Estrogenic activity of zearalenone, \( \alpha \)-zearalenol and \( \beta \)-zearalenol assessed using the E-Screen assay in MCF-7 cells

Elena Tatay, Silvia Espín, Antonio-Juan García-Fernández & María-José Ruiz

To cite this article: Elena Tatay, Silvia Espín, Antonio-Juan García-Fernández & María-José Ruiz (2017): Estrogenic activity of zearalenone, \( \alpha \)-zearalenol and \( \beta \)-zearalenol assessed using the E-Screen assay in MCF-7 cells, Toxicology Mechanisms and Methods, DOI: 10.1080/15376516.2017.1395501

To link to this article: http://dx.doi.org/10.1080/15376516.2017.1395501
Short communication

Estrogenic activity of zearalenone, α-zearalenol and β-zearalenol assessed using the E-Screen assay in MCF-7 cells

Elena Tatay\textsuperscript{a}, Silvia Espín\textsuperscript{b,c}, Antonio-Juan García-Fernández\textsuperscript{b}, María-José Ruiz\textsuperscript{1a}

\textsuperscript{a}Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Avda. Vicent Andrés Estellés s/n, 46100 – Burjassot (Valencia), Spain

\textsuperscript{b}Laboratory of Toxicology, Department of Health Sciences, Biomedical Research Institute of Murcia (IMIB-UM-Arrixaca), University Clinical Hospital “Virgen de la Arrixaca”, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain

\textsuperscript{c}Department of Biology, University of Turku, 20014 Turku, Finland

Abstract

Mycotoxins, including zearalenone (ZEA), can occur worldwide in cereals. They can enter the food chain and cause several health disorders. ZEA and its derivatives (α-zearalenol, α-ZOL and β-zearalenol, β-ZOL) have structural analogy to estrogen, thus they can bind to estrogen receptors (ERs). In order to characterize the estrogenic activity of ZEA, α-ZOL and β-ZOL, the proliferation of ER-positive human breast cancer cells (MCF-7) exposed to these mycotoxins was measured. After exposure at levels ranging from 6.25 to 25 µM, cell proliferation was evaluated by using the E-Screen bioassay. In accordance with previous studies, our results show the estrogenic activity of ZEA, α-ZOL and β-ZOL in MCF-7 cells. This effect is related to ZEA and its metabolites being flexible enough to bind to mammalian ERs. The relative proliferative effect (RPE) ranged from 10% to 91%. The α-ZOL induced the highest proliferative effect due to its higher affinity for the ERs compared to the other mycotoxins.

Keywords: Zearalenone, metabolites, estrogenic activity, E-Screen.

\textsuperscript{1}Corresponding author:
Maria-Jose Ruiz, Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andres Estelles, s/n, 46117, Burjassot, Valencia, Spain. Phone: +34 963.543.055; Fax: +34 963.544.954; e-mail: M.Jose.Ruiz@uv.es; ORCID: 0000-0003-4174-6688; Research ID: F-6551-2015
1. Introduction

Certain chemical compounds may mimic or antagonize the action of natural estrogens such as 17β-estradiol (E2) \textit{in vitro} and \textit{in vivo}. These compounds are defined as substances with estrogenic or antiestrogenic activity (Bittner et al. 2014). Many of these substances have little obvious structural similarity to natural estrogens, but they are able to bind to estrogen receptors (ERs), influence the expression of estrogen-regulated genes, regulate the growth of estrogen-dependent cells and produce physiological estrogen responses \textit{in vivo} (Darbre et al., 2002; EFSA, 2013).

Since these substances can interfere with the normal functioning of endocrine processes, they may cause several hormone-related health disorders in humans and animals, including early puberty in females, sperm count reduction, alteration of the functions of reproductive organs, obesity, altered sexual behaviors, and increased incidence of some breast, ovarian, testicular and prostate cancers (Bittner et al., 2014).

Mycotoxins are biologically active metabolites produced by fungal species that are toxic to humans and other animals. They represent a serious problem since mycotoxin contamination of agricultural products can occur worldwide and cause several health disorders. Zearalenone (ZEA) is a resorcylic acid lactone derivate produced by \textit{Fusarium} fungi. ZEA and its derivatives (α-zearalenol [α-ZOL]; β-zearalenol [β-ZOL]) have structural analogy to estrogen, and their structures are flexible enough to bind to mammalian ERs (Parveen et al., 2009). Thus, they are able to mimic the activity of naturally occurring estrogens (Gajecka, 2012). The estrogenic activity of ZEA and its metabolites has been determined both \textit{in vivo} and \textit{in vitro} (Le Guevel and Pakdel, 2001; Minervini et al., 2005; Caloni et al., 2009; Parveen et al 2009; Frizzell et al 2011; Busk et al., 2012; Prouillac et al., 2012; Cortinovis et al., 2013). It is known that these mycotoxins, also called mycoestrogens, induce their toxicity by competitive
binding to the ER and modifying steroid metabolism and producing morphological and functional changes in the reproductive system (Salem et al. 2017). It has been shown that ZEA decreases fertility due to reproductive tract disorders and abnormal fetal development, reduces the size and weight of the adrenal and pituitary glands in animals, and alters the ovulation cycle (Parveen et al., 2009; Cortinovis et al., 2013; EFSA, 2016). Due to the anabolic activity of ZEA, it is used as a hormonal growth-promoter in food animals in the United States and Canada, whereas any use of ZEA as a growth promoter is forbidden in the EU (Le Guevel and Pakdel, 2001).

The aim of the present study was to characterize the estrogenic activity of ZEA and its major metabolites, α-ZOL and β-ZOL, in MCF-7 cells. For this purpose, cells were exposed to ZEA, α-ZOL or β-ZOL standards at different concentrations (6.25, 9.37, 12.5, 18.75 and 25 µM) and the proliferative effect (PE) on the target cells was assessed using the E-Screen bioassay. The E-screen bioassay is based on the measurement of the increased proliferation of the target cells caused by the presence of estrogen active substances (Soto et al. 1995). This test is considered one of the most useful screening bioassays for evaluating estrogenic activity due to its high sensitivity, standardized conditions and ease in performance and because it makes it possible to screen a large number of compounds in a short period of time (Andersen et al. 1999, Minervini et al., 2005). The MCF-7 human breast cancer cell line is commonly used for E-screen bioassays because it contains both ER subtypes (ERα and ERβ), responding to the presence of estrogens with a PE (Minervini et al. 2005). When MCF-7 cells are grown in medium without estrogens, proliferation is prevented, and when estrogens are added, the cells are seen to proliferate (Schirilo et al., 2012). Moreover, this cell line shows stable estrogen dependency compared to other human cell lines (Minervini et al., 2005; Schirilo et al., 2009). On the basis of the previous information available
(Minervini et al., 2005; Molina-Molina et al., 2014), we expect to find that ZEA and its metabolites exhibit a marked estrogenic activity in MCF-7 cells.

2. Materials and methods

2.1 Reagents and equipment

Dulbecco’s Modified Eagle’s Medium (DMEM), methanol, 17β-estradiol (E2), non-essential amino acids (NEAAs), glutamine, pyruvate, insulin, sulforhodamine B (SRB), trichloroacetic acid (TCA) and acetic acid were provided by Sigma Chemical Co. (St Louis, MO, USA). The mycotoxin standards ZEA (318.36 g/mol), α-ZOL (320.38 g/mol) and β-ZOL (320.38 g/mol) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Charcoal/dextran treated fetal bovine serum and fetal bovine serum (FBS) were from Cambrex (Belgium). Deionized water (resistivity <18 MΩ cm) was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA). Stock solutions of mycotoxins were prepared in methanol and maintained at -20°C in darkness. Stock solution of E2 (10^{-3}M) was prepared in ethanol and stored at -20°C in darkness. The final concentrations tested were obtained by adding the culture medium with mycotoxins or E2, and the final solvent concentration in medium was ≤ 1% (v/v).

2.2 Cell and culture conditions

The MCF-7 (ATCC-HTB-22) cell line obtained from the American Type Culture Collection was used between passages 50 and 90. The MCF-7 cells were grown in polystyrene tissue culture flasks at pH 7.4, 37°C, 5% CO₂ and 95% relative humidity. The MCF-7 cells were maintained in DMEM medium with 15 mg/l phenol red and supplemented with 5% FBS, 1% glutamine, 1% NEAA, 1% pyruvate and 0.1% insulin. Because of the hormonal activity of phenol red and FBS, experiments in MCF-7 cells
were performed in an experimental medium (phenol red-free culture medium DMEM) supplemented with 5% dextran-coated charcoal-treated FBS (5%), 1% glutamine, 1% NEAA, 1% pyruvate and 0.1% insulin. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St. Louis, MO, USA).

2.3 E-screen assay

The E-screen assay was carried out according to the method described by Körner et al. (1999) and modified by Schirilo et al. (2009). Before the assay, the activity of MCF-7 cells was checked comparing estrogenic response and cell proliferation in the presence of absence of E2 (10⁻⁹ M) to be sure that the endocrine activity of the mycotoxins would be accurately evaluated. Briefly, MCF-7 cells were trypsinized and plated into 96-well plates with experimental medium at a concentration of 2x10³ cells/well. Cells were allowed to attach for 24 h, and the seeding medium was removed, washed with PBS and replaced by the experimental medium containing serial dilutions of ZEA, α-ZOL or β-ZOL (from 6.25 to 25 µM), E2 as positive control and negative control (methanol). A wide range of mycotoxin concentrations were selected according to the different cytotoxic effects observed for the compounds tested. The range of E2 concentrations tested was from 10⁻³ to 10⁻⁹ M. It has been demonstrated that E2 induces maximal proliferation at a concentration of 10⁻⁹ M (Molina-Molina et al., 2014). Previous studies in our laboratory indicate that the late exponential phase of MCF-7 cell proliferation in response to E2 concentration is reached after 144 h, so the bioassay was ended on day 6. Following the incubation time, the medium was carefully aspirated to avoid cell detachment, and the cells were incubated at 4°C for 1 h in 10% TCA (w/v). The TCA was removed and the wells were washed under a gentle stream of tap water and thoroughly air-dried. The TCA-fixed cells were stained with 0.4% (w/v) SRB
dissolved in 1% acetic acid. Following incubation, the supernatant was discarded and unbound dye was removed by rinsing the wells with 1% acetic acid. After thorough drying, bound dye was solubilized with 10 mM Tris base, pH 10.5, for 20-30 min. The optical density (OD) was measured at 570 nm with a reference filter at 690 nm using a fluorimeter (Multiskan MCC/340 plate reader, Thermo Fisher Scientific, Marietta, OH, USA). Three independent experiments were performed.

The basic endpoint of the E-screen assay is the cell proliferation relative to the hormone-free control. The proliferative effect (PE) of ZEA, its metabolites and E2 is the ratio of the maximum OD value obtained with each mycotoxin or E2 to that of the negative control (equation 1).

\[
PE = \frac{OD \text{ (mycotoxins or E2)}}{OD \text{ (negative control)}}
\] (equation 1)

The estrogenic activity of ZEA, α-ZOL and β-ZOL was evaluated by determining the relative proliferative effect (RPE, %), which compares the maximum proliferation induced by the mycotoxin to that induced by E2 (equation 2).

\[
RPE \text{ (\%)} = \frac{[(PE-1) \text{mycotoxin}/(PE-1)\text{E2}]}{100}
\] (equation 2)

Thus, full agonistic activity (RPE = 100%) can distinguished from partial agonistic activity (RPE < 100%). Mycotoxins can be classified according to the strength of their RPE as total agonists (when RPE ranges from 80% to 100%), partial agonists (between 25% and 80%) or weak agonists (between 10% and 25%). A RPE < 10% was defined as not determinable and considered a negative result.

2.4 Statistical analyses

The statistical analysis of the data was carried out using the SPSS version 19 statistical package (SPSS, Chicago, IL, USA). All values are expressed as the mean ± standard error of the mean (SEM) of three independent experiments. Data were
analyzed by one-way analysis of variance (ANOVA) followed by the Tukey’s HDS test for post hoc for pairwise comparisons. A $p$-value $\leq 0.05$ was considered statistically significant.

3. Results

The estrogenic activity of ZEA and its major metabolites, $\alpha$-ZOL and $\beta$-ZOL, in MCF-7 cells was evaluated by using the E-Screen assay. Table 1 and Figure 1 show the PE and RPE (%) of ZEA and its metabolites on MCF-7 cells.

The mycotoxins exhibited estrogenic activity, showing a significant increase in RPE (%) at the tested concentrations compared to the control. The PE induced by ZEA and its metabolites ranged from 1.05 to 1.62, except for the highest concentrations of $\alpha$-ZOL tested (18.75 and 25 µM) with lower PE values since they showed saturation in the proliferative effect on MCF-7 cells (Table 1, Figure 1). The highest RPE (90.5%) was observed at 9.37 µM $\alpha$-ZOL exposure.

4. Discussion

The E-screen assay is used for the detection and quantification of the estrogenic activity of single chemicals by their direct binding to ERs, stimulating the growth of estrogen dependent cell lines, commonly MCF-7. In order to evaluate the estrogenic activity produced by ZEA and its metabolites ($\alpha$-ZOL and $\beta$-ZOL), we determined the PE and RPE (%) induced by different concentrations of these mycotoxins in MCF-7 cells. The results obtained show that the three mycotoxins tested increase the RPE (%) values compared to the solvent control, and $\alpha$-ZOL estrogenic potency is greater than that of ZEA or $\beta$-ZOL. In accordance with our results, Minervini et al. (2005) reported a more active PE and estrogenic potency in $\alpha$-ZOL than in other mycotoxins. However,
for all mycotoxins evaluated (ZEA, α-ZOL and β-ZOL), the highest concentrations tested showed saturation in PE in MCF-7 cells, showing a decrease in the RPE (%). In this sense, higher concentrations have shown to produce toxic effect according to the IC₅₀ values (ZEA >100 µM; α-ZOL=27±4 µM and β-ZOL >100 µM; Tatay et al., 2014a, 2014b). Our results show that α-ZOL at high concentrations produces increased cell death and decreased proliferation, reaching eventually even negative values. The results obtained in our work are similar to those obtained by other authors (Kuiper et al., 1998; EFSA, 2016).

Shier et al. (2001) classified ZEA and 16 structural analogs according to their proliferative potency in MCF-7 cells. Their data suggest that the functional group at the 6 position has the greatest effect on estrogenicity; α-ZOL (α-OH at the 6 position) demonstrated greater potency than other mycotoxins in stimulating the growth of MCF-7 cells (Shier et al., 2001). According to Parveen et al. (2009), this effect may be due to the fact that ZEA and its metabolites have a flexible structure which is able to bind to ERs as strongly as the natural estrogen E2. The greater estrogenic potency of α-ZOL compared to ZEA and β-ZOL reflects its greater affinity for ERs in MCF-7 cells. On the other hand, and in contrast to the hormones, α-ZOL does not bind to the carrier protein - thereby increasing its ability to produce toxic effects (Parveen et al., 2009). In view of our results and according to the classification showed in section 2.3, ZEA and β-ZOL could be classified as partial agonists at a concentration of 6.25 and 9.37 µM, while α-ZOL would be classified as total agonists at those concentrations. ZEA and β-ZOL could be classified as total agonists when the concentrations reach 12.5 µM.

The estrogenic properties of ZEA and its isomers have also been studied in other cell lines using different assays (EFSA, 2016). Prouillac et al. (2012) determined the estrogenic potency of ZEA, α-ZOL and β-ZOL on adenosine triphosphate (ARP)-
binding cassette (ABC) transporters which are regulated by hormonal influence, i.e. estrogen and progesterone. According to our findings, they observed that all mycotoxins induced alterations of ABC transporter expression in human placental BeWo cells at concentration of 0.1 µM. Frizzell et al. (2011) and Ehrlich et al. (2015) used the activation of reporter gene assay (RGA assay) and CALUX assay (an ERα chemical activated luciferase reporter gene assay) to assess the estrogenicity of ZEA and its metabolites, and they demonstrated that α-ZOL was more estrogenic than ZEA and almost as estrogenic as β-ZOL.

In conclusion, the E-screen assay in MCF-7 cells can be used to evaluate the estrogenic activity of ZEA and its metabolites. The estrogenic effect of the three mycotoxins may be related to ZEA and its metabolites having a flexible structure (Parveen et al. 2009), being able to bind to ERs. The highest RPE of α-ZOL is due to its higher affinity for the ERs compared to the other mycotoxins. However, as reported in previous studies (Tatay et al., 2017), it is important to consider that estrogenic activity is not the only mechanism whereby ZEA, α-ZOL and β-ZOL produce their toxic effects.

Acknowledgements

This study was supported by the Spanish Ministry of Economy and Competitiveness (AGL2016-77610-R). Silvia Espín is financially supported by the Academy of Finland (project 265859 to Dr Tapio Eeva) and by Fundación Séneca-Agencia de Ciencia y Tecnología de la Región de Murcia (20031/SF/16 to Dr Silvia Espín).

Declaration of interest statement

The authors report no declaration of interest.
5. References


Legend of Figures and Tables

Figure 1. Relative proliferative effect (RPE, %) on MFC-7 cells exposed to different concentrations of ZEA, α-ZOL and β-ZOL (6.25–25 µM). The concentration tested for E2 was $10^{-9}$ M. Results are the means ± SEM from three independent experiments. (*)Significant differences versus control ($p \leq 0.05$).

Table 1. Proliferative effect (PE) and relative proliferative effect (RPE, %) (mean ± SD) of mycotoxins on MCF-7 cells
Figure 1. Relative proliferative effect (RPE, %) on MFC-7 cells exposed to different concentrations of ZEA, α-ZOL and β-ZOL (6.25-25 µM). The concentration tested for E2 was $10^{-9}$ M. Results are the means ± SEM from three independent experiments. (*)Significant differences versus control ($p \leq 0.05$).

Table 1. Proliferative effect (PE) and relative proliferative effect (RPE, %) (mean ± SD) of mycotoxins on MCF-7 cells

<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>ZEA</th>
<th>PE</th>
<th>RPE (%)</th>
<th>α-ZEA</th>
<th>PE</th>
<th>RPE (%)</th>
<th>β-ZEA</th>
<th>PE</th>
<th>RPE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td></td>
<td>1.13 ± 0.01</td>
<td>26.24 ± 2.01</td>
<td>1.57 ± 0.02</td>
<td>82.38 ± 1.05</td>
<td>1.16 ± 0.02</td>
<td>46.08 ± 2.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.25</td>
<td></td>
<td>1.30 ± 0.02</td>
<td>59.62 ± 1.75</td>
<td>1.62 ± 0.01</td>
<td>90.50 ± 1.02</td>
<td>1.25 ± 0.04</td>
<td>68.96 ± 1.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.37</td>
<td></td>
<td>1.42 ± 0.01</td>
<td>82.68 ± 1.60</td>
<td>1.50 ± 0.02</td>
<td>72.83 ± 1.10</td>
<td>1.32 ± 0.02</td>
<td>87.70 ± 1.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td></td>
<td>1.43 ± 0.01</td>
<td>84.81 ± 1.59</td>
<td>0.98 ± 0.03</td>
<td>-2.07 ± 1.68</td>
<td>1.28 ± 0.02</td>
<td>77.22 ± 1.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.75</td>
<td></td>
<td>1.05 ± 0.03</td>
<td>10.36 ± 2.17</td>
<td>0.46 ± 0.02</td>
<td>-77.23 ± 3.57</td>
<td>1.11 ± 0.03</td>
<td>31.52 ± 2.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>1.05 ± 0.03</td>
<td>10.36 ± 2.17</td>
<td>0.46 ± 0.02</td>
<td>-77.23 ± 3.57</td>
<td>1.11 ± 0.03</td>
<td>31.52 ± 2.17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*C: concentration in µM*