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Molecular insights on xenoestrogenic potential of zearalenone-14-glucoside through a mixed *in vitro/in silico* approach

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List of abbreviation:
E2
17-β-estradiol
ER
Estrogen receptor
LBD
Ligand binding domain
ZEN
Zearalenone
ZEN14Glc
Zearalenone-14-glucoside
ZEN14GlcA
Zearalenone-14-glucuronide

ABSTRACT

The mycotoxin zearalenone may contaminate food and feed worldwide upon infections by *Fusarium* spp. of plants and raw materials intended for human and animal consumption. Currently, contamination by zearalenone and congeners pose concern for health due to xenoestrogenic effects. However, while zearalenone and the main reduced metabolites are well-known xenoestrogens, some plant metabolites that may enter the food chain have been observed aside. Among them, zearalenone-14-glucoside may be abundant in the edible parts of infected plants, thereby entering significantly the human diet and animal feeding. On the basis of previous works, the lack of xenoestrogenicity for this compound *per se* was taken for granted, while neglecting the direct proof of estrogenic activity and considering the hydrolysis as a possible source of estrogenically active metabolites. The present work investigated the xenoestrogenicity of zearalenone-14-glucoside, in comparison to zearalenone, deepening the underlying molecular mechanisms through an integrated *in vitro/in silico* approach. On the basis of our results, zearalenone-14-glucoside effectively stimulated a xenoestrogenic response in cells, but such stimulus can be entirely attributable to the hydrolysis phenomenon, as the glycosylated form turned out to be unable to effectively bind and activate the estrogens receptors.

1. Introduction

Zearalenone (ZEN) is a toxic and low-molecular weight secondary metabolite produced by fungi belonging to *Fusarium* spp., mainly *F. culmorum* and *F. graminearum*, upon infection of small grains and maize pre-harvest (Gromadzka et al., 2008). It is chemically described as 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-beta-resorcylic acid lactone (Fig. 1A), and it has been commonly considered the reference compound for several structurally related derivatives arising from fungal, human and plant metabolism. Besides cytotoxic and genotoxic effects, ZEN may pose a health risk for humans and animals on account of its xenoestrogenic activity (EFSA, 2011). The main molecular mechanism requires the direct binding and activation of estrogen receptors (ERs), which are ligand-induced intracellular transcriptional factors belonging to nuclear receptor superfamily (Brzozowski et al., 1997; Spyrakis and Cozzini, 2009).

Because of the major public health concern due to the entry of ZEN and analogues into animal feeding and human diet, intensive study are devoted to assess the biological activity (primarily including the capability to bind and activate ERs) and the occurrence of this group of mycotoxins in grains. In the past decades, studies were actually limited to seek and assess solely the reduced forms of ZEN (namely, zearalenols and zearalanols), thereby neglecting the majority of metabolites arising from human and

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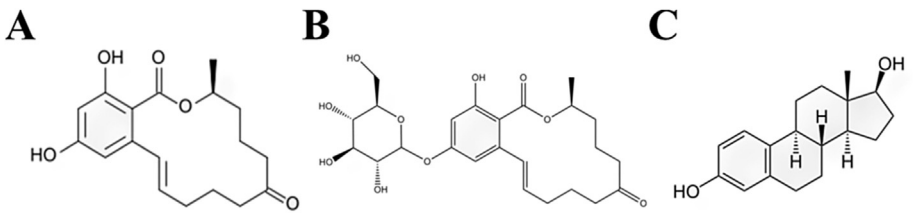

Fig. 1. Chemical structures of compounds under investigation are reported. Zearalenone (**A**), zearalenone-14-glucosideEN-14-Glc (**B**), 17-β-estradiol (**C**).

plant metabolism. As a consequence, the underestimation of the ZEN-dependent toxic load may be possible, also on the account of the fact that the bioconversion might determine activity enhancement or reversion to the parent compound. Just in the recent years, studies have addressed the detection and evaluation of oxidized and conjugated forms (e.g. ref (Ehrlich et al., 2015; Frizzell et al., 2015; Poppenberger et al., 2006).), thus pointing out the need for their evaluation *per se* in the aim of defining a more informed scenario for risk assessment.

In this framework, the plant modified forms are of particular concern. De facto, plants and animals carry out detoxifying mechanisms but, in contrast to animals, plants lack excretion apparatus and chemically modified mycotoxins may be compartmented in the edible parts (Berthiller et al., 2013). In particular, such modified forms can be found in contaminated commodities in a comparable or even higher amount than ZEN (De Boevre et al., 2012; Nathanail et al., 2015), and the 14-β-D-glucoside (ZEN14Glc; Fig. 1B) may accumulate in infected plants and plant tissues entering the food chains (EFSA, 2016; Engelhardt et al., 1988; Schneweis et al., 2002). However, the hazard concerning the xenoestrogenic activity of ZEN14Glc is still poorly characterized as the data collected so far on the ADME/Tox (i.e. the toxicity following the Absorption, Distribution and Metabolism before the Excretion) are scarce and controversial. Indeed, the absorption has been observed in a Caco-2 cells model (Cirlini et al., 2016) but, while the hydrolysis to ZEN and the further release of zearalenols have been observed in several *in vitro, in vivo* and *ex vivo* experiments (Dellafiora et al., 2016a, 2016b; Gareis et al., 1990), the capability to directly bind the ERs has been ruled out in cell-free conditions (Poppenberger et al., 2006). This scenario ultimately makes hard to define a clear declaration of concern for ZEN14Glc and, therefore, the hazard has been inferred on the basis of the possible conversion to toxicologically active aglycones, while lacking the direct proof of xenoestrogenicity. A propos the shortage of evidence, also the European Food Safety Authority (EFSA) pointed out the need for collecting further data on modified mycotoxins (EFSA, 2014a), particularly considering that the effects of metabolic transformation on toxicity have to be considered in the setting of appropriate health-based guidelines (EFSA, 2016).

In this framework, for the first time, the present work addressed the characterization of xenoestrogenic activity of ZEN14Glc deepening the underlying molecular basis and assessing the key steps needed for the ER-dependent response in the light of the presumptive hydrolysis. More in detail, the ability to bind the ERs has been characterized using a hybrid *in vitro/in silico* approach to further investigate the reasons behind the low binding activity previously reported. Then, yeast-based functional assay has been used to assess the capability of ZEN14Glc to recruit co-activator proteins as mandatory to advance the estrogenic stimulus, even not always occurring after the ER binding. Also, the ability to promote the estrogen-dependent gene transcription has been assessed by means of RT-Real Time qPCR and an estrogenic functional assay, both based on MCF-7 cells. Finally, the human phase-II ZEN metabolite zearalenone-14-glucronide (ZEN14GlcA) has been assessed *in silico* to find analogies with ZEN14Glc in interacting with ER.

2. Materials and methods

2.1. Chemicals

ZEN (10 mg, crystalline) was purchased from RomerLabs (Tulln, Austria). ZEN-14-β-D-glucoside was synthesized as previously described (Dall'Erta et al., 2013). The identity and structure of ZEN-14-β-D-glucoside were confirmed by nuclear magnetic resonance (details reported in the Supporting Information), while the purity was checked by using UHPLC-MS/MS analysis (99.8%; details reported in the Supporting Information). Cell culture medium and reagents were from BioWhittaker (Lonza, Milan, Italy) and Celbio (Milan, Italy). 17-β-estradiol (E2), solvents, salts and other general-use chemicals were purchased from Sigma-Aldrich (Milan, Italy). The ERα-LBD was purchased from Life Technologies™ (A15677). Stock solutions of E2, ZEN and ZEN14Glc were prepared in dimethylsulfoxide.

2.2. Cell line

The human breast adenocarcinoma cell line MCF-7 (ATCC® HTB-22™ and repTOP^{tm} B17 clone) retains several characteristics of differentiated mammary epithelium including the ability to process E2 via cytoplasmic estrogen receptors. Therefore, it is considered to as an appropriate model to investigate the activity of compounds under investigation due to the consistency with the toxicological endpoint.

Prior to the experiments, cells were thawed and grown in tissue culture flasks as monolayer in DMEM, supplemented with 1% glutamine, 0.5% penicillin/streptomycin and 10% fetal bovine serum at 37 °C in a humidified (95%) CO_2 (5%) incubator. The cultured cells were trypsinized with trypsin/EDTA for a maximum of 7 min and seeded with a subcultivation ratio of 1:3–1:8.

2.3. Cell-free competitive binding assay

The ability of E2 (as positive control), ZEN, and ZEN14Glc to competitively bind the ERα-LBD has been assayed *in vitro* by using the HitHunter EFC Estrogen Chemiluminescence Assay Kit (DiscoveRx Corp., Fremont, CA, USA). The assay is based on the complementation of β-galactosidase enzyme by two separate fragments, the enzyme acceptor (EA) and the enzyme donor peptide (ED). The reconstitution of active β-galactosidase is prevented in the absence of competitor binders due to the sequestration of ED by the ERα LBD. A competitor binder competes for the binding site thus resulting in β-galactosidase complementation (which can be measured by the hydrolysis of a chemiluminescent substrate). The assay was performed according to the manufacturer's instructions. Chemiluminescent emission was measured with the luminometer LumiLITE™ Microplate Reader (DiscoveRx Corp., Fremont, CA, USA.). Each measure was carried out in triplicate.