (ZEN) is produced by *Fusarium* species contaminating various agriculture crops. In this study, the effects of ZEN and its metabolites α-zearalenol (α-ZEL), and β-zearalenol (β-ZEL) on the formation of carcinogenic oestrogen-catechols in MCF-7 cells were investigated. To assess the effects of mycoestrogens on the activity of cytochrome P450 1A1 and CYP1B1, the rate of ethoxresorufin O-deethylation (EROD-assay) was measured. The effects of mycoestrogens on the expression of CYP 1A1, CYP 1B1, aryl-hydrocarbon receptor (AhR), and oestrogen receptor alpha (ER α) were determined by qPCR. The catechol-O-methyltransferase (COMT) activity was measured as the ratio of the methoxy metabolites of oestradiol. Results show that mycoestrogens inhibited significantly the CYP1-dependent EROD activities. In the presence of selective inhibitors, mycoestrogens reduced CYP 1A1 and enhanced CYP 1B1 activity. Quantitative PCR analyses demonstrated the upregulation of AhR and confirmed the selective effect of mycoestrogens on CYP1 expression levels and the decline of the CYP 1A1/CYP 1B1 ratio. Mycoestrogens increased the ratio of 4-MeOE to 2-MeOE2 formation significantly ($P < 0.05$). Our results suggest that the tested mycoestrogens increase the production of CYP1B1-mediated oestrogen catechol metabolites, directing the biotransformation of E2 towards 4-OHE2, which has been identified earlier as a crucial factor in oestrogen-induced tumour initiation.

Keywords Zearalenone · Mycoestrogens · Oestrogen metabolism · Catechol oestrogen · Carcinogenicity

Abbreviations

AhR Aryl-hydrocarbon receptor
COMT Catechol-O-methyltransferase

CYP Cytochrome P450
ER Oestrogen receptors
TCDD 2,3,7,8-Tetrachlorodibenzo-p-dioxin
ZEN Zearalenone
α-ZEL α-Zearalenol
β-ZEL β-Zearalenol

✉ Hassan Malekinejad
hassanmalekinejad@yahoo.com; malekinejad.h@umsu.ac.ir

Faezeh Malekinejad
faez.mknj@gmail.com

Johanna Fink-Gremmels
J.fink@uu.nl

¹ Experimental & Applied Pharmaceutical Sciences Research Center, Urmia University of Medical Sciences, Urmia, Iran

² Department of Clinical Biochemistry, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran

³ Department of Veterinary Pharmacology, Pharmacy and Toxicology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

⁴ Department of Pharmacology and Toxicology, School of Pharmacy, Urmia University of Medical Sciences, Urmia, Iran

Introduction

The mycotoxin zearalenone (ZEN) is produced as a secondary metabolite by various *Fusarium* species, which are found in foods and feed materials (EFSA 2017). ZEN is a stable compound and is not degraded or inactivated by common food and feed processing procedures. Following consumption of contaminated food/feed, ZEN and/or its metabolites act as an oestrogenic compound and via binding to oestrogen receptors (ERs) produce hyperoestrogenism (Fruhauf et al. 2019; Malekinejad et al. 2005). ZEN is metabolised mainly by steroid dehydrogenases, yielding as main metabolites

α -ZEL and β -ZEL. Minor metabolites originate from a CYP450 catalysed oxidation resulting in the catechols including 6-OH-ZEL, as well as 8-OH-ZEL, 13-OH-ZEL, and 15-OH-ZEL, respectively (Drzymala et al. 2014).

Many environmental contaminants including polycyclic aromatic hydrocarbons and polyhalogenated dioxins, furans, and biphenyls exert their toxic effects by binding and activating aryl hydrocarbon receptors (AhR) (Moorthy et al. 2015; Spink et al. 2003a, b; Whitlock 1999). The AhR is an intracellular protein, which was originally characterised as a xenobiotic receptor and acts as a ligand-activated transcription factor (Gargaro et al. 2021). This intracellular protein binds to the inducing compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the cytoplasm, and then, the inducer-receptor complex releases associated heat-shock 90 proteins and other chaperons. The inducer-receptor complex enters the nucleus and forms an active heterodimer with ARNT (Ah receptor nuclear translocator) protein. This complex in the nucleus binds to the 5' –regulatory regions of several genes and consequently alters the rate of their transcription (Fallarino et al. 2014; Roberts et al. 2000).

Among the inducible target genes for AhR are the biotransformation enzymes cytochrome P450 1A1 and 1B1 (CYP1A1 and CYP1B1). The induction of CYP1A1 and 1B1 in human breast cancer MCF-7 cells has already been reported in response to the TCDD exposure (Christou et al. 1994; Shimada and Fujii-Kuriyama 2004). Moreover, CYP1A1 and 1B1 both are expressed in normal human breast epithelial cells and CYP1B1 is the dominant one in non-induced native cells (Larsen et al. 1998).

In the breast epithelium, CYP1A1 and 1B1 play a major role in oestrogen (estrone (E1) and 17 β -oestradiol (E2)) metabolism and catalyse the formation of oestrogen catechol (Li et al. 2021; Spink et al. 1994, 1997). The main catechols produced by CYP1A1 and 1B1 are 2-hydroxy and 4-hydroxy-E2 (2OH- and 4-OHE2), respectively, which both can be further oxidised to form reactive quinones. Quinones built from 2-OH-E2 are considered to react with DNA to form stable adducts without mutations. In contrast, quinones derived from 4-OH-E2 form depurinating adducts resulting in mutational changes (Stack et al. 1996). Thus, 4OH-E2 seems to be the most genotoxic metabolite of E2 as oestradiol 4-hydroxylation activity is enhanced in breast and uterine tumours in comparison to normal tissues (Liehr et al. 1995).

Previous reports indicated that ZEN enhanced the TCDD-induced CYP1B1 activity but not mRNA expression in MCF-7 cells. The authors showed that ZEN reduced CYP1A1 activity and mRNA levels in the TCDD-induced MCF-7 cells (Yu et al. 2004). However, there is lack of knowledge about any effect(s) of ZEN metabolites on CYP1A1 and 1B1 expression and on E2 metabolism. Moreover, previous investigations postulated that ER α is possibly involved in the activities of AhR in oestrogen-responsive cells (Klinge et al. 2000; Swedenborg and Pangratz 2010). In this study, we aimed to investigate the

possible effect of ZEN and its major (reductive) metabolites in comparison with E2 on CYP1A1 and CYP1B1 activity. Subsequently, alterations in mRNA level of CYP1A1, CYP1B1, ER α , and AhR in TCDD-induced and control MCF-7 cells were investigated to identify potential transcriptional links. Finally, the effects of ZEN and its metabolites on E2 metabolism, which is catalysed by CYP1A1 and 1B1, were addressed to show potential implications of such an interaction for a selective DNA adduct formation and carcinogenesis of endogenous oestrogens.

Material and methods

Chemicals

Zearalenone (ZEN), α -zearalenol (α -ZEL), β -zearalenol (β -ZEL), 7-ethoxyresorufin, magnesium chloride, sodium chloride, dicumarol, α -naphthoflavone, pyrene, S-adenosyl-L-methionine (SAM), and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St Louis, MO, USA). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was obtained from SUPELCO (Bellefonte, PA, USA). Alamar Blue (AB) was purchased from Biosource International, Biosource Netherlands, B.V. E2, 2-OHE2, and 4-OHE2 have been purchased from Steraloids, Inc. (Newport, Rhode Island 02,840, USA). Methanol, acetone, acetonitrile, hexane, and dichloromethane (DCM) were of HPLC grade and obtained from J.T. Baker (Deventer, the Netherlands). Analytical grade acetic acid was from Merck (Darmstadt, Germany). Solid-phase extraction (SPE) cartridges C₁₈ (500 mg; 3 mL) were purchased from J.T. Baker (Bergen op Zoom, the Netherlands). Dulbecco's modified Eagle's tissue culture medium (DMEM) was supplied by Combrex (Bio Science Verviers, Belgium).

Cell culture

Human breast cancer cells (MCF-7) were cultured in DMEM, supplemented with 10% bovine calf serum (BCS), 1% penicillin (100 units/mL) streptomycin (100 μ g/mL), 1% L-glutamine, and 1% sodium pyruvate (100 mM). Cells were cultured in standard culture flasks, kept at 37 °C in a humidified atmosphere of 5% CO₂ in air. Every 4–5 days' subcultures were performed using trypsin. Prior to chemical exposure, cells were grown in phenol red-free DMEM containing 10% charcoal-stripped BCS to avoid any influence of phenol red and normal BCS oestrogenic activity.

Cell viability

For cytotoxicity assessment, MCF-7 cells were seeded in 96-well tissue culture plates at a density of 2×10^4 cells/well.

Cells were treated for 48 h with test compounds at various concentrations (0, 1, 10, 50, 100, and 250 nM). The control cells received only the same concentration of test compound's solvent (DMSO), and the final concentration of solvent was not more than 0.1% of the culture medium. After the given treatment period of time, Alamar Blue (AB) solution (10: 1, v/v) was added to the cell suspension and incubated for 3 h. AB assay was used as an indicator of living cells in the reduction of resazurin to fluorescent resorufin. The fluorescence of the medium, due to the reduced AB, was measured by using an excitation wavelength of 560 nm and an emission wavelength of 590 nm (Fluostar, Fluorescence Measurement System, BMG Labtechnologies, GmbH, Germany). The cell viability was calculated:

$$\% \text{ cell viability} = \frac{\text{AB reduction activity of treated cells}}{\text{AB reduction activity of control cells}}$$

Ethoxresorufin O-deethylation (EROD) assay

The CYP1 EROD activity assay was measured according to the method described by Burke and Mayer (1983) with minor modification (Burke and Mayer 1983). In short, MCF-7 cells were seeded in 96-well plates and incubated for 24 h to attach and then treated with 10-nM TCDD and various concentrations of E2, ZEN, α -ZEL, and β -ZEL or DMSO (0.1% v/v) for 48 h. The medium was removed after treatment, and cells were washed twice with pre-warmed PBS and then incubated with 50-mM Tris buffer (pH 7.8) containing 0.9% NaCl (w/v), 6.25 mM MgCl₂, 5 μ M 7-ethoxresorufin, and 10 μ M dicumarol at 37 °C for 90 min. IC₅₀ values of E2 and mycoestrogen-treated cells relative to the TCDD-received group were calculated by CompuSyn software (Combosyn, Inc., Paramus, USA).

For inhibition studies, after exposure of the MCF-7 cells to TCDD and E2 or different mycoestrogens (100 nM), cells were treated with α -naphthoflavone (ANF) as a specific inhibitor of CYP1A1 and/or pyrene (PYN), a specific inhibitor of CYP1B1, at 10- μ M concentrations for 6 h. The formation of resorufin, a metabolite with fluorescence characteristic, was measured fluorometrically with an excitation wavelength of 530 nm and emission at 590 nm.

Cytosolic fraction preparation and catechol-O-methyltransferase (COMT) activity measurement

In order to investigate any possible effect of ZEN and its metabolites on the O-methylation activity, the TCDD-induced MCF-7 cells were homogenised in a 2-mL cold phosphate buffer (pH 7.2) containing 0.1-mM EDTA by using a Potter–Elvehjem Teflon-glass homogeniser. Thereafter cytosolic part of the cells was prepared through two

times centrifugation (Beckman L7-55): first at 10,000 \times g for 10 min at 4 °C to remove any debris and second time at 100,000 \times g for 75 min at 4 °C to separate the supernatant as the cytosolic part of cells. The obtained cytosolic parts were stored at –70 °C for further analyses.

COMT activity was measured according to a previously described method (van Duursen et al. 2004). Briefly, equal amounts of cytosolic proteins (200 μ g) from TCDD-induced (10 nM) MCF-7 cells were incubated with 50-mM phosphate buffer (pH 7.6), 5-mM MgCl₂, 150- μ M SAM, 1-mM DTT, and various concentrations of ZEN, α -ZEL, and β -ZEL or the solvent vehicle (DMSO 0.1%). Reaction was started by adding 2-OHE2 and 4-OHE2 (10 μ M) and incubating at 37 °C for 30 min. The reaction was stopped by placing the reaction tubes on ice. After the addition of the internal standard (20- μ L equilin, 10 μ M), metabolites were extracted using dichloromethane and solid-phase extraction (SPE), respectively. Subsequently, the eluted solutions of the SPE were evaporated under the stream of N₂ till dryness. The extracted samples were derivatized using trimethylsilyl, and the peak areas for 2- and 4-MeOE2 at m/z 446 and equilin at m/z 340 were identified by GC/MS.

RNA isolation and RT-PCR

Total RNA was isolated from MCF-7 cells using the standard TRIZOL method. For RT-PCR, cDNA was synthesized in a 20- μ L reaction mixture containing 4 μ L of 5 \times iScript Reaction Mix, 1- μ L iScript reverse transcriptase, and 15- μ L nuclease-free water containing 1- μ g RNA template, according to the protocol of the manufacturer (iScript TM cDNA Synthesis Kit, BIO RAD). The synthesized cDNA was amplified by a standard PCR mixture. Cycling protocols for 20- μ L reaction mix were 5 min at 25 °C, followed by 30 min at 45 °C, and 5 min at 85 °C.

Quantitative-PCR analysis for hs CYP1A1, 1B1, ER α , and AhR in control and treated MCF-7 cells

Using the iCycler MyiQ system (Bio-Rad), cDNA from control and treated MCF-7 cells with E2, ZEN, α -ZEL, and β -ZEL at 0, 50, 100, and 250 nM, respectively, for 48 h were subjected to qPCR in total 25- μ L reaction mix, which contains 10- μ L cDNA template, 0.75- μ L forward primer, 0.75- μ L reverse primer, 12.5- μ L IQ SYBR Green supermix solution, and 1- μ L DNA-free water. qPCR conditions were run as the following protocol: general denaturation at 95 °C for 3 min, 1 cycle, followed by 40 cycles of denaturation, 95 °C for 20 s; annealing, 64.2 °C for 30 s; and elongation, 72 °C for 30 s. The specific primers for CYP1A1, 1B1, AhR, and ER α were designed and manufactured by Invitrogen (Invitrogen, Life technologies, Paisley, Scotland, UK). Primer pairs for qPCR are as listed in Table 1.

Table 1 Nucleotide sequence of the primers used in qPCR

Target gene	GenBank accession number	Primers (5'- 3')
CYP1A1	NM_000499	FWD: TATGACCACAAACCACCAAGAACT REV: GTAGGTAGCGAAGAATAGGGATGA
CYP1B1	NM_000104	FWD: TTGGTTATTCTGAAGGTAGCA REV: CTTCTTCATTCACTAGTAGTTGGTGT
AhR	NM_001621	FWD: TGTATAACCCAGCCAGATTCT REV: TCCAGCAGACACCTTAGACGA
ER α	NM_000125	FWD: CCGCTCATGATCAAACGCTCT REV: ATCCAACAAGGCAGTGAACCATCT
β -Actin	NM_001101.5	FWD: CTT CTA CAA TGA GCT GCG TG REV: CAT GAG GTA GTC AGT CAG G

Statistical analysis

Results were analysed with GraphPad Prism (Version 7.0; GraphPad software Inc., San Diego, USA). Results were presented as mean \pm standard deviation. To compare the differences between various groups, analysis of variance (ANOVA) was performed and followed by Bonferroni post hoc test. A P value <0.05 was considered significant.

Results

Effects of E2 and mycoestrogens on cell viability

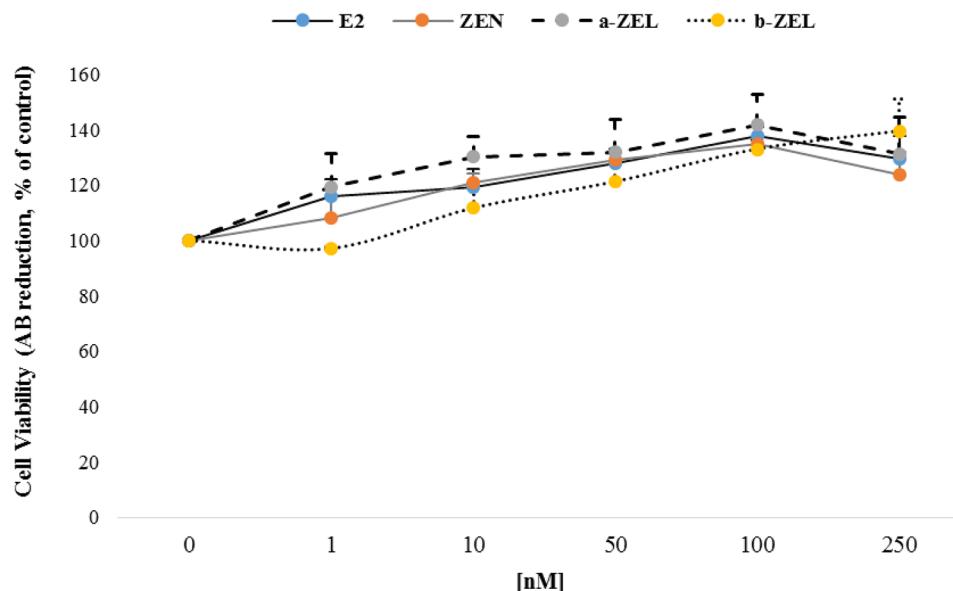
To exclude any cytotoxic effects of E2 and mycoestrogen treatment on MCF-7 cells, the given concentrations (1–250 nM) were tested in the Alamar Blue reduction assay. None of the used compounds resulted in cytotoxicity after 48-h treatment time. By contrast, E2 and mycoestrogens at

the tested concentrations increased the proliferation rate by 10 to 40% (Fig. 1).

Effect of E2 and mycoestrogens on TCDD-induced EROD activity

The EROD assay was used to determine any effect of E2 and mycoestrogens on CYP1 activities. Exposing MCF-7 cells to 10-nM TCDD resulted in an almost sixfold induction in CYP1 activities in comparison to control (DMSO treated) cells. Moreover, exposing TCDD-induced cells subsequently to E2, ZEN, α -ZEL, and β -ZEL at various concentrations (0, 1, 10, 50, 100, and 250 nM) for 48 h reduced these EROD activities significantly ($P < 0.05$). Surprisingly, β -ZEL as being a weak mycoestrogen in terms of oestrogenic potency reduced CYP1 activities more than the other studied mycoestrogens and even 17 β -estradiol (Fig. 2). Calculated IC50 values in nanomolar scale resulted in the following ranking:

Fig. 1 Effect of E2 and mycoestrogens on MCF-7 cell viability and proliferation. Results of the Alamar Blue reduction assay. Data are expressed as percentage of untreated controls. Data points represent results of triplicates



$$\beta - ZEL \ 1.57 \pm 0.53 < ZEN \ 3.47 \pm 0.25 < \alpha < E2 \ 27.03 \pm 9.34 \\ \pm 9.34. - ZEL \ 24.1 \pm 3.9 < E2 \ 27.03 \pm 9.34$$

Effect of E2 and mycoestrogens on EROD activity in the presence of CYP selective inhibitors

In order to evaluate and determine the distinct effect of E2 and mycoestrogens on CYP1A and CYP1B1 activities, the TCDD-induced MCF-7 cells were co-incubated with either 1- μ M α -naphthoflavone (ANF) as a selective CYP1A1 inhibitor or 1- μ M pyrene (PYN) as a selective CYP1B1 inhibitor for 6 h. ANF reduced significantly ($P < 0.05$) the EROD activity in TCDD-pre-treated cells. Combining the ANF treatment with E2 and mycoestrogens at 0.1- μ M concentration further decreased the inducing effect of TCDD on EROD activity. β -ZEL and E2 exerted the strongest inhibitory effect, respectively (Fig. 3A).

In a comparable experimental design, the effect of a pre-treatment of the cells with 1- μ M PYN, as a selective CYP1B1 inhibitor, was analysed. Results show that E2 and ZEN partly reversed the PYN inhibition of EROD activity, significantly ($P < 0.05$), while no significant difference was measured for α -ZEL and β -ZEL (Fig. 3B).

Mycoestrogens downregulated the expression of CYP 1A1 and 1B1 in TCDD-induced MCF-7 cells

Since both CYP1A1 and 1B1 display EROD activity, the expression of both enzymes was measured by qPCR in control and TCDD-induced MCF-7 cells. Results show that

CYP1B1 gene expression was 25-fold higher than CYP1A1 (Fig. 4A) in intact MCF-7 cells, while CYP1A1 expression was induced by a factor of 7.7-fold higher after 48-h exposure to 10-nM TCDD (Fig. 4B).

Treatment of TCDD-induced cells with various concentrations of E2 and mycoestrogens collectively decreased the CYP1A1 mRNA level in a concentration-dependent manner. E2, ZEN, α -ZEL, and β -ZEL at the highest given concentration (100 nM) lowered the TCDD-induced CYP1A1 mRNA level by 58, 62, 66, and 68%, respectively (Fig. 5A).

In the intact MCF-7 cells, the CYP1B1 gene was expressed and significantly induced by 10-nM TCDD. Treatment of TCDD-induced cells with E2 and mycoestrogens caused a downregulation of CYP1B1 expression at the mRNA level (Fig. 5B). However, the degree of downregulation in the expression of CYP 1B1 was found significantly lower than that of CYP 1A1.

Since the expression of CYP1A1 and 1B1 was downregulated under E2 and mycoestrogen exposure with significant differences, we calculated the ratio of CYP1A1/1B1 expression. As shown in Table 2, calculation of the ratio of CYP1A1/1B1 following exposure to the test compounds and comparing with control (TCDD-induced) cells highlighted a concentration-dependent and significant decrease of the ratio for E2, ZEN, and β -ZEL but not α -ZEL. While the overall ratio for α -ZEL was lower than the TCDD reference ratio, a reversed response to increasing concentrations was observed. The lowest ratio was obtained for 100-nM β -ZEL treatment and followed by ZEN, E2, and α -ZEL at an equimolar concentration.

Fig. 2 Effects of E2 and mycoestrogens on CYP1 activity (EROD assay) in TCDD-induced MCF-7 cells: asterisks indicate a significant ($P < 0.001$) difference between the control and TCDD-received cells and ### represents significant ($P < 0.001$) differences between the TCDD-exposed and TCDD plus E2 and/or mycoestrogen-treated MCF cells. Data points show means of triplicates

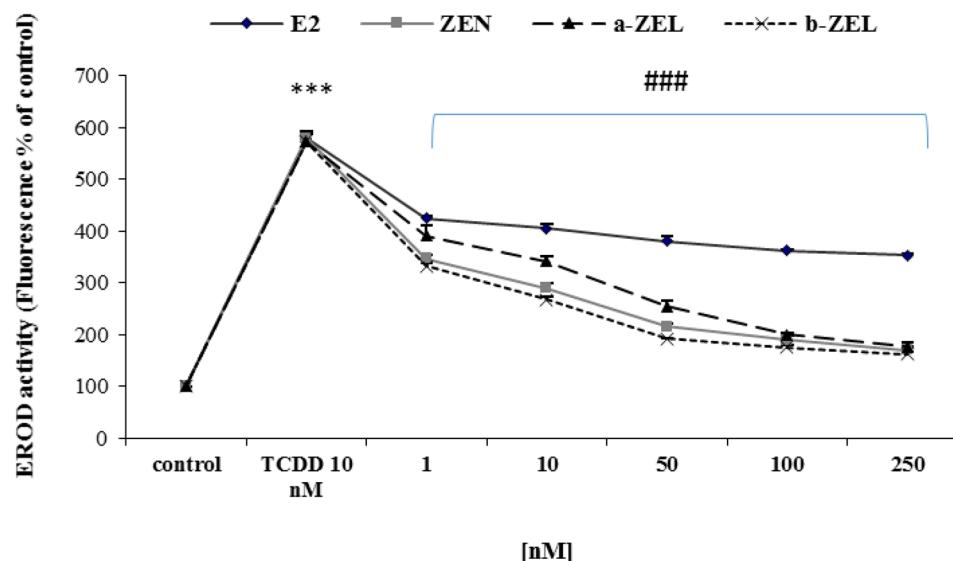


Fig. 3 Effects of **A** the specific CYP1A1 inhibitor α -naphthoflavone (ANF; 1 μ M) and **B** the specific CYP1B1 inhibitor pyrene (PYN, 1 μ M) alone and in combination with E2 and different mycoestrogen compounds (0.1 μ M) on EROD activity in MCF-7 cells; data represent means of triplicates. Asterisks show significant ($P < 0.001$) difference between the control and TCDD-received cells, # represents significant ($P < 0.01$) difference between the TCDD-received and specific CYP1A1 and CYP1B1 inhibitor-treated cells and \$ indicates significant ($P < 0.05$) difference between the TCDD plus specific inhibitor-received cells and those cells which in addition to TCDD and specific inhibitors were treated with E2 and/or ZEN, α -ZEL, and β -ZEL

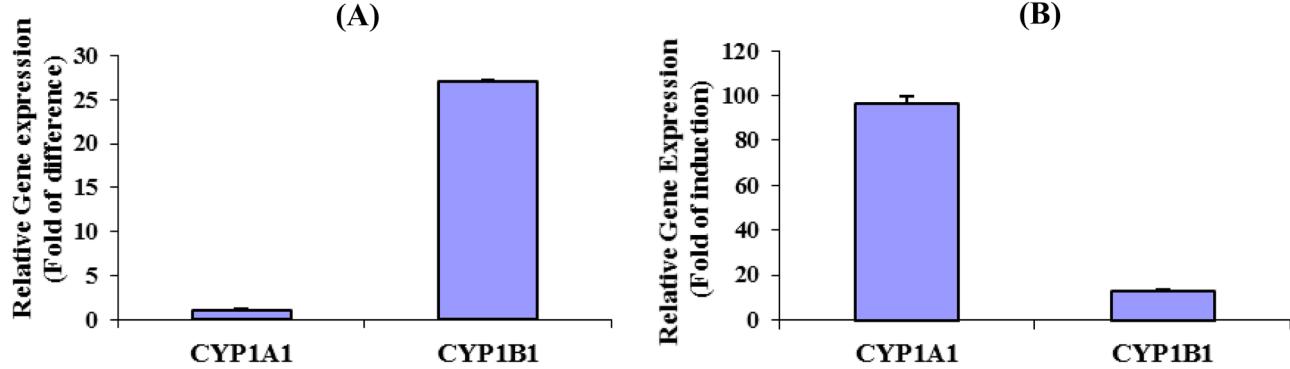
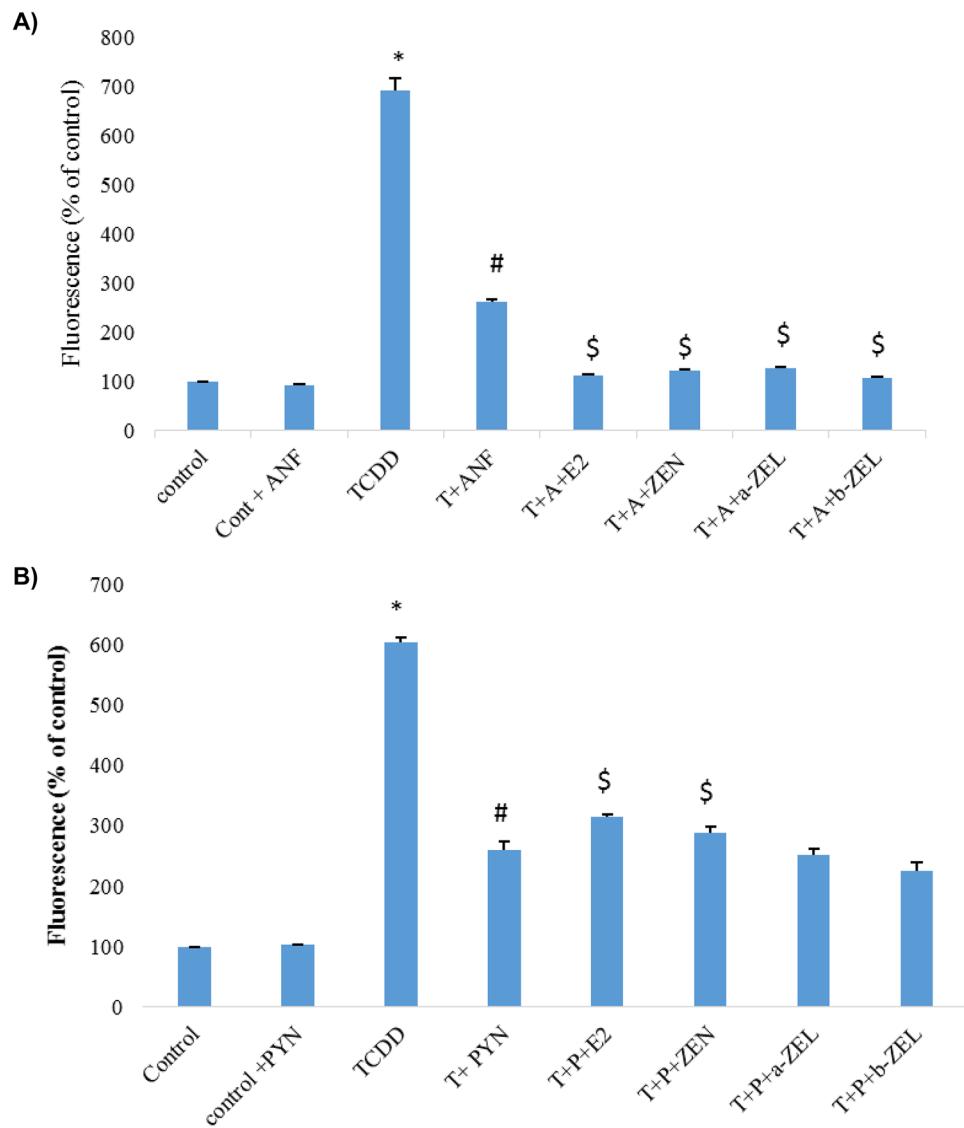
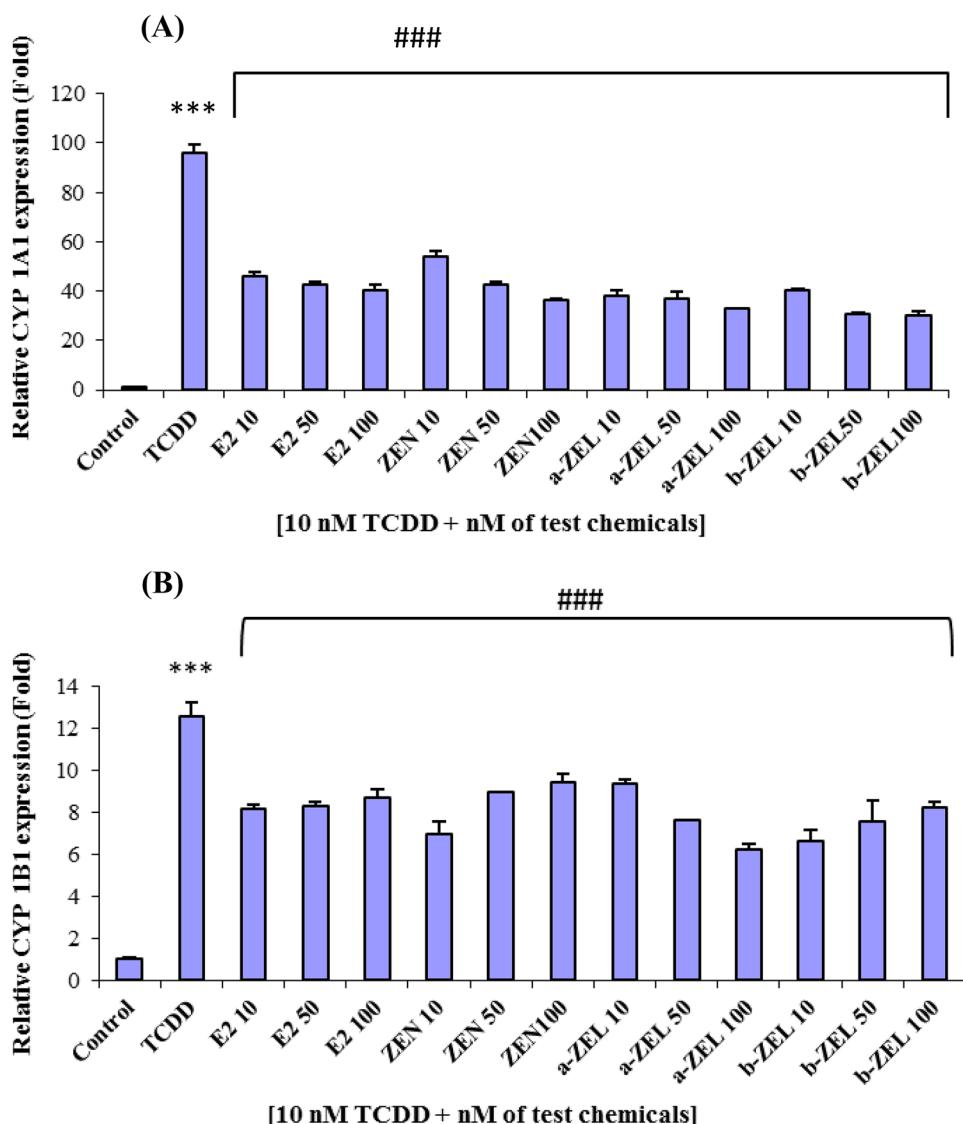


Fig. 4 Relative expression of cytochrome P450 1A1 and 1B1 mRNA in **A** control and **B** TCDD-induced MCF-7 cells

Fig. 5 Effects of E2 and mycoestrogens on **A** CYP 1A1 and **B** CYP 1B1 expression in TCDD-induced MCF-7 cells. Asterisks indicate a significant ($P < 0.05$) difference between the control and TCDD-received cells and ### represents significant ($P < 0.001$) differences between the TCDD-exposed and TCDD plus E2 and/or mycoestrogen-treated MCF cells. Data points are representing means of triplicates



Mycoestrogens altered the expression of AhR in TCDD-exposed MCF-7 cells

In a similar series of experiments, we tested the effect of TCDD and oestrogenic compounds on the expression of the AhR. Following exposure of MCF-7 cells to 10-nM TCDD, the expression of AhR at the mRNA level was slightly upregulated. Treatment of TCDD-exposed MCF-7 cells with 100-nM E2, ZEN, and α -ZEL for 48 h resulted in a significantly increased ($P < 0.05$) upregulation of AhR expression.

No significant difference was found between TCDD-exposed cells and TCDD plus β -ZEL-treated cells (Fig. 6A).

E2 and mycoestrogens downregulated the expression of ER α in TCDD-exposed MCF-7 cells

The levels of expression of ER α in TCDD-pre-treated cells were slightly upregulated in comparison to control cells. E2 and mycoestrogen treatment resulted in a significant downregulation of ER α expression in TCDD-exposed cells (Fig. 6B).

Table 2 Effects of mycoestrogens on the ratio of CYP 1A1/CYP 1B1 expression in TCDD-induced MCF-7 cells

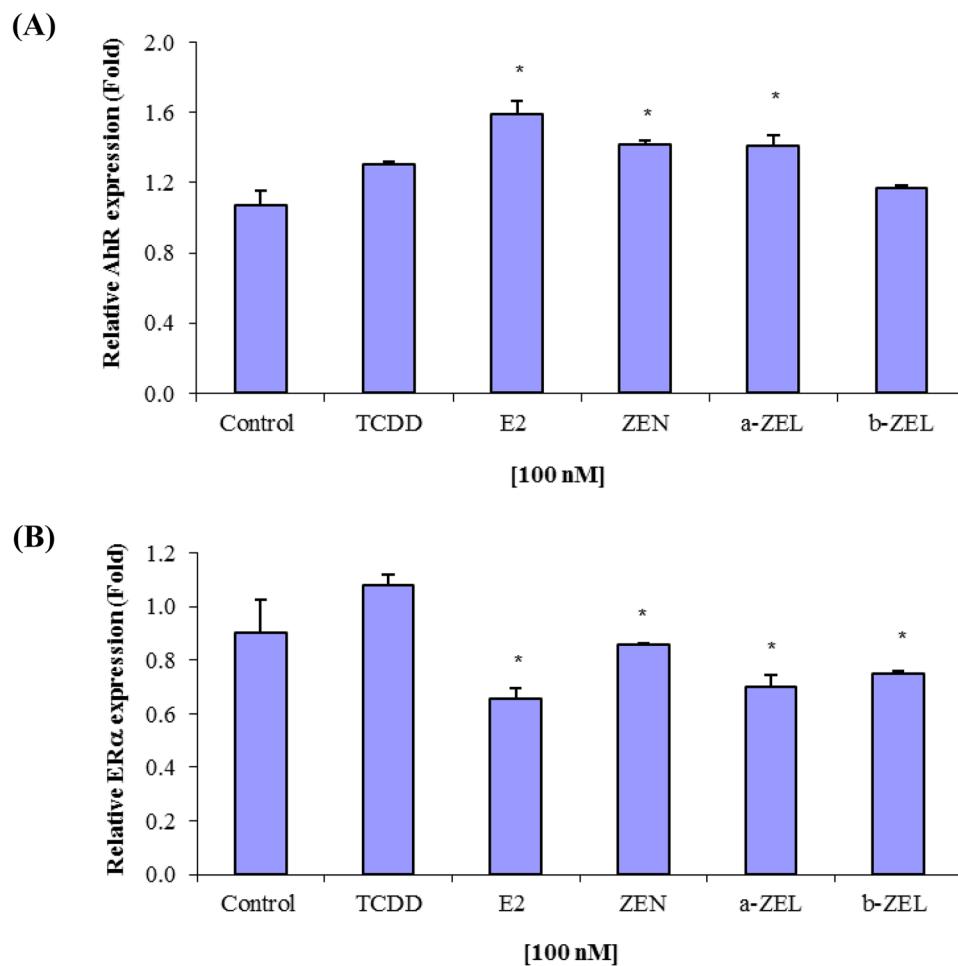
Treatment group	Ratio of CYP 1A1/ CYP 1B1 expression
TCDD (T)	7.59±0.08
T+E2 10 nM	5.55±0.10*
T+E2 50 nM	5.10±0.01*
T+E2 100 nM	4.39±0.37*
T+ZEN 10 nM	8.17±0.51
T+ZEN 50 nM	4.79±0.07*
T+ZEN 100 nM	4.23±0.53*
T+ α -ZEL 10 nM	4.17±0.14*
T+ α -ZEL 50 nM	4.98±0.14*
T+ α -ZEL 100 nM	5.24±0.02*
T+ β -ZEL 10 nM	5.89±0.26*
T+ β -ZEL 50 nM	4.35±0.40*
T+ β -ZEL 100 nM	3.68±0.02*

A significant ($P<0.05$) difference (asterisks) between the TCDD-treated and TCDD plus E2 and/or mycoestrogen-exposed cells have been recorded

E2 and mycoestrogens changed COMT activity

To evaluate the effect of mycoestrogens on E2 metabolism and to clarify the processing of the phase I catechol metabolites, the formation of the stable methoxyestrogens 2- and 4-MeOE2 was measured. The production of 2-and 4-MeOE2 in non-treated, control MCF-7 cells was not detectable as the applied method was not sensitive enough (calculated LOD for 2- and 4-MeOE2 were 0.5 and 0.1 ng/mL, respectively). However, under TCDD induction, both metabolites were detected, and 2-MeOE2 was found to be the major metabolite. The effects of mycoestrogens at 100-nM concentration on E2 metabolism in TCDD-induced MCF-7 cells are depicted in Fig. 7. All tested compounds including endogenous oestrogen decreased the level of 2-MeOE2 (Fig. 7). The observed decrease in the level of 2-MeOE2 could be ranked as follows: β -ZEL>ZEN>E2> α -ZEL, which is consistent with reduction in the mRNA level of CYP1A1 under TCDD induction.

Fig. 6 Effects of E2 and mycoestrogens on **A** AhR and **B** ER α expression in TCDD-induced MCF-7 cells; asterisks are representing significant ($P<0.05$) differences between TCDD-exposed and TCDD-exposed plus E2 and/or mycoestrogens received MCF-7 cells. Data are indicating means of triplicates



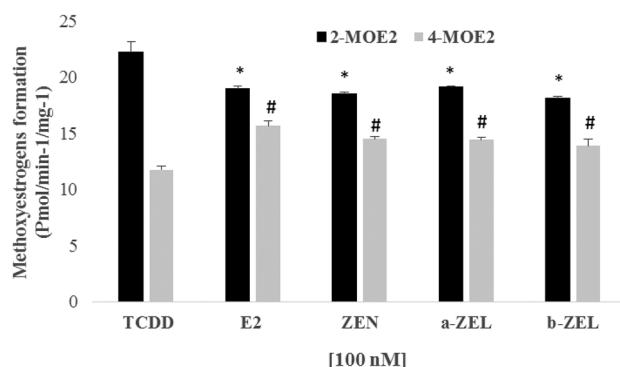


Fig. 7 Effects of mycoestrogens on methoxyestrogen formation; asterisks and # are representing significant ($P < 0.05$) differences between TCDD-exposed and TCDD-exposed plus E2 and/or mycoestrogen-treated MCF-7 cells. Data are indicating means of triplicates

In contrast to 2-MeOE2 formation, the production of 4-MeOE2 under mycoestrogen exposure increased significantly ($P < 0.05$). E2 caused the highest elevation in 4-MeOE2 formation followed by ZEN and β -ZEL (Fig. 7). α -ZEL exerted the weakest effect on increasing of 4-MeOE2 level, which is in accordance with the lowest increase of CYP1B1 expression under α -ZEL treatment. The ratio of 4-to 2-MeOE2 formation under mycoestrogen exposure was significantly increased ($P < 0.05$) in comparison to the TCDD reference control (Table 3).

Discussion

The main objective of this study was to elucidate the potential effects of mycoestrogen zearalenon and its major metabolites α -ZEL and β -ZEL on E2 metabolism. Biotransformation of E2 is catalysed by enzymes of the CYP1 family, and subsequent processing of hydroxylated metabolites by COMT is considered as a critical step in

Table 3 The ratio of 4-MeOE2/2-MeOE2 formation by cytosolic proteins of the TCDD-induced MCF-7 cells

Treatment group	Ratio of 4-MeOE2/2-MeOE2 formation
TCDD	0.53 ± 0.01
T + E2 100 nM	0.82 ± 0.03*
T + ZEN 100 nM	0.77 ± 0.01*
T + α -ZEL 100 nM	0.73 ± 0.04*
T + β -ZEL 100 nM	0.76 ± 0.04*

Asterisks are representing significant ($P < 0.05$) differences between TCDD-exposed and TCDD-exposed plus E2 and/or mycoestrogen-treated MCF-7 cells. Data are indicating means of triplicates

the carcinogenesis of E2. Results obtained in MCF-7 cells suggest that mycoestrogens not only are substrates but also modulate the activity of CYP1A1 and CYP1B1, particularly after upregulation of these enzymes by TCDD. These findings were confirmed by qPCR analysis of the respective expression level of the CYP1A1 and CYP1B1 enzymes as well as AhR, the associated transcription factor. Results suggest that ZEN and its main metabolites direct E2 metabolism into an increased formation of 4-OHE2 as the metabolite considered to be responsible of mutational DNA damage, which can be the initiating factor in tumorigenesis.

The human breast cancer MCF-7 cell line is recognized as a valuable model for studying the effects of oestrogen and oestrogen-like compounds on cell proliferation and mechanism(s) regulating gene expression following exposure to either endogenous oestrogens or exogenous oestrogenic chemicals including environmental pollutants such as TCDD and phytoestrogens, and increasing evidence indicates that oestrogens possess mitogenic, cancer-promoting, and cancer-initiating effects (Spink et al. 2003a, b; Yue et al. 2003). Mycoestrogens, such as ZEN and its major metabolites, bind to oestrogen receptors like E2 and provoke cell proliferation in MCF-7 cells, as demonstrated again in the current study. They also show a high affinity to enzymes, which are involved in the processes of steroidogenesis in target tissues (Malekinejad et al. 2006). This stimulated the hypothesis that mycoestrogens might interfere also with other metabolic pathways of endogenous oestrogen processing.

Cytochrome P450s catalyse the metabolism of steroids, sterols, and a number of other endocrine and autocrine substances such as eicosanoids and retinoids (Guengerich and Yoshimoto 2020; Nebert 1991). In humans, CYP1A1 and 1B1 play a major role in the biotransformation of endogenous oestrogens to catechol oestrogens (Badawi et al. 2001; Kim et al. 1998). Catechol-oestrogens escaping further processing by COMT will be converted into reactive imines and are therefore considered as a critical step in mutagenesis and tumorigenesis. To assess CYP1A1 and 1B1 enzyme activity in the presence of oestrogenic compounds in this study, we performed an EROD activity assay, which preferentially reflects CYP1A1 activity but also CYP1A2 and CYP1B1 activities. Our results show that like E2, mycoestrogens decreased profoundly EROD activity. Surprisingly, we identified β -ZEL as the most potent compound in the inhibition of EROD activity, indicating strong inhibition of CYP1 activity. This finding is not consistent with the oestrogenic potency of given compounds as E2 exerts the strongest oestrogenicity, followed by α -ZEL and β -ZEL, respectively (Malekinejad et al. 2005). One explanation for this finding could be the involvement of other pathway(s) in the modulation of CYP1 activities under oestrogen and mycoestrogen

treatment along with oestrogen receptor (ER) signalling. To confirm the stimulatory and inhibitory effects of E2 and mycoestrogens on CYP 1B1 and CYP 1A1 respectively, EROD activity assessment was performed in the presence of selective inhibitors for these isoenzymes. Results show that E2 and mycoestrogens reduce CYP1A1 and enhanced CYP 1B1 activities thereby creating favourable conditions for CYP 1B1-mediated E2 biotransformation.

It is well known that human CYP1A1 predominantly catalyse E2 conversion into 2-OHE2, while CYP1B1 primarily catalyse E2 to 4-OHE2 hydroxylation. Increasing evidence indicates that E2 carcinogenicity is mainly associated with the formation of 4-OHE2 (Lanxiang et al. 2019; Park 2018; Yager 2000). There is a variety of studies showing that E2 itself in a concentration- and time-dependent manner increases 4-MeOE2 formation and in turn decreases the 2-MeOE2 production in TCDD-induced MCF-7 cells (Spink et al. 2003a, b). Both metabolites, 2- and 4-MeOE2, are stable end products of E2 metabolism. In the present study, we investigated the effect of mycoestrogens on E2 metabolism and our observations indicate that ZEN and its metabolites follow the E2 pattern by increasing and decreasing 4- and 2-MeOE2 formation, respectively, albeit with some differences. E2 was found to be the strongest stimulator of CYP1B1, and β-ZEL is the most potent inhibitor of CYP1A1. These findings are in accordance with activity measurement of CYP1A1 under E2 and mycoestrogen exposure as E2 and β-ZEL exerted the weakest and strongest inhibition, respectively, in the EROD assay. However, the 4-MeOE2 formation under E2 and mycoestrogen treatment revealed another feature indicating that E2 exert the potent stimulatory effect, followed equally by α-ZEL and ZEN, while β-ZEL caused the weakest stimulation of CYP1B1. These differences in the regulation of CYP1A1 and 1B1 by E2 (in a concentration-dependent manner) and by mycoestrogens indicate again that there is more than one pathway to regulate these two important genes. Our finding that E2 itself effects TCDD-induced E2 metabolism confirmed previous reports (Spink et al. 2003a, b). Moreover, the estimated ratio of 4-MeOE2/2-MeOE2 formation, as a critical determinant of DNA adduct formation, indicated that E2 caused the highest ratio, followed without significant differences equally by all tested mycoestrogens.

To identify whether the observed changes in CYP1A1 and 1B1 activities in EROD assay and E2 metabolism were due to any changes in the transcriptional activities of these genes, we performed a series of studies to measure the expression of CYP1A1 and 1B1. Our data indeed demonstrated consistent results with the enzyme activities (EROD assay) for CYP1A1 as the relative expression of CYP1A1 was significantly lowered ($P < 0.05$) following treatment with E2 and mycoestrogens, while CYP 1B1 expression increased slightly but significantly after exposure of TCDD-induced MCF-7 cells to the test compounds, indicating that

the observed results from E2 metabolism might be associated with changes in CYP1A1 and 1B1 expression. These finding is in consistence with previous reported data concerning E2 and ZEN (Tsuchiya et al. 2004a, b; Yu et al. 2004), while data on the effects of α-ZEL and β-ZEL were lacking as yet and are reported here for the first time.

The difference between oestrogenic potency and CYP1A1 regulation by endogenous oestrogen and mycoestrogens indicates that there might be other pathways such as AhR involvement or ERα-AhR interactions, which modulate CYP1A1 regulation. This phenomenon has been shown for E2 and TCDD in MCF-7 cells (Beischlag and Perdew 2005; Tijet et al. 2006).

The CYP1A1 and 1B1 genes are indeed under the transcriptional control of a ligand-activated transcription factor of AhR. However, there is also increasing evidence indicating that ERα pathway alone or via a cross-talk between AhR and ERα is a potential regulator of oestrogenic compounds such as TCDD and its effect on CYP1A1 and 1B1 expression and activities (Otake et al. 2003; Tsuchiya et al. 2005). To gain insight and understand how CYP1A1 and 1B1 are regulated under E2 and mycoestrogen exposure, the expression of two crucial transcription factors was determined. According to obtained results, except β-ZEL, all other tested compounds increased significantly AhR mRNA levels, while all compounds including β-ZEL lowered ERα expression significantly. These findings may explain that while β-ZEL could diminish the CYP1 activity more than all other tested compounds, it had almost the same effect on AhR expression. The decrease in CYP1A1 induction following exposure to E2 has been reported in human endometrial and breast cancer cells. The molecular mechanism for this decrease appears to be oestrogen receptor dependent, and earlier studies demonstrated that endogenous E2 reduces CYP1A1 expression by squelching available nuclear factor-1, a transcriptional factor that interacts with both AhR and ERα (Matthews et al. 2005; Ricci et al. 1999). ERα occupation by ligands, such as ERα agonists and antagonists, provides an opportunity to proteasome-mediated proteolysis to degrade ERα (Preisler-Mashek et al. 2002). However, as yet, there is no available data to show this mechanism also for mycoestrogens. An alternative explanation for increased AhR expression and decreased ERα expression in cells exposed to E2 or mycoestrogens is the inhibition of AhR-ER crosstalk in MCF-7 cells, whereby the ligand-activated AhR induces proteasome-dependent downregulation of ERα (Hanieh et al. 2016; Safe et al. 2000).

In conclusion, the findings of the present study suggest that exogenous oestrogenic compounds like as the mycoestrogens ZEN and its major endogenous metabolites α-ZEL and β-ZEL show similar effects as E2 on CYP1A1, CYP1B1, AhR, and ERα expression in MCF-7 cells. This strengthens the hypothesis that these food-borne mycoestrogens may affect the pathogenesis and prevalence of oestrogen-related carcinogenicity.

Acknowledgements The authors wish to appreciate Dr. Pashaie Belal for his generous technical support.

Author contribution Faezeh Malekinejad: conceptualization, methodology, investigation, and writing original draft preparation; Johanna Fink-Gremmels: validation, reviewing, and editing; Hassan Malekinejad: data curation, visualization, investigation, and supervision.

Data Availability All data related to this study have been provided in the current article.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no conflict of interest.

References

- Badawi AF, Cavalieri EL, Rogan EG (2001) Role of human cytochrome P450 1A1, 1A2, 1B1, and 3A4 in the 2-, 4-, and 16 [alpha]-hydroxylation of 17 [beta]-estradiol. *Meta-Cli Exper* 50:1001–1003
- Beischlag TV, Perdew GH (2005) ER α -AHR-ARNT protein-protein interactions mediate estradiol-dependent transrepression of dioxin-inducible gene transcription. *J Biol Chem* 280:21607–21611
- Burke MD, Mayer RT (1983) Differential effects of phenobarbitone and 3-methylcholanthrene induction on the hepatic microsomal metabolism and cytochrome P-450-binding of phenoxyazone and a homologous series of its n-alkyl ethers (alkoxyresorufins). *Chem Biol Interact* 45:243–258
- Christou M, Savas Ü, Spink DC, Gierthy JF, Jefcoate CR (1994) Co-expression of human CYP1A1 and a human analog of cytochrome P450-EF in response to 2, 3, 7, 8-tetrachloro-dibenzo-p-dioxin in the human mammary carcinoma-derived MCF-7 cells. *Carcinogenesis* 15:725–732
- Drzymala SS, Herrmann AJ, Maul R, Pfeifer D, Garbe LA, Koch M (2014) In vitro phase I metabolism of cis-zearalenone. *Chem Res Toxicol* 27:1972–1978
- EFSA Panel on Contaminants in the Food Chain (CONTAM), Knutson HK, Alexander J, Barregård L, Bignami M, Brüschweiler B, Oswald IP (2017) Risks for animal health related to the presence of zearalenone and its modified forms in feed. *EFSA J* 15(e04851):2
- Fallarino F, Romani L, Puccetti P (2014) AhR: far more than an environmental sensor. *Cell Cycle* 13:2645–2646
- Fruhauf S, Novak B, Nagl V, Hackl M, Hartinger D, Rainer V, Labudová S, Adam G, Aleschko M, Moll WD, Thamhesl M (2019) Biotransformation of the mycotoxin zearalenone to its metabolites hydrolyzed zearalenone (HZEN) and decarboxylated hydrolyzed zearalenone (DHZEN) diminishes its estrogenicity in vitro and in vivo. *Toxins* 11:481
- Gargaro M, Scalisi G, Manni G, Mondanelli G, Grohmann U, Fallarino F (2021) The landscape of AhR regulators and coregulators to fine-tune AhR functions. *Int J Mol Sci* 22:757
- Guengerich FP, Yoshimoto FK (2020) Cytochrome P450 metabolism leads to novel biological sterols and other steroids. In: *Mammalian Sterols* (145–171). Springer, Cham
- Hanieh H, Mohafez O, Hairul-Islam VI, Alzahrani A, Bani Ismail M, Thirugnanasambantham K (2016) Novel aryl hydrocarbon receptor agonist suppresses migration and invasion of breast cancer cells. *PLoS ONE* 11:e0167650
- Kim JH, Stansbury KH, Walker NJ, Trush MA, Strickland PT, Sutter TR (1998) Metabolism of benzo [a] pyrene and benzo [a] pyrene-7, 8-diol by human cytochrome P450 1B1. *Carcinogenesis* 19:1847–1853
- Klinge CM, Kaur K, Swanson HI (2000) The aryl hydrocarbon receptor interacts with estrogen receptor alpha and orphan receptors COUP-TFI and ERR α 1. *Arch Biochem Biophys* 373:163–174
- Lanxiang W, Bin W, Ge X, Yutang H, Chunjie W, Honghao Z (2019) Long-term exposure of 4-hydroxyestradiol induces the cancer cell characteristics via upregulating CYP1B1 in MCF-10A cells. *ToxMech Meths* 29:686–692
- Larsen MC, Angus WG, Brake PB, Eltom SE, Sukow KA, Jefcoate CR (1998) Characterization of CYP1B1 and CYP1A1 expression in human mammary epithelial cells: role of the aryl hydrocarbon receptor in polycyclic aromatic hydrocarbon metabolism. *Cancer Res* 58:2366–2374
- Li Y, Cui J, Jia J (2021) The activation of procarcinogens by CYP1A1/1B1 and related chemo-preventive agents: a review. *Curr Cancer Drug Targets* 21:21–54
- Liehr JG, Ricci MJ, Jefcoate CR, Hannigan EV, Hokanson JA, Zhu BT (1995) 4-Hydroxylation of estradiol by human uterine myometrium and myoma microsomes: implications for the mechanism of uterine tumorigenesis. *Proceed Nat Acad Sci* 92:9220–9224
- Malekinejad H, Maas-Bakker RF, Fink-Gremmels J (2005) Bioactivation of zearalenone by porcine hepatic biotransformation. *Vet Res* 36:799–810
- Malekinejad H, Van Tol HTA, Colenbrander B, Fink-Gremmels J (2006) Expression of 3 α -and 3 β -hydroxy steroid dehydrogenase mRNA in COCs and granulosa cells determines zearalenone biotransformation. *Toxicol in Vitro* 20:458–463
- Matthews J, Wihlén B, Thomsen J, Gustafsson JA (2005) Aryl hydrocarbon receptor-mediated transcription: ligand-dependent recruitment of estrogen receptor α to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin-responsive promoters. *Mol Cell Bio* 25:5317–5328
- Moorthy B, Chu C, Carlin DJ (2015) Polycyclic aromatic hydrocarbons: from metabolism to lung cancer. *Toxicol Sci* 145:5–15
- Nebert DW (1991) Minireview: Proposed role of drug-metabolizing enzymes: regulation of steady state levels of the ligands that effect growth, homeostasis, differentiation, and neuroendocrine functions. *Mol Endocrinol* 5:1203–1214
- Ohtake F, Takeyama KI, Matsumoto T, Kitagawa H, Yamamoto Y, Nohara K, Tohyama C, Krust A, Mimura J, Chambon P, Yanagisawa J (2003) Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature* 423:545–550
- Park SA (2018) Catechol estrogen 4-hydroxyestradiol is an ultimate carcinogen in breast cancer. *Biomed Sci Lett* 24:143–149
- Preisler-Mashek MT, Solodin N, Stark BL, Tyriver MK, Alarid ET (2002) Ligand-specific regulation of proteasome-mediated proteolysis of estrogen receptor- α . *Am J Physiol Endocrinol Metab* 282:E891–E898
- Ricci MS, Toscano DG, Mattingly CJ, Toscano WA (1999) Estrogen receptor reduces CYP1A1 induction in cultured human endometrial cells. *J Biol Chem* 274:3430–3438
- Roberts EA, Harper PA, Wong JM, Wang Y, Yang S (2000) Failure of Ah receptor to mediate induction of cytochromes P450 in the CYP1 family in the human hepatoma line SK-Hep-1. *Arch Biochem Biophys* 384:190–198
- Safe S, Wormke M, Samudio I (2000) Mechanisms of inhibitory aryl hydrocarbon receptor-estrogen receptor crosstalk in human breast cancer cells. *J Mammary Gland Biol Neoplasia* 5:295–306
- Shimada T, Fujii-Kuriyama Y (2004) Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. *Cancer Sci* 95:1–6

- Spink DC, Hayes CL, Young NR, Christou M, Sutter TR, Jefcoate CR, Gierthy JF (1994) The effects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin on estrogen metabolism in MCF-7 breast cancer cells: evidence for induction of a novel 17 β -estradiol 4-hydroxylase. *J Steroid Biochem Mol Biol* 51:251–258
- Spink DC, Spink BC, CaoJQ GJF, Hayes CL, Li Y, Sutter TR (1997) Induction of cytochrome P450 1B1 and catechol estrogen metabolism in ACHN human renal adenocarcinoma cells. *J Steroid Biochem Mol Biol* 62:223–232
- Spink BC, Hussain MM, Katz BH, Eisele L, Spink DC (2003a) Transient induction of cytochromes P450 1A1 and 1B1 in MCF-7 human breast cancer cells by indirubin. *Bioch Pharmacol* 66:2313–2321
- Spink DC, Katz BH, Hussain MM, Pentecost BT, Cao Z, Spink BC (2003b) Estrogen regulates Ah responsiveness in MCF-7 breast cancer cells. *Carcinogenesis* 24:1941–1950
- Stack DE, Byun J, Gross ML, Rogan EG, Cavalieri EL (1996) Molecular characteristics of catechol estrogen quinones in reactions with deoxyribonucleosides. *Chem Res Tox* 9:851–859
- Swedenborg E, Pongratz I (2010) AhR and ARNT modulate ER signaling. *Toxicology* 268:132–138
- Tijet N, Boutros PC, Moffat ID, Okey AB, Tuomisto J, Pohjanvirta R (2006) Aryl hydrocarbon receptor regulates distinct dioxin-dependent and dioxin-independent gene batteries. *Mol Pharma* 69:140–153
- Tsuchiya Y, Nakajima M, Kyo S, Kanaya T, Inoue M, Yokoi T (2004a) Human CYP1B1 is regulated by estradiol via estrogen receptor. *Cancer Res* 64:3119–3125. <https://doi.org/10.1158/0008-5472.can-04-0166>
- Tsuchiya Y, Nakajima M, Yokoi T (2004b) Regulation of human CYP1B1 and its role in carcinogenesis and estrogen homeostasis. *Seikagaku Jpn Biochem Soc* 76:1565–1569
- Tsuchiya Y, Nakajima M, Yokoi T (2005) Cytochrome P450-mediated metabolism of estrogens and its regulation in human. *Cancer Lett* 227:115–124
- van Duursen MB, Sanderson JT, de Jong PC, Kraaij M, van den Berg M (2004) Phytochemicals inhibit catechol-O-methyltransferase activity in cytosolic fractions from healthy human mammary tissues: implications for catechol estrogen-induced DNA damage. *Toxicol Sci* 81:316–324
- Whitlock JP Jr (1999) Induction of cytochrome P4501A1. *Annu Rev Pharmacol Toxicol* 39:103–125
- Yager JD (2000) Chapter 3: endogenous estrogens as carcinogens through metabolic activation. *JNCI Monographs* 2000:67–73
- Yu Z, Hu D, Li Y (2004) Effects of zearalenone on mRNA expression and activity of cytochrome P450 1A1 and 1B1 in MCF-7 cells. *Ecoto Environ Saf* 58:187–193
- Yue W, Santer RJ, Wang JP, Li Y, Verderame MF, Bocchinfuso WP, Korach KS, Devanesan P, Todorovic R, Rogan EG, Cavalieri EL (2003) Genotoxic metabolites of estradiol in breast: potential mechanism of estradiol induced carcinogenesis. *J Steroid Biochem Mol Bio* 86:477–486

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.