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Preliminary evaluation of ER- and AhR-mediated gene expression patterns in rainbow trout (*Oncorhynchus mykiss*) liver after short-term exposure to zearalenone in binary mixtures

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ABBREVIATIONS

3α-HSD – 3α-hydroxysteroid dehydrogenase
γ-BHC – gamma 1,2,3,4,5,6 hexachlorocyclohexane
AhR – aryl hydrocarbon receptor
B[*a*]P – benzo[*a*]pyrene
Ct – threshold cycle
CYP1A – cytochrome P450 1A
DDT – dichlorodiphenyltrichloroethane
E2 – 17β-estradiol
ER – estrogen receptor
EREs – estrogen responsive elements
HepG2 – human liver carcinoma cell line
HPG axis – hypothalamic-pituitary-gonadal axis
NTCs – no template control
PXR – pregnane X receptor
qPCR – quantitative PCR
RPL19 – 60S ribosomal protein L19
vtg – vitellogenin
XREs – xenobiotic response elements
ZEA – zearalenone
ZRP – *zona radiata* protein

ABSTRACT

While estrogenic properties of mycotoxin zearalenone (ZEA) has been an extensively studied issue, little is known about molecular background of its biological responses that cannot be simply explained by the estrogenic potential. The present study describes effects of ZEA (10mg·kg^{-1} body weight) in binary mixtures either with benzo[*a*]pyrene (B[*a*]P), or with 17β-estradiol (E2) on ER- and AhR-dependent gene expression in juvenile rainbow trout liver, evaluated using Real-Time qPCR. The study revealed dual nature of ZEA, as the treatment with this compound alone increased mRNA levels of both ER- and AhR-mediated gene expression. However, our results did not show any synergistic or additive effect of ZEA in binary mixures with E2 or B[*a*]P on studied gene expression levels. Whether the intriguing potential of ZEA to elicit distinct signals was a result of binding affinity to AhR or/and ER and AhR mutual receptor interactions, should be investigated in further experiments.

INTRODUCTION

Zearalenone (ZEA; known as F-2 toxin) is a nonsteroidal mycoestrogen produced by various fungi belonging to the genus *Fusarium*. These fungi are frequently found in cereal crops worldwide, resulting in mycotoxin contamination of animal feed material, food, and even dairy (Fink-Gremmels 2008; Zinedine et al. 2007). The structure of ZEA (Figure 1), chemically described as 6-[10-hydroxy-6-oxo-E-1-undecenyl]-B-resorcyclic acid lactone, was first determined by Urry et al. (1966). In view of biological studies, the most crucial feature of the chemical structure of ZEA is its ability to bind to estrogen receptor (ER), and the potential to induce expression of estrogen responsive genes. In fact, in recent years it has become apparent that exposure to ZEA leads to a number of disorders of the reproductive system in mammals, e.g. decreased libido, anovulation, infertility, or neoplasmic lesions, which all derive from the mechanisms that alter transcription of ER-dependent genes in ER-positive cells (Jakimiuk et al. 2009; Minervini and Aquila 2008; Shier et al. 2001; Tiemann et al. 2003; Zinedine et al. 2007). In oviparous animals, including fish, ZEA mimics the action of 17β-estradiol (E2) by binding to ERs and activates

estrogen responsive genes (EREs) including vitellogenin (*vtg*) or *zona radiata* protein (*zrp*) (Arukwe et al. 1999; Chen et al. 2010; Olsen et al. 2005). In this manner, ZEA acts similarly to other known and putative xenoestrogens, such as diethylstilbestrol (Folmar et al. 2002), 4-nonylphenol (Flouriot et al. 1995), bisphenol A (Letcher et al. 2005), and *o,p'*-DDT (Okoumassoun et al. 2002).

While most of the biological properties of ZEA are attributed to the agonistic effect on the ER, the compound was found to produce certain biological responses that cannot be explained simply by its estrogenic potential. For example, Ding and co-workers (2006) reported on ZEA induction of CYP3A, a drug-metabolizing enzyme, through activation of the pregnane X receptor (PXR) in HepG2 cells. In our earlier study (Woźny et al. 2008) we have found that ZEA up-regulates CYP1A gene expression levels *in vivo* in rainbow trout liver, what suggests the possibility that ZEA impacts aryl hydrocarbon receptor (AhR)-dependent gene expression. The AhR is a transcription factor that binds to xenobiotic response elements (XREs), upstream of genes classified as "Ah gene battery", thus it mediates majority of the cell adaptive responses (Nebert et al. 2000). The rationale underlying the findings described above may be a simple one: a cell may need to rapidly metabolize ZEA, as it does with other foreign chemical species, and convert it into a molecule that can be readily excreted. While the available data do not exclude the possibility of ZEA to be an AhR ligand, other more subtle mechanisms, through the estrogen receptor (Woźny et al. 2008) may also be responsible for increased transcription of some AhR-mediated genes.

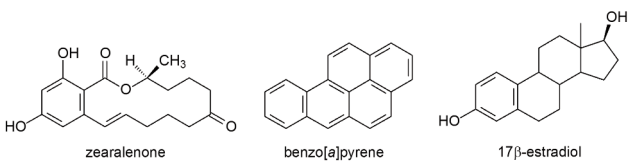

Figure 1. Chemical structure of compounds used in the study.

Based on the findings summarized above, it can be deduced that ZEA toxicity almost certainly involves a complex web of altered gene expression, so far however, the full array of components modulating the effects of ZEA activity within the eukaryotic cell is not yet known. No attempts were undertaken, based on the transcription profiles of marker genes, to compare the effects of ZEA with those of other model chemicals that are known to induce distinct cell physiologic responses. Such data, yielding information on both direction and magnitude of altered mRNA transcription, should give a clue about the effects of ZEA on the cells. In this *in vivo* study we examined the effects of ZEA in rainbow trout liver (major site of biotransformation) on expression levels of several marker genes representing either ER- or AhR-signaling pathways and we compared them with those of two model chemicals (Figure 1): benzo[*a*]pyrene (B[*a*]P, model xenobiotic, AhR inducer), and 17β-estradiol (natural ligand for ER). Furthermore, to gain knowledge on the possible interactive effects with other model compounds, we examined effects of ZEA in binary mixtures with E2 or with B[*a*]P.

MATERIAL AND METHODS

Fish exposure and samples collection

The fish were maintained in accordance with the regulations set forth by the Local Ethical Commission No. 64/2008 issued on 18th of September 2008 (conforming to principles of Laboratory Animal Care, NIH publication No. 86-23, revised in NIH 1985). Juvenile all-female rainbow trout (*Oncorhynchus mykiss*) individuals with an average body weight of 50.3g and an average length of 16.9cm were obtained from the Department of Salmonid Research in Rutki (Inland Fisheries Institute in Olsztyn, Poland). All fish were acclimated to experimental conditions for a minimum of 2 weeks prior to exposure.

The time span of the study and dosing concentrations of examined compounds were selected based on our earlier observations in rainbow trout (Woźny et al. 2008). For this study the individuals were randomly sampled, anesthetized by immersion in etomidate solution (Propiscin®; Poland), and injected intraperitoneally with tested compounds dissolved in corn oil (Sigma; Schnelldorf, Germany) as a carrier solution, or corn oil alone (control sample). Juvenile trout were exposed to zearalenone (ZEA; Sigma), benzo[*a*]pyrene (B[*a*]P; Sigma), and 17β-estradiol (E2; Sigma) administered singly at a dose of 10mg·kg^{-1} each, and administered as mixtures: ZEA+B[*a*]P, ZEA+E2, B[*a*]P+E2 (at doses of 10+10mg·kg^{-1}). The 24h treatment period was set based on the pilot study of Ziółkowska (2008). After 24h of exposure (T=7°C), five individuals were randomly taken from each of the experimental group, then the fish were anesthetized and decapitated by severance of the spinal cord. Liver tip was excised and immediately immersed in the RNA*Later*™ solution (Sigma) according to the manufacturer recommendations and stored at −20°C.

RNA isolation and cDNA synthesis

RNA*Later*™ preserved liver tissues were homogenized and extracted for total RNA isolation using Total RNA Mini isolation kit (A&A Biotechnology; Gdynia, Poland) according to the manufacturer's protocol. To prevent genomic DNA contamination, RNA samples were incubated with RNase-free DNase I (Roche Diagnostics; Mannheim Germany). Total RNA quality and quantity of all samples were estimated using BioPhotometer (Eppendorf; Hamburg, Germany).

Real-Time qPCR

Primer pairs for Real-Time qPCR (Table 1) were either chosen from the literature or designed using Primer Express v.2.0 software (Applied Biosystems; Branchburg, NJ, USA). The assay was performed on ABI 7500 Real-Time PCR system thermocycler (Applied Biosystems) in singleplex mode, and all

Table 1. Details of primer pairs and their amplicons used in the study.

Primer pair		Sequence (5'→3')	Melting temperature (°C)	Amplicon length (bp)	Reference
ERα1	Forward	ccctgctggtgacagagagaa	82.3	107	Nagler et al. 2007
	Reverse	atcctccaccaccattgagact			
ERβ2	Forward	ctgaccccagaacagctgatc	81.2	125	Nagler et al. 2007
	Reverse	tcggccaggttggtaagtg			
VTG	Forward	acaaggactctcaatccacccc	79.4	148	this paper
	Reverse	tggtcagctctcactaaacgga			
AhR2α	Forward	gagaggaacttcgtgtgtcgg	81.7	99	this paper
	Reverse	ctggcaatggaggaacttcaa			
AhR2β	Forward	cgtccattttggagatcagaact	79.8	110	this paper
	Reverse	tgagtagcccaggacaaccttc			
CYP1A	Forward	tcaacttacctctgctggaagc	78.0	68	Rees and Li 2004
	Reverse	ggtgaacggcaggaagga			
RPL19	Forward	gtcacggtgcactctcgcgc	84.0	101	Brzuzan et al. 2007
	Reverse	cgggcattggctgtaccctt			

samples were analyzed in duplicates. Each PCR reaction tube contained 10μl of Power SYBR® Green master mix (Applied Biosystems), 2.5pmol of each (forward and reverse) primer, 1μl of cDNA as a template, and PCR-grade H_2O to a final volume of 20μl. The reaction was performed in standard thermal conditions: 95°C for 10min, then 45 cycles of 95°C for 15s and 60°C for 1min. On the plate, negative water controls (NTCs) were included to rule out the possibility of cross-contamination. To verify the quality of PCR products, a melting curve analysis and agarose gel electrophoresis was performed after each run. In addition, representative PCR fragments produced by designed primer pair were sequenced under contract (Genomed, Warsaw, Poland) and their sequences were compared to the data deposited in GenBank® (BLAST; http://www.ncbi.nlm.nih.gov).

Data analysis and statistics

Data obtained from the assay (Table 2) was used to compute mRNA expression ratios of ERα1, ERβ2, VTG, AhR2α, AhR2β and CYP1A, relative to RPL19 as the endogenous control. Briefly, calculation of the expression ratio was based on gene individual Real-Time qPCR efficiency (E), and the threshold cycle difference (ΔCt) of an unknown sample versus a control ($\Delta Ct_{control-sample}$) according to the mathematical model:

$$\text{Ratio} = [(E_{target})^{\Delta Ct\ target}]\cdot[(E_{reference})^{\Delta Ct\ reference}]^{-1}$$

given by Pfaffl (2001). The efficiencies for each gene were estimated by running reactions with a dilution series of cDNA template with primer pairs used in the study, and the threshold cycle (Ct) vs. cDNA concentrations was plotted to calculate respective slope values (data not shown). The corresponding Real-Time qPCR efficiencies were calculated according to the equation:

$E = 10^{[-1/slope]}$ (Pfaffl 2001).

The resulting E-values for each gene examined were the basis for the calculation and further randomization tests with REST© (Pfaffl et al. 2002). Differences in gene expression between control and treated samples were assessed in group means for statistical significance by randomization tests (REST©2008; Pfaffl et al. 2002) and were considered significant at $P<0.05$, $P<0.01$, or $P<0.001$. For pairwise comparisons between treatment groups, levels of significance were adjusted according to Miller method (Miller 1981), by dividing the significance level by the number of tests performed on the same data set. In this study, the critical level of significance was set by dividing the standard significance level ($P=0.05$) by 15, the number of group comparisons for respective genes. This method yielded a critical significance level (P) of 0.0033 for each comparison performed.

Table 2. Variation in the number of threshold cycles (Cts) for target and endogenous genes determined by real-time PCR in liver of rainbow trout after 24h of the experiment.

Experimental group	Factor	24h						
		ERα1	ERβ2	VTG	AhR2α	AhR2β	CYP1A	RPL19
Control	Mean	27.54	26.65	31.39	31.54	33.46	25.98	20.46
	S.E.	0.51	0.49	0.98	0.41	0.68	0.55	0.55
	CV(%)	4.15	4.15	6.99	2.92	4.55	4.71	5.99
ZEA	Mean	24.46	26.66	25.39	30.94	32.60	23.69	20.70
	S.E.	0.32	0.39	0.22	0.42	0.74	0.23	0.41
	CV(%)	2.92	3.31	1.94	3.06	5.06	2.17	4.38
B[*a*]P	Mean	26.51	26.52	29.57	29.26	30.92	20.25	20.78
	S.E.	0.61	0.54	0.47	0.89	0.81	0.60	0.34
	CV(%)	5.18	4.54	3.54	6.81	5.88	6.61	3.66
E2	Mean	25.82	27.00	24.52	32.25	33.65	26.93	20.75
	S.E.	0.34	0.42	0.26	0.35	0.61	0.56	0.69
	CV(%)	2.93	3.50	2.35	2.42	4.05	4.65	7.43
ZEA+B[*a*]P	Mean	24.13	26.67	26.69	29.19	31.81	19.47	20.89
	S.E.	0.37	0.51	0.16	0.26	0.38	0.38	0.51
	CV(%)	3.39	4.30	1.37	2.01	2.69	4.39	5.45
ZEA+E2	Mean	23.55	25.24	24.24	29.96	31.75	23.58	20.33
	S.E.	0.18	0.25	0.34	0.42	1.10	0.43	0.48
	CV(%)	1.75	2.23	3.12	3.16	7.76	4.11	5.30
B[*a*]P+E2	Mean	23.66	25.57	23.37	28.70	30.73	19.49	19.56
	S.E.	0.30	0.40	0.13	0.34	0.69	0.78	0.58
	CV(%)	2.83	3.50	1.21	2.62	5.00	8.96	6.57

* Given are the mean, standard error (S.E.), coefficient of variation (CV), each one based on $n=5$ for controls and treated fish 24h.

RESULTS

Real-Time qPCR reaction efficiency, specificity, and the Cts variation

Real-Time qPCR reactions efficiency were high ranging from 0.95 to 0.98, and their specificity was confirmed with melting curve analysis and agarose gel electrophoresis. Amplicon for each primer pair was visualized as a single band at the expected length and its melting curve analysis resulted in a single specific melting temperature (Table 1). In addition, sequence analysis of the PCR products revealed 99-100% homology to the respective mRNA sequences deposited in GenBank (data not shown). No primer–dimer formations were generated during the applied 40 real-time PCR amplification cycles.

Table 2 summarizes results of the real time PCR assay for target and reference genes examined in all experimental groups. Variations of mean Cts for investigated transcripts remained stable between 1.21 and 8.96% in each sample. Furthermore, Cts values of the reference gene, RPL19 measured after 24h of the experiment in treated samples were not significantly different to those in the control group (REST©, $P>0.05$).

Chemical-induced changes in ER-mediated gene expression

Figure 2 and Table 3 show the gene expression results obtained after pairwise comparisons of control vs. treatment, or treatment vs. treatment groups, respectively. All data were calculated from Table 2 and normalized by the reference gene, RPL19.

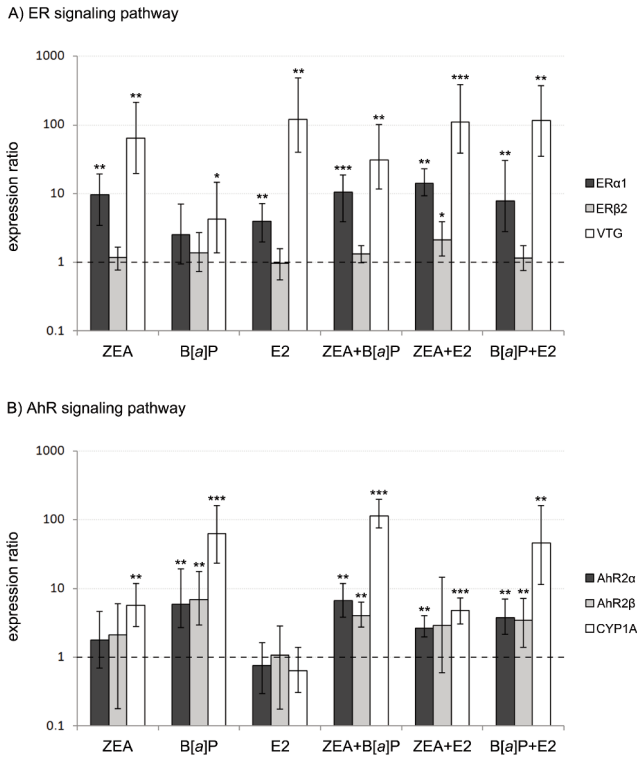

Figure 2. Genes expression pattern of A) estrogen receptor (ER) and B) aryl hydrocarbon receptor (AhR) signaling pathways in liver of rainbow trout after 24h exposure with: ZEA, B[*a*]P, E2 (10mg·kg^{-1} each), and their mixtures: ZEA+B[*a*]P, ZEA+E2, B[*a*]P+E2 (10+10mg·kg^{-1} each). Bars represent mean values of expression ratios with their respective standard errors of the mean (S.E., *n*=5), normalized by RPL19 as an endogenous reference relative to a control sample (ratio=1.00, dashed line). The expression ratio values were calculated by REST©2008 software. Asterisks indicate significant difference between the control and treated group of fish (* *P*<0.05, ** *P*<0.01, * *P*<0.001).**

Figure 2A shows the mean expression ratios (with standard errors) of genes representing ER-signaling pathway (ERα1, ERβ2, VTG) in each experimental group of fish. The exposure of trout to ZEA alone significantly increased mRNA expression of ERα1 (9.6-fold, $P<0.01$) and VTG (64.7-fold, $P<0.01$), while leaving the ERβ2 levels unchanged ($P>0.05$). Similarly, E2 alone increased the ERα1 expression (3.9-fold increase, $P<0.01$) and VTG mRNA (120.3-fold, $P<0.01$) but not the ERβ2 level. The fish treated with B[*a*]P showed elevated expression of VTG (4.2-fold, $P<0.05$).

For the genes representing ER-signaling pathway, treatment of fish with binary mixtures resulted in gene expression changes similar to those observed in trout exposed to single chemicals (Figure 2A). When compared to controls, the exposure to ZEA+B[*a*]P, ZEA+E2, and B[*a*]P+E2 increased the ERα1 (about 10-fold) and VTG (about 100-fold) mRNA expression but not the ERβ2 mRNA level, except the ZEA+E2 group where the modest induction of ERβ2 was observed upon treatment. However, a significant difference in ERβ2 expression (1.8-fold, $P<0.0033$) between the groups ZEA+E2 and ZEA+B[*a*]P was noticed (Table 3). The trout exposed to a mixture of ZEA and B[*a*]P had higher levels of ERα1 mRNA (3.5-fold, $P<0.0033$) than those treated with E2 alone (Table 3).

Chemical-induced changes in AhR-mediated gene expression

Figure 2B shows the mean expression ratios (with standard errors) of genes representing AhR-signaling pathway (AhR2α, AhR2β, CYP1A) in each experimental group of fish. B[*a*]P-treated group of trout showed significant up-regulation of expression of both AhR genes (AhR2α: 5.9-fold, AhR2β: 6.9-fold, $P<0.01$) and CYP1A (62.5-fold increase, $P<0.001$). The fish exposed to ZEA alone had higher levels of CYP1A mRNA (5.7-fold) than controls. The mRNA expression levels of AhR2α, AhR2β, and CYP1A mRNA were not significantly altered after exposure to E2 ($P>0.05$), as compared to controls. However, the mRNA expression of CYP1A and AhR2α observed in trout exposed to E2 were significantly lower than those in fish treated with ZEA and B[*a*]P, respectively (Table 3).

Treatment of fish with binary mixtures resulted in AhR-dependent genes expression changes, similar to those observed in trouts exposed to single chemicals (Figure 2B). The exposure to ZEA+B[*a*]P, ZEA+E2, and B[*a*]P+E2 increased the AhR2α (3 to 9-fold), AhR2β (about 2 to 6-fold), and CYP1A (about 6 to 100-fold) mRNA expression, with the exception of the ZEA/E2 group where the modest induction of AhR2β observed upon the treatment was not significant (Figure 2B). Significant difference in the transcript levels of AhR2α and CYP1A (8.8-fold and 179.4-fold, $P<0.0033$) was noticed between fish exposed to ZEA with B[*a*]P and those exposed to E2 (Table 3). Finally, the amounts of CYP1A mRNA quantified in trout exposed to a mixture of ZEA and E2, were significantly lower than those observed in groups B[*a*]P and ZEA+B[*a*]P (Table 3).

DISCUSSION

In the present study we investigated the effects of a short-term exposure to ZEA injected alone or as binary mixture with model compounds on ER- and AhR-mediated gene expression level in juvenile rainbow trout liver.

Our results confirmed the estrogenic potential of ZEA as it was capable to up-regulate mRNA levels of ERα1, VTG, but also showed its potential to elevate CYP1A expression (Figure 2). While previous reports seem to fully correlate with agonistic effect of ZEA on ER-mediated gene transcription (Arukwe et al. 1999; Chen et al. 2010; Parveen et al. 2009; Woźny et al. 2008), little is known about a possible interactions of ZEA with AhR signaling pathway. Two possible mechanisms responsible for this phenomenon were proposed in our recent paper (Woźny et al. 2008), explaining the dual nature of ZEA action: i) mycoestrogen ZEA is at least a partial AhR ligand, and/or ii) to some extent, ER regulates the transcription of AhR-mediated genes.

Table 3. Genes with significantly ($P<0.0033$) altered mRNA expression (fold change) after multiple comparisons between experimental groups.

Experimental group	vs.	Experimental group				
		B[*a*]P	**E2**	**ZEA+B[*a*]P**	**ZEA+E2**	**B[*a*]P+E2**
Experimental group	**ZEA**	VTG (0.07)	CYP1A (0.11)	–	–	–
	B[*a*]P		AhR2α (0.13)	–	CYP1A (0.08)	–
	E2			ERα1 (3.47) AhR2α (8.76) CYP1A (179.37)	–	–
	ZEA + B[*a*]P				ERβ2 (1.81) CYP1A (0.04)	–
	ZEA + E2					–

The first explanation seems to be a favorable hypothesis when we take into account the molecular geometry of ZEA (Figure 1). The carbonyl group might be responsible for estrogenic properties of the parent molecule, as it is reduced to hydroxyl group by 3α-HSD in biotrasformation pathway, which significantly increases binding affinity to ER of the derivative (Zinedine et al. 2007). On the other hand, numerous studies of the past decades show that Ah receptor may be activated by a vast spectrum of endo- and exogenous ligands, also the ones that exceed standard molecular dimensions (15·12·5Å) of a typical AhR ligand (Denison and Heath-Pagliuso 1998). Planar orsellinic acid moiety and the dimensions of the molecule (Panneerselvam et al. 1996) suggest that ZEA may have at least partial agonistic properties for AhR binding site. In addition, recent paper reports on high involvement of human CYP1A2 in ZEA oxidative biotransformation into catechols (Pfeiffer et al. 2009). If AhR "gene battery" is thought to be aimed at an elimination of the inducing agent or its metabolites, then it seems that the novel finding of Pfeiffer and co-workers should strengthen the hypothesis of the possible ZEA binding affinity to Ah receptor, which now should be experimentally proven for fish species.

As for the second proposed mechanism, high ERα1 expression ratio and the lack of both AhR genes induction after exposure to ZEA suggest that the CYP1A expression ratio could be regulated via ER. Currently, there are known at least several "cross-talk" interferences of ER with other receptor systems (Pascussi et al. 2008), and the ER-AhR bi-directional interaction is one of the best examined (Bemanian et al. 2004; Mortensen and Arukwe 2007; Ohtake et al. 2003; Safe and Wormke 2003). We hypothesize that this mechanism may account, at least partially, for significant increase of CYP1A expression ratio upon treatment with ZEA. Other cross-talk interactions should also be taken into consideration. For example, Gerbal-Chaloin and co-workers (2006) reported on cross-talk between PXR and AhR. Results revealed that omeprazole-sulphide (AhR antagonist) is efficiently converted to omeprazole (AhR activator) by CYP3A4, after treatment with rifampicin, PXR agonist (Gerbal-Chaloin et al. 2006). Therefore, further studies including multiple doses and different time points are necessary to provide an effective assessment of the ER-AhR cross-talk hypothesis.

Although exposure of fish to E2, as a model compound, have met our expectations concerning the estrogenic effect (and lack of it on XREs), the response of fish to treatment with B[*a*]P may be worth mentioning. Apart from its expected xenobiotic effect, exposure of fish to B[*a*]P also resulted in VTG mRNA over-expression. It has been shown that exposure of zebrafish to B[*a*]P may result in

mRNA level modulation of VTG with no significant effect on ER mRNA (Hoffmann and Oris 2006). While it is widely accepted that several xenoestrogens mimic the action of E2 by binding to ERs and activating estrogen responsive genes including VTG, the gene may also be induced through other mechanisms. It has been suggested that VTG may be induced by elevation of endogenous free plasma estrogen levels generated through several non-ER pathways along the HPG axis (Huggett et al. 2003; Thompson et al. 2000; Tilton et al. 2002; Xie et al. 2005). For example, chemicals like Aroclor 1254 (Khan et al. 2001), ketoconazole (Villeneuve et al. 2007a, 2007b), malathion and γ-BHC (Singh and Singh 1992) interfere with the expression and/or secretion of releasing hormones, trophic hormones or steroidogenic enzymes, which may result in an imbalance of sex steroids and the disturbance of VTG production. Based on the data obtained in this study, it is likely that B[*a*]P induces VTG production by a non-ER-mediated pathway, namely elevation of endogenous estrogen levels.

Most of the past research were focused on studying effects of individual pollutants, however in the environment, mycotoxins most often exist as a complex mixture of different compounds, which display a diverse array of biological effects induced via many cellular pathways. The aim of this study was to evaluate any possible interactions on gene expression level of ZEA in binary mixtures with E2 or B[*a*]P, as the model compounds. Interestingly, a higher mRNA level of both ERs and AhR2α was observed in group treated with mixture of ZEA+E2 when compared to groups exposed with these compounds alone (Figure 2), which may be considered as a slight additive effect. However, pairwise comparison of the treated groups (ZEA+E2 vs. E2 as well as ZEA+B[*a*]P vs. B[*a*]P) did not reveal any genes with significantly altered expression ($P>0.0033$, Table 3). Therefore, none of the provided results confirmed an explicit thesis about what is meant by synergistic or even additive action of ZEA with model compounds. Further research with mechanistic concepts that entails an expected combined effect are necessary in order to perform a prospective risk assessment of mycotoxin mixtures. By using equitoxic and equimolar concentrations of the compounds, it would be possible to assess which of the effects of ZEA are additive or antagonistic. More genes should be involved in such an assay as well as other responses, e.g. protein levels should be examined.

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