Assessment of estrogenic and anti-androgenic activities of the mycotoxin zearalenone and its metabolites using in vitro receptor-specific bioassays

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ABSTRACT

Zearalenone (ZEN) is a well-known mycotoxin present in numerous agricultural products. Humans and animals are therefore at a risk of exposure to zearalenone through consumption of contaminated food. After intake, ZEN is reduced to α- and β-zearalenol (α-ZEL and β-ZEL), zearalanone (ZAN), and α- and β-zearalanol (α-ZAL and β-ZAL). Although their estrogenicity has been well characterized, much less is known about their interaction with other nuclear receptors. This study was undertaken to investigate interactions of ZEN and its five metabolites, with the human androgen receptor (hAR) and estrogen receptor alpha (hERα). Their ability to induce hAR-mediated reporter gene expression was examined in androgen-sensitive PALM cells, whereas the effects on hERα function were assessed in MCF-7 cells using the E-Screen bioassay. We confirm that ZEN and its metabolites are full agonists for hERα and demonstrate that all six compounds tested possess hAR-mediated antagonistic activity in PALM cells, in which ZAN, α-ZAL, and β-ZAL were the most effective hAR antagonists. Overall, the observed estrogenic and anti-androgenic potencies of ZEN and its metabolites suggest that these compounds may interfere with the endocrine system by various modes of action and that further investigation is warranted into their role as endocrine disrupters in animals and humans

Keywords: Mycotoxins; Zearalenone (ZEN); PALM cell line; E-Screen bioassay; Human androgen receptor (hAR)

Abbreviations: E2, 17β-estradiol; MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; AhR, aryl hydrocarbon receptor; CPP, central precocious puberty; CAR, constitutive androstane receptor; DCC, dextran-coated charcoal; DMSO, dimethyl sulfoxide; ERs, estrogen receptors; FBS, fetal bovine serum; Flut, flutamide; G418, geneticin; hAR, human androgen receptor; hCG, human chorionic gonadotropin; hERα, human estrogen receptor alpha; hPXR, human pregnane X receptor; HSD, hydroxysteroid dehydrogenases; ICI, ICI 182,780; R1881, methyltrienolone; NRs, nuclear receptors; POPs, persistent organic pollutants; Proc, procymidone; PE, proliferative effect; rt, rainbow trout; StAR, steroidogenic acute regulatory protein; SRB, sulforhodamine B; TCA, trichloroacetic acid; Vin, vinclozolin; ZAN, zearalanone; ZEN, zearalenone; RAL, β-resorcyclic acid lactone; β-ZAL, β-zearalanol; β-ZEL, β-zearalenol; α-ZAL, α-zearalanol; α-ZEL, α-zearalenol

1. Introduction

Mycotoxins are a structurally diverse group of small-molecularweight compounds produced by secondary metabolism of some filamentous fungi, especially those belonging to the genus Aspergillus, Penicillium, or Fusarium. Under suitable temperature and humidity conditions, they can contaminate various foods and feeds, posing serious risks to human and animal health (Sweeney and Dobson, 1998). Zearalenone (ZEN, Fig. 1), chemically described as a β-resorcyclic acid lactone (RAL), is a non-steroidal estrogenic mycotoxin biosynthesized by several Fusarium species (Bennett and Klich, 2003) that is commonly found in the soil in temperate and hot countries and is a frequent contaminant of cereal crops worldwide (Zinedine et al., 2007). Despite its relatively low acute toxicity, this mycotoxin is the most prominent RAL produced by Fusarium species and is known for its estrogenic and anabolic properties in several animal species (Gromadzka et al., 2008; Takemura et al., 2007). In most of them, ZEN is rapidly metabolized after ingestión and absorption, mainly by hepatic and intestinal cells. Hydroxysteroid dehydrogenases (HSD) convert ZEN by 7-cetone reduction into two stereoisomeric metabolites α- and β-zearalenol (α-ZEL and β-ZEL). A further reduction of the pathway (11–12 double bound reduction) yields two minor metabolites α- and β-zearalanol (α-ZAL and β-ZAL). Another route involves reduction of ZEN to zearalanone (ZAN) which can be also further reduced to α-ZAL and β-ZAL (Malekinejad et al., 2006a). ZEN is principally metabolized by conjugation via detoxifying pathways, and its reduced metabolites are then conjugated with sulfonic or glucuronic acid, followed by extensive biliary excretion and entero-hepatic recirculation, and are finally eliminated, mainly in the urine (Duca et al., 2012; Gromadzka et al., 2008; Zinedine et al., 2007). Species-dependent differences in the metabolic conversion of ZEN have been observed, and these have been linked to its differential susceptibility to the adverse effects of ZEN exposure (Malekinejad et al., 2006b). Thus, concentration ratios of its major metabolites (α-ZEL and β-ZEL) in liver samples (microsomes and the postmitochondrial fractions) vary considerably among animal species. For example, pigs are among the most susceptible species, with a reported predominance of the α-ZEL metabolite (Malekinejad et al., 2005), whereas β-ZEL is more prevalent in poultry and ruminants. α-ZEL metabolite has also been described as the most frequent metabolite in humans (Videmann et al., 2008). Surprisingly, little is known about human exposure to these compounds and

their effects on human health, except for some studies in cancer patients (Pillay et al., 2002) and on estrogenic mycotoxins suspected as triggering factor for precocious pubertal development in exposed prepubertal girls (Massart and Saggese, 2010). Thus, serum mycoestrogen levels were assessed in a small clinical study of precocious puberty in Hungary (Szuets et al., 1997), detecting ZEN in 5/36 subjects with serum levels ranging from 18.9 to 103 μg/l (59.3 to 323.5 nM). In a later study in the North-West region of Tuscany (Italy), Massart et al. (2008) investigated serum mycoestrogen levels in 32 girls with idiopathic central precocious puberty (CPP); they found elevated serum ZEN and α-ZEL levels [Means of 933.7 ng/l and 106.5 ng/l, respectively (approximately 2.93 and 0.33 nM)] in 35% of the girls suggesting a possible relationship between environmental mycoestrogen exposure and the development of precocious puberty in females. The authors pointed to the anabolic growth effects of mycotoxins in the exposed girls. An epidemiologic study in New Jersey (Bandera et al., 2011) on the possible relationship of urinary mycoestrogens with body size and development detected mycoestrogens in 78.5% of 163 girls aged 9 and 10 years [ΣZEN and metabolites ranging from 0.03 to 29.8 μg/l (approximately 0.09 to 93.3 nM)]. The girls with detectable urinary mycoestrogen levels tended to be shorter and were less likely to have started breast development. The authors suggested that mycoestrogens may act as anti-estrogenic agents, delaying the height spurt, which generally occurs at the age of around 9 years in US girls, at the same time as breast development onset. We recently quantified ZEN and metabolites in the urine of 42 Tunisian adult women in a collaborative study on breast cancer, using a sensitive ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) approach (Belhassen et al., 2014). ZEN, α-ZAL, and β-ZAL were detected in 19% of the analyzed samples and quantified in 16.7% of them at concentrations ranging from 0.76 to 3.17 μg/l (2.35 to 9.84 nM). These values are similar to those reported by Bandera et al. in healthy girls and lower than the mean serum ZEN concentrations observed by Pillay et al. (2002) in breast and cervical cancer patients (range 150 to 457 μg/l, approximately 0.46 to 1.42 μM).

Despite its non-steroidal structure, ZEN and its metabolites have been shown to exert their estrogenic effects through their ability to bind to estrogen receptors (ERs) (Kuiper et al., 1998). Thus, they have demonstrated in vitro estrogenic activity in MCF-7 human breast cancer cells (Malekinejad et al., 2005; Shier et al., 2001; Takemura et al., 2007), recombinant cell lines (Frizzell et al., 2011), and recombinant yeast systems carrying human estrogen receptor alpha (hERα) or rainbow trout ERα (rtERα) (Le Guevel and

Pakdel, 2001), finding that the estrogenic activity of α-ZEL was higher than that of ZEN or β-ZEL. Estrogenic activity has also been observed in vivo in female mice (Takemura et al., 2007). Moreover, ZEN and its major metabolites (α-ZEL and β-ZEL) have also been shown to act as potential endocrine-disrupting chemicals (EDCs) by altering hormone production (Frizzell et al., 2011).

Although ZEN and some of its metabolites (mainly α-ZEL and β-ZEL) have been well characterized as estrogen agonists, much less is known about their interactions with the human androgen receptor (hAR). The objective of this study was to investigate interactions of ZEN and its five metabolites, α-ZEL, β-ZEL, ZAN, α-ZAL, and β-ZAL, with the hAR and hERα. Their estrogenic potencies were evaluated in MCF-7 cells using the E-Screen bioassay, and their effects on the hAR-mediated induction of transcription were investigated in a reporter gene assay in the androgen-sensitive PALM cells, a stable prostatic cell line.

2. Materials and methods

2.1. Chemicals and materials

Culture medium and fetal bovine serum (FBS) were obtained from Gibco (Invitrogen, Barcelona, Spain). Reference standards, including 17β-estradiol (E2), ICI 182,780 (ICI), methyltrienolone (R1881), ZEN, α-ZEL, β-ZEL, ZAN, α-ZAL, β-ZAL, vinclozolin (Vin), procymidone (Proc), flutamide (Flut), puromycin, geneticin (G418), luciferin (sodium salt), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT), sulforhodamine B (SRB), and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). Stock solutions (10 mM) of E2 (≥98% purity), R1881 (≥98% purity), Vin (≥97% purity), Proc (≥98% purity), Flut (≥98% purity), ZEN (≥99% purity), α-ZEL (≥98% purity), β-ZEL (>95% purity), ZAN (98% purity), α-ZAL (≥97% purity) and β-ZAL (98% purity) were prepared in ethanol (>99.7% purity), and successive dilutions were performed in culture medium. Stock solutions were kept at −20 °C, and dilution series were freshly prepared before each experiment. All other chemicals were of the highest quality available from commercial sources. For cell proliferation assays, the absorbance was read in a Titertek Multiscan apparatus (Flow, Irvine, CA), while an infinite M200 luminometer (Tecan, Barcelona, Spain) was used to detect luciferase

activity in intact cells. Finally, all cell culture plastics were obtained from Falcon (VWR International Eurolab, Barcelona, Spain).

2.2. Cells and culture conditions

Cell lines used in this study were cultured as previously described (Molina-Molina et al., 2013). In brief, human breast cancer MCF-7 cells were cultured for routine maintenance in DMEM with phenol red supplemented with 10% FBS, while PALM cells, a human androgen-dependent stable transfected cell line (Terouanne et al., 2000), were cultured in Ham's F12 supplemented with 10% FBS, 1 mg/ml G418, and 1 μg/ml puromycin. Because of the hormonal activity of phenol red and FBS, experiments were performed in a test culture medium, i.e., phenol red-free DMEM supplemented with 10% dextran-coated charcoal-FBS (10% DCC-FBS) for MCF-7 cells or Ham's F12 supplemented with 6% DCC-FBS and 1% antibiotic for PALM cells, in a 5% CO2 humidified atmosphere at 37 °C.

2.3. E-Screen bioassay

Assay procedure and data analysis were conducted as previously described (Molina-Molina et al., 2013). Briefly, MCF-7 cells were trypsinized and plated in 96- well culture plates at initial concentrations of 4 × 103 cells per well. Cells were allowed to attach for 24 h, and the seeding medium was then removed and replaced with the test culture medium. A range of concentrations of the test compound was added to this medium in the sample wells. In each experiment, a dose–response curve (0.1 pM–1000 pM) for E2 and a negative control (cell treated only with hormonefree medium) were included. The bioassay was ended on day 6 (late exponential phase) by removing the media from the wells, fixing the cells, and staining them with SRB. Finally, bound dye was solubilized and the absorbance was read at 492 nm. Linearity of the SRB assay with cell number was verified prior to cell growth experiments.

Agonistic assays were performed with concentrations of the test compounds ranging from 0.001 to 1000 nM. For each compound, the ratio between the cell yield obtained and the proliferation of hormone-free control cells (negative control) was calculated. Tests were done in triplicate for each concentration. Results were expressed as proliferative effect (PE) [MCF-7 cell proliferation (-fold over control)]. Finally, the dose resulting in half-maximal MCF-7 cell proliferation (EC50 value) was calculated for each compound..

2.4. Living cell luciferase assay

Luciferase assays were conducted by using the stably transfected luciferase reporter PALM cell line according to previously described protocols (Molina-Molina et al., 2006, 2008, 2013). Cells were seeded at a density of 5 × 104 cells per well in 96-well white opaque tissue culture plates in 150 μl test culture medium. Test compounds were prepared 4x concentrated in the same medium, and 50 μl was added per well 8 h after seeding. Cells were incubated with the compounds for 40 h at 37 °C. At the end of incubation, the medium containing test compounds was removed and replaced with test culture medium containing 0.3 mM luciferin. Next, the 96-well plate was introduced into a luminometer, and luminescence was measured in intact living cells for 2 s.

Agonistic activities of hAR in PALM cells were tested in the presence of increasing concentrations (0.01–10 μM) of the test compounds. Tests were performed in quadruplicate for each concentration. Results were expressed as percentage of maximal luciferase activity. Maximal luciferase activity (100%) was obtained in the presence of 10 nM R1881. For each compound, the potency corresponding to the concentration yielding half-maximal luciferase activity (EC50 value) was calculated. The antagonistic activities of these compounds were determined by coincubation with R1881 (0.3 nM) agonist. At this concentration, activity reaches approximately 80% of maximal luciferase activity. Each plate included serial dilutions of the antagonists Proc, Flut, or Vin (0.01–10 μM) as a positive control and a negative control (test culture medium alone) alongside the test samples. Data were expressed as halfmaximal inhibitory concentration (IC50 value) for each compound tested.

2.5. MTT assay for evaluating cell toxicity

The effects of ZEN, α-ZEL, β-ZEL, ZAN, α-ZAL, and β-ZAL on cell viability were assessed with the MTT test, using Denizot and Lang's modified technique (Denizot and Lang, 1986). Briefly, cell lines (MCF-7 and PALM) were seeded at a density of 5 × 104 cells per well in 96-well tissue culture-grade plates for 8 h, followed by treatment with different concentrations (0.001–10,000 nM) of each compound for a further 24 h. Cells were washed with PBS three times, and 100 μl of MTT solution (0.5 mg/ ml) were then added to each well. After incubation (2 h), viable cells cleaved the MTT tetrazolium ring into a dark blue formazan reaction product, whereas dead cells remained colorless. The MTT-containing medium was gently removed, and DMSO was added to each well. After

shaking, the plates were read in absorbance at 540 nm. Medium alone with no cells served as an additional control. Data were expressed as the mean of three wells.

2.6. Data analysis

For all assays, each compound was tested at various concentrations in at least three independent experiments, and data were expressed as mean ± SD. Individual dose–response curves, in the absence and presence of agonist, were fitted using the sigmoid dose–response function of a graphics and statistics software package (GraphPad Prism, version 4.0, 2003, Graph-Pad Software Inc., San Diego, CA, USA). Results are presented as EC50 and IC50 values. Data were analyzed for significant differences using one-way ANOVA followed by Dunnett's post-comparison test (vs. control). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Cell viability

The MTT test used to assess the cytotoxicity of ZEN, α-ZEL, β-ZEL, ZAN, α-ZAL, and β-ZAL was assessed for the two cell lines used in this study. In all assays, the tested mycotoxins were devoid of any cytotoxicity in either cell line (cell survival ranging from 95 to 100%) in the 0.001–10,000 nM range (data not shown).

3.2. Estrogenic effects of ZEN and its metabolites in the E-Screen bioassay

The estrogenic potential of ZEN, α-ZEL, β-ZEL, ZAN, α-ZAL, and β-ZAL was characterized by using the E-Screen bioassay to investigate their ability to stimulate cell proliferation in MCF-7 cells. As previously reported, the natural estrogen E2 strongly induces significant proliferation in a dose-dependent manner in this cell line, with an EC50 value of 0.018 nM (Molina-Molina et al., 2013). When the six mycoestrogens were applied to MCF-7 cells, all exhibited a marked estrogenic activity, showing full dose–response curves but with different potencies, as indicated by their EC50 values (Table 1). As depicted in Fig. 2, the α-stereoisomers α-ZEL and α-ZAL were the most potent agonists, being only around 3- and 6-fold (respectively) less potent than E2. ZEN, ZAN, and the β-stereoisomers β-ZEL and β-ZAL also behaved as full hERα agonists, although the concentration needed for maximal activity varied according to the test compound (0.1

μM for ZAN and β-ZAL, and 1 μM for ZEN and β-ZEL), with a potency that was 15- to 470-fold lower than that of E2. Finally, we explored whether the estrogenic effects of the tested compounds observed in the E-Screen bioassay were ER-mediated. As expected, co-incubation with the pure anti-estrogen ICI (10 nM) abolished the estrogen response of all studied compounds, confirming that the ability of these compounds to act as full agonists derives from ER binding (data not shown).

3.3. Effect of ZEN and its metabolites on transcriptional activation through hAR

Prompted by a report (Frizzell et al., 2011) on the weak antiandrogenic activities of ZEN and some metabolites, ZEN, α-ZEL, β-ZEL ZAN, α-ZAL, and β-ZAL were tested for their ability to activate hAR in PALM cells. As in previous studies (Molina-Molina et al., 2006, 2008, 2013), the synthetic androgen R1881 was found to exert marked androgenic activity in this cell line, with an EC50 value of 0.1 nM (Fig. 3A) whereas the well-known anti-androgens Proc, Flut and Vin (used as positive controls in the antagonistic assay) showed a potent anti-androgenic activity (Fig. 3B). When the antagonistic activities of ZEN and its metabolites were examined, all six compounds exhibited a manifest anti-androgenic activity, with IC50 values in the micromolar range (Table 1). α-ZAL was the most effective hAR antagonist, exhibiting a full dose–response curve, followed by β-ZAL and ZAN, which at the highest concentrations tested (10 μM) also proved to be hAR antagonists that strongly inhibited the luciferase activity induced by 0.3 nM of R1881 (Fig. 4). ZEN, α-ZEL, and β-ZEL were significantly less effective in PALM cells, exhibiting a significant but weak antagonistic activity at concentrations of 10 μM.

By contrast, none of the tested compounds were able to activate luciferase expression in PALM cells after 40 h of exposure at concentrations up to 10 μM (data not shown), indicating that they are devoid of androgenic activity. Finally, in order to further characterize the antagonistic properties of the tested compounds, competitive reversal assays were performed with PALM cells to determine whether the anti-androgenic activities observed in transactivation assays reflect the ability of ZEN and its metabolites to bind to hAR. The antagonistic activity of all test compounds was completely reversed by co-incubation with excess (1000 times the EC50 value) of the synthetic androgen R1881 (100 nM), demonstrating the specificity of the response (Fig. 4).

4. Discussion

Most studies on the hormonal activity of ZEN and its metabolites (mainly α-ZEL and β-ZEL) have focused on their interaction with hERs, and there has been much less research on their interaction with other nuclear receptors (NRs), such as hAR. In this study, we show that ZEN and its five metabolites exhibit manifest and differential anti-androgenic activities in androgen-sensitive PALM cells, a stable prostatic bioluminescent cell line. The anti-androgenic potency of these compounds appears to depend on their chemical structure. Thus, the α-stereoisomer of ZAN (α-ZAL), with a α-OH group at the 7-position in the macrocyclic lactone ring, exhibited the strongest anti-androgenic potency, followed by β-ZAL and ZAN. Hence, the position of hydroxyl substituent appears to play a significant role in the anti-androgenic activity of these ZEN metabolites, with compounds α-hydroxylated at the 7-position showing the highest activity. Interestingly, the IC50 values found for α-ZAL and ZAN were in the same range as that reported for the well-known fungicide vinclozolin (Molina-Molina et al., 2006). In contrast, ZEN and its major metabolites (α-ZEL and β-ZEL) were less effective to inhibit the luciferase activity induced by 0.3 nM of R1881 in this cell line. These findings suggested that in addition to the position of the hydroxyl group, a further reduction of the compounds (by 11–12 double bound reduction) is also a factor in their antiandrogenic activity via hAR. These results are in agreement with previous findings by Frizzell et al. (2011) in a reporter gene assay using the androgen-responsive TARM-Luc cell line, in which ZEN and its major metabolites α-ZEL and β-ZEL demonstrated weak antagonist effects. The greater potency obtained in the present cell line may be attributable to differences in the concentrations tested (i.e., a maximum concentration of 10 μM in our study versus a concentration of only 2.5 μM) or to a difference in the sensitivity of the cell lines. Fang et al. (2003) used a rat recombinant AR protein in a study of 202 natural, synthetic, or environmental chemicals in receptor competition assays and reported a greater binding ability to this receptor for some metabolites of ZEN (mainly β-ZAL) in comparison to β-ZEL and ZAN and other known anti-androgenic compounds such as flutamide.

We also studied the effects of the ZEN and its five metabolites on cell proliferation, using the E-screen bioassay to characterize the estrogenic response of these compounds to hERα. All six compounds exhibited a potent estrogenic activity in MCF-7 cells (αZEL > α-ZAL > ZAN > β-ZAL > ZEN > β-ZEL) that was only 3- to 470- fold less than that of

E2, with EC50 values in the nanomolar range (0.06 to 8.49 nM). Similar EC50 values were also reported by Shier et al. (2001) when ZEN and 16 structural analogs were ranked by proliferative potency in MCF-7 cells. Their experimental data suggested that the functional group at the 7-position has the greatest effect on estrogenicity, which was strongest for α-ZEL (α-OH at the 7 position), as observed in the present study. It has been reported that increased flexibility in the macrocyclic lactone ring may favor a tighter binding of ZEN analogs to hERs (Shier et al., 2001). Takemura et al. (2007) also showed that α-ZEL had a significantly higher ability than ZEN to compete for binding to hERα in classical competitive receptor binding assays. Likewise, previous studies using reporter gene assays of recombinant hERα or rtERα (Le Guevel and Pakdel, 2001) observed a higher estrogenic potency for α-ZEL than for ZEN.

Finally, in a more recent study (Frizzell et al., 2011), α-ZEL again exhibited the strongest estrogenic potency (EC50 value of 0.022 nM) in a mammalian cell line (MMV-Luc cells) reporter gene assay, which was only slightly less potent than E2 (EC50 value of 0.015 nM). They reported that ZEN was approximately 70-fold less potent than α-ZEL and twice as potent as β-ZEL. These data are largely in agreement with our findings on the differential estrogenic potencies of these compounds for hERα activity. Although higher potencies were observed for hERα agonistic activity (EC50 = 0.06–8.49 nM) than for hAR antagonistic activity (IC50 = 0.41–23.10 μM), these observations merit a short comment. First, reported human serum concentrations (range: 0.06 to 0.323 μM) in precocious puberty (Szuets et al., 1997) and breast and cervical cancer (0.46 to 1.42 μM; Pillay et al., 2002) fall well within the IC50 range (0.41–23.10 μM) observed for ZEN and metabolites in PALM cells. Second, in contrast to endogenous hormones, ZEN and metabolites exhibit limited or no binding to carrier proteins, facilitating their easier access to NRs target sites and resulting in a much larger potential potency than is suggested by their actual concentrations (Leffers et al., 2001).

The in vitro and in vivo effects of ZEN and its metabolites cannot be solely explained as a function of their affinities to hERs, and there is increasing evidence that these compounds disrupt hormonal homeostasis by mechanisms other than ER interactions, such as interference in steroidogenesis or binding to other receptors, as it has been demonstrated in the present work. Thus, ZEN was reported to induce activation of the human pregnane X receptor (hPXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (AhR) in HepG2 cells (Ayed-Boussema et al., 2001), indicating that it may act by binding directly to these receptors. Both hPXR and AhR activation

induce the expression of enzymes involved in the metabolism of endogenous hormones as well as xenobiotics (Ma, 2008). hPXR activation has been associated with decreased androgen levels (Zhang et al., 2010) and increased corticosterone and aldosterone levels (Zhai et al., 2007). Hence, activation of these receptors by ZEN may add to the overall endocrine disruption potential by increasing or decreasing the removal of endogenous hormones in vivo, thereby altering the hormonal homeostasis. ZEN and its major metabolites (α-ZEL and β-ZEL) have also been shown to act as potential EDCs by affecting hormone production (progesterone, estradiol, testosterone and cortisol) in the H295R steroidogenesis assay, with the most striking observation being the induction by β-ZEL of progesterone production (Frizzell et al., 2011). More recently, Zatecka et al. (2014) investigated the effects of two ZEN concentrations on reproductive parameters and testicular gene expression in male mice. The mice exposed to a low ZEN dose showed a decreased expression of the gene for AR and its component Fkbp5, a decreased expression of the Grth gene (expressed in the germinal cell line), and an increased expression of the Mas1 gene (expressed in Sertoli cells). The authors suggested that ZEN exerted an effect on the androgen hormonal system, consistent with previous studies on the specific localization of ZEN at the site of testosterone synthesis in the interstitial region of the testes (Appelgren et al., 1982). Furthermore, in vitro experiments indicated that ZEN may be a potent inhibitor of testosterone production in mouse human chorionic gonadotropin (hCG)-stimulated Leydig cells by blocking the expressions of 3β-HSD-1, cytochrome P450 side chain cleavage enzyme (P450scc), and steroidogenic acute regulatory protein (StAR) at mRNA levels (Yang et al., 2007a). In addition, serum testosterone concentrations in male mice were found to be significantly affected by ZEN and α-ZEL (Yang et al., 2007b), indicating that both mycotoxins develop negative effects downstream of ERs. Interestingly, many in vivo effects observed after ZEN treatment, e.g., the induction of apoptosis of testicular germ cells in a time-dependent and stage-specific manner, were previously described after in utero exposure to the anti-androgen fungicide vinclozolin (Uzumcu et al., 2004).

Food safety can be adversely affected by chemical contaminants frequently found as residues (Peshin et al., 2002). Historically, the attention of toxicologists was initially drawn to pesticides and persistent organic pollutants (POPs) present as residues in food (Carreño et al., 2007; Fernandez et al., 2008), quickly followed by concerns about mycotoxins, due to their implication in multiple human and animal diseases worldwide. To date, it has been established that ZEN and/or its metabolites are EDCs that can have

estrogenic effects and alter reproductive tracts in animals and humans (Zinedine et al., 2007). In the present study, we concluded that ZEN and its five metabolites evidenced an effect on hAR as well as on hERα activity, suggesting that these compounds may interfere with the endocrine system via more than one mechanism of action. Inadvertent exposure to estrogenic and antiandrogenic chemicals, especially during periods of greater vulnerability (Fudvoye et al., 2014), has been associated with impaired function of the male reproductive system (Andersson et al., 2014; Sharpe and Skakkebaek, 2008). The compounds tested in the present work may contribute to these effects, given that they all exhibited estrogenic and anti-androgenic potential. However, EDCs often act through multiple endocrine pathways, and it is not possible to predict the overall endocrine disruptive effect based on in vitro studies. Further research is needed to confirm the contribution of hAR activation to the overall in vivo effects of ZEN and/or its metabolites.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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References

Andersson, A.M., Frederiksen, H., Grigor, K.M., Toppari, J., Skakkebæk, N.E., 2014. Special issue on the Impact of endocrine disrupters on reproductive health. Reproduction 147, E1.

Appelgren, L.E., Arora, R.G., Larsson, P., 1982. Autoradiographic studies of [3H]zearalenone in mice. Toxicology. 25, 243–253.

Ayed-Boussema, I., Pascussi, J.M., Maurel, P., Bacha, H., Hassen, W., 2001. Zearalenone activates pregnane X receptor, constitutive androstane receptor and aryl hydrocarbon receptor and corresponding phase I target genes mRNA in primary cultures of human hepatocytes. Environ. Toxicol. Pharmacol. 31, 79–87.

Bandera, E.V., Chandran, U., Buckley, B., Lin, Y., Isukapalli, S., Marshall, I., et al., 2011. Urinary mycoestrogens, body size and breast development in New Jersey girls. Sci. Total Environ. 409, 5221–5227.

Belhassen, H., Jiménez-Díaz, I., Ghali, R., Ghorbel, H., Molina-Molina, J.M., Olea, O., et al., 2014. Validation of a UHPLC–MS/MS method for quantification of zearalenone, α-zearalenol, β-zearalenol, α-zearalanol, β-zearalanol and zearalanone in human urine. J. Chromatogr. B. Analyt Technol Biomed Life Sci. 962, 68–74.

Bennett, J.W., Klich, M., 2003. Mycotoxins. Clin. Microbiol. Rev. 16, 497–516.

Carreño, J., Rivas, A., Granada, A., Jose Lopez-Espinosa, M., Mariscal, M., Olea, N., et al., 2007. Exposure of young men to organochlorine pesticides in Southern Spain. Environ. Res. 103, 55–61.

Denizot, F., Lang, R., 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods 89, 271–277.

Duca, R.C., Mabondzo, A., Bravin, F., Delaforge, M., 2012. In vivo effects of zearalenone on the expression of proteins involved in the detoxification of rat xenobiotics. Environ. Toxicol. 27, 98–108.

Fang, H., Tong, W., Branham, W.S., Moland, C.L., Dial, S.L., Hong, H., et al., 2003. Study of 202 natural, synthetic, and environmental chemicals for binding to the androgen receptor. Chem. Res. Toxicol. 16, 1338–1358.

Fernandez, M.F., Kiviranta, H., Molina-Molina, J.M., Laine, O., Lopez-Espinosa, M.J., Vartiainen, T., et al., 2008. Polychlorinated biphenyls (PCBs) and hydroxy-PCBs in adipose tissue of women in Southeast Spain. Chemosphere 71, 1196–1205.

Frizzell, C., Ndossi, D., Verhaegen, S., Dahl, E., Eriksen, G., Sørlie, M., et al., 2011. Endocrine disrupting effects of zearalenone, alpha- and beta-zearalenol at the level of nuclear receptor binding and steroidogenesis. Toxicol. Lett. 206, 210–217.

Fudvoye, J., Bourguignon, J.P., Parent, A.S., 2014. Endocrine-disrupting chemicals and human growth and maturation: a focus on early critical windows of exposure. Vitam. Horm. 94, 1–25.

Gromadzka, K., Waskiewicz, A., Chelkowski, J., Goliski, P., 2008. Zearalenone and its metabolites: occurrence, detection, toxicity and guidelines. World Mycotoxin J. 1, 209–220.

Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., et al., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139, 4252–4263.

Le Guevel, R., Pakdel, F., 2001. Assessment of oestrogenic potency of chemicals used as growth promoter by in-vitro methods. Hum. Reprod. 16, 1030–1036.

Leffers, H., Naesby, M., Vendelbo, B., Skakkebaek, N.E., Jørgensen, M., 2001. Oestrogenic potencies of Zeranol, oestradiol, diethylstilboestrol, Bisphenol-A and

genistein: implications for exposure assessment of potential endocrine disrupters. Hum. Reprod. 16, 1037–1045.

Ma, Q., 2008. Xenobiotic-activated receptors: from transcription to drug metabolism to disease. Chem. Res. Toxicol. 21, 1651–1671.

Malekinejad, H., Maas-Bakker, R.F., Fink-Gremmels, J., 2005. Bioactivation of zearalenone by porcine hepatic biotransformation. Vet. Res. 36, 799–810.

Malekinejad, H., Van Tol, H.T., Colenbrander, B., Fink-Gremmels, J., 2006a. Expression of 3a- and 3b-hydroxy steroid dehydrogenase mRNA in COCs and granulosa cells determines zearalenone biotransformation. Toxicol. In Vitro 20, 458–463.

Malekinejad, H., Maas-Bakker, R., Fink-Gremmels, J., 2006b. Species differences in the hepatic biotransformation of zearalenone. Vet. J. 172, 96–102.

Massart, F., Saggese, G., 2010. Oestrogenic mycotoxin exposures and precocious pubertal development. Int. J. Androl. 33, 369–376.

Massart, F., Meucci, V., Saggese, G., Soldani, G., 2008. High growth rate of girls with precocious puberty exposed to estrogenic mycotoxins. J. Pediatr. 152, 690–695.

Molina-Molina, J.M., Hillenweck, A., Jouanin, I., Zalko, D., Cravedi, J.P., Fernandez, M.F., et al., 2006. Steroid receptor profiling of vinclozolin and its primary metabolites. Toxicol. Appl. Pharmacol. 216, 44–54.

Molina-Molina, J.M., Escande, A., Pillon, A., Gómez, E., Pakdel, F., Cavaillès, V., et al., 2008. Profiling of benzophenone derivatives using fish and human estrogen receptor-specific in vitro bioassays. Toxicol. Appl. Pharmacol. 232, 384–395.

Molina-Molina, J.M., Amaya, E., Grimaldi, M., Sáenz, J.M., Real, M., Fernández, M.F., et al., 2013. In vitro study on the agonistic and antagonistic activities of bisphenol-S and other bisphenol-A congeners and derivatives via nuclear receptors. Toxicol. Appl. Pharmacol. 272, 127–136.

Peshin, S.S., Lall, S.B., Gupta, S.K., 2002. Potential food contaminants and associated health risks. Acta Pharmacol. Sin. 23, 193–202.

Pillay, D., Chuturgoon, A.A., Nevines, E., Manickum, T., Deppe, W., Dutton, M.F., 2002. The quantitative analysis of zearalenone and its derivatives in plasma of patients with breast and cervical cancer. Clin. Chem. Lab. Med. 40, 946–951.

Sharpe, R.M., Skakkebaek, N.E., 2008. Testicular dysgenesis syndrome: mechanistic insights and potential new downstream effects. Fertil. Steril. 89, e33–e38.

Shier, W.T., Shier, A.C., Xie, W., Mirocha, C.J., 2001. Structure-activity relationships for human estrogenic activity in zearalenone mycotoxins. Toxicon 39, 1435–1438.

Sweeney, M.J., Dobson, A.D.W., 1998. Mycotoxin production by Aspergillus, Fusarium and Penicillium species. Int. J. Food Microbiol. 43, 141–158.

Szuets, P., Mesterhazy, A., Falkay, G., Bartok, T., 1997. Early telarche symptoms in children and their relations to zearalenon contamination in foodstuffs. Cereal Res. Commun. 25, 429–436.

Takemura, H., Shim, J.Y., Sayama, K., Tsubura, A., et al., 2007. Characterization of the estrogenic activities of zearalenone and zeranol in vivo and in vitro. J. Steroid Biochem. Mol. Biol. 103, 170–177.

Terouanne, B., Tahiri, B., Georget, V., Belon, C., Poujol, N., Avances, C., et al., 2000. A stable prostatic bioluminescent cell line to investigate androgen and antiandrogen effects. Mol. Cell. Endocrinol. 160, 39–49.

Uzumcu, M., Suzuki, H., Skinner, M.K., 2004. Effect of the anti-androgenic endocrine disruptor vinclozolin on embryonic testis cord formation and postnatal testis development and function. Reprod. Toxicol. 18, 765–774.

Videmann, B., Mazallon, M., Tep, J., Lecoeur, S., 2008. Metabolism and transfer of the mycotoxin zearalenone in human intestinal Caco-2 cells. Food Chem. Toxicol. 46, 3279–3286.

Yang, J., Zhang, Y., Wang, Y., Cui, S., 2007a. Toxic effects of zearalenone and alphazearalenol on the regulation of steroidogenesis and testosterone production in mouse Leydig cells. Toxicol. In Vitro 21, 558–565.

Yang, J.Y., Wang, G.X., Liu, J.L., Fan, J.J., Cui, S., 2007b. Toxic effects of zearalenone and its derivatives alpha-zearalenol on male reproductive system in mice. Reprod. Toxicol. 24, 381–387.

Zatecka, E., Ded, L., Elzeinova, F., Kubatova, A., Dorosh, A., Margaryan, H., et al., 2014. Effect of zearalenone on reproductive parameters and expression of selected testicular genes in mice. Reprod. Toxicol. 45C, 20–30.

Zhai, Y., Pai, H.V., Zhou, J., Amico, J.A., Vollmer, R.R., Xie, W., 2007. Activation of pregnane X receptor disrupts glucocorticoid and mineralocorticoid homeostasis. Mol. Endocrinol. 21, 138–147.

Zhang, B., Cheng, Q., Ou, Z., Lee, J.H., Xu, M., Kochhar, U., et al., 2010. Pregnane X receptor as a therapeutic target to inhibit androgen activity. Endocrinology 151, 5721–5729.

Zinedine, A., Soriano, J., Molto, J.C., Manes, J., 2007. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an estrogenic mycotoxin. Food Chem. Toxicol. 45, 1–18. J.

Fig. 1. Chemical structures and metabolic pathways of ZEN and its metabolites.

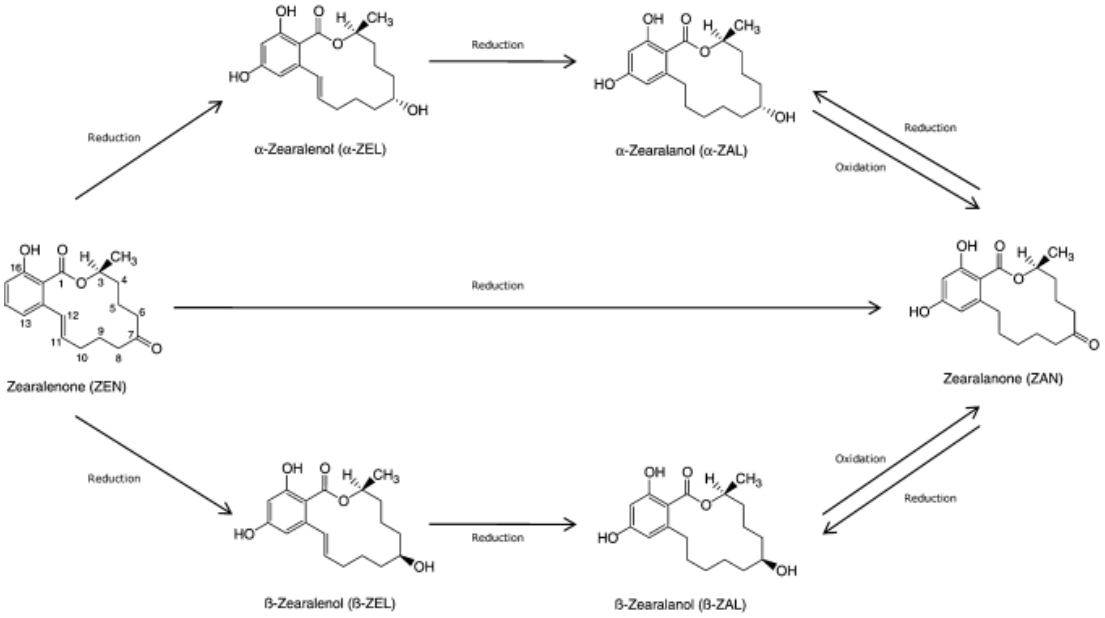

Fig. 2. Dose–proliferative response curves on MCF-7 cells in the E-Screen bioassay.

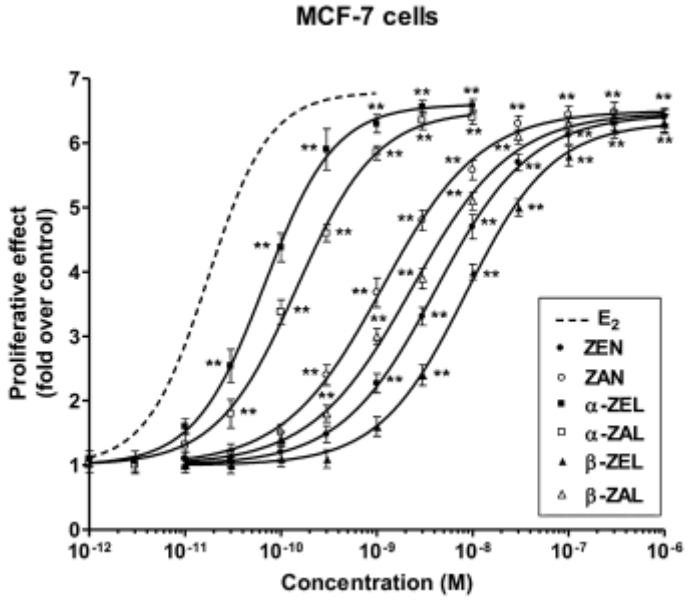

Fig. 3. Dose–response curves of R1881, procymidone, flutamide and vinclozolin in PALM cells.

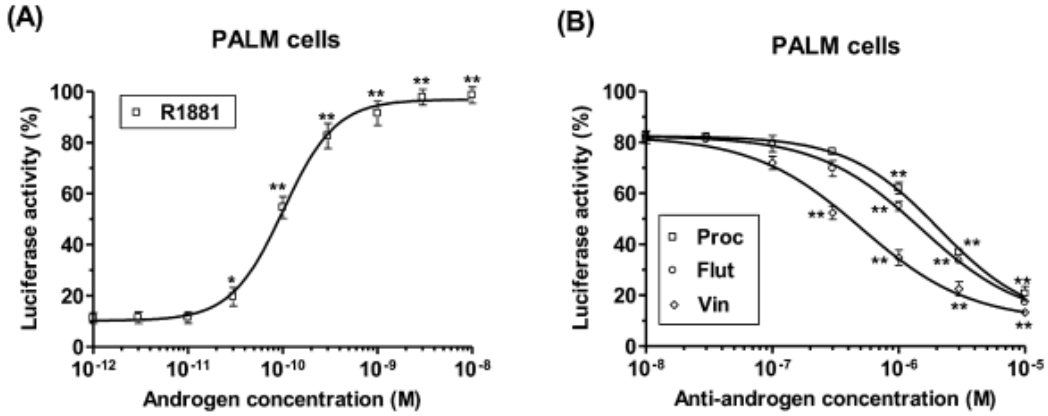

Fig. 4. Transcriptional activity of hAR in response to ZEN and its metabolites.

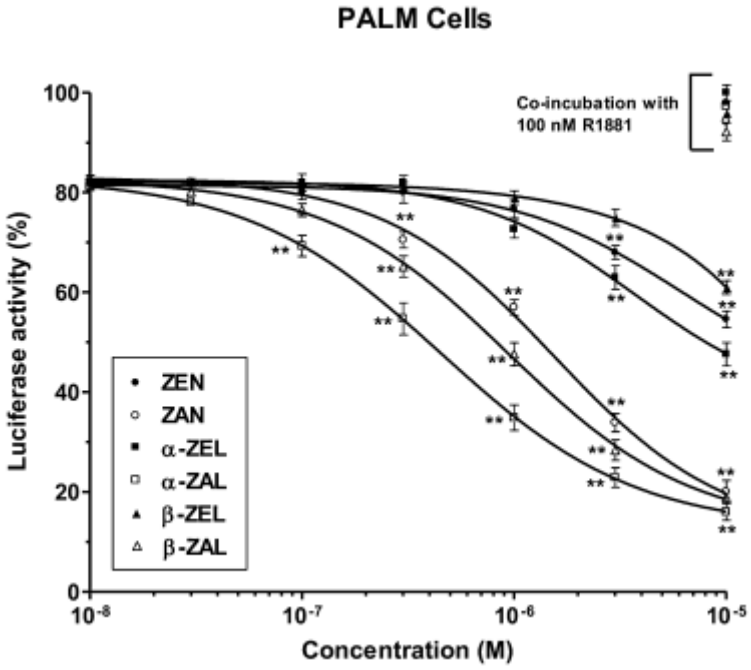

Table 1. Calculated EC50 (effective concentrations for half-maximal proliferative effect on MCF-7 cell proliferation and for half-maximal luciferase activity on PALM cells) and IC50 (inhibitory concentrations for half-maximal luciferase activity on transcriptional activation through hAR) values of tested compounds.

Compounds	MCF-7 cells (hERα)	PALM cells (hAR)	PALM cells (hAR)
	EC_{50} (nM)	EC_{50} (nM)	IC_{50} (μM)[a]
E_2	0.018 ± 0.04	nt	nt
R1881	nt	0.10 ± 0.05	nt
Proc	nt	nt	1.97 ± 0.31
Flut	nt	nt	1.65 ± 0.18
Vin	nt	nt	0.35 ± 0.02
ZEN	3.81 ± 0.25	ne	6.16 ± 2.43
α-ZEL	0.06 ± 0.01	ne	4.17 ± 1.09
β-ZEL	8.49 ± 1.21	ne	23.10 ± 5.28
ZAN	1.21 ± 0.14	ne	1.54 ± 0.41
α-ZAL	0.14 ± 0.04	ne	0.41 ± 0.11
β-ZAL	2.29 ± 0.35	ne	0.93 ± 0.24

a The antagonistic activities of these compounds were determined by coincubation with 0.3 nM R1881 agonist.

ne, no effect.

nt, not tested.