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Short communication

Nonsteroidal mycotoxin alternariol is a full androgen agonist in the yeast reporter androgen bioassay

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ABSTRACT

Alternariol (AOH) is a toxic metabolite of phytopathogenic fungi of the *Alternaria* spp. and important contaminant of agricultural commodities.

According to the recent studies, AOH has a potential to modulate the endocrine system of humans and animals. In the view of these reports, our study addressed the effects of AOH on human estrogen receptor (hERα) and androgen receptor (hAR) signaling with the use of the yeast estrogen and androgen reporter bioassays.

Our results show that, apart from a weak estrogenic response, AOH induces full androgenic response of the bioassay with the EC50 of 269.4 μM. The androgenic potency of AOH relative to testosterone (T) is 0.046%. Moreover, in the presence of T, AOH at 5 μM acts as a weak antiandrogen, whereas at higher concentrations AOH sum up with the androgenic activity of T in a dose-dependent manner, suggesting additive effect.

To our knowledge it is the first report of the androgenic potency of natural, nonsteroidal substance and may have the impact on the direction of the further studies. Further research is warranted to clarify the role of AOH in disruption of AR signaling in humans and animals.

1. Introduction

Alternariol (AOH) is a dibenzo-α-pyrone secondary metabolite of phytopathogenic fungi of the genus *Alternaria*. Due to its widespread occurrence at relatively high (μg/kg) levels, in food of plant origin (grains, nuts, fruits and vegetables), AOH may pose a threat to human health (Ostry, 2008; Dall'Asta et al., 2014). Nonetheless, as yet, no regulation concerning exposure limits for AOH in food and feed is available (EFSA, 2011).

Over the past decades, there has been a growing concern about exogenous substances, that may alter the normal functions of the endocrine systems of humans and animals and consequently cause endocrine-related adverse health outcomes (Diamanti-Kandrakis et al., 2009).

Recent *in vitro* studies has demonstrated the potential of AOH to modulate endocrine system through interference with nuclear receptor signaling (Lehmann et al., 2006; Frizzell et al., 2013; Vejdovszky et al., 2016; Demaegdt et al., 2016), upregulation of steroid hormone receptors (Frizzell et al., 2013), altering hormone production and interfering with gene expression of some of the steroidogenic enzymes (Frizzell et al., 2013; Kalayou et al., 2014).

In the view of these reports, our study addressed the effects of AOH on the human estrogen receptor α (hERα) and androgen receptor (hAR) signaling *in vitro* with the use of the yeast estrogen and androgen reporter gene bioassays.

2. Material and methods

2.1. Chemicals

Dimethyl sulfoxide (DMSO) for spectroscopy (Uvasol®) of 99.8% purity (GC) was purchased from Merck (Germany). Difco™ Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulfate (YNB), Bacto™ Agar and D-(+)-glucose (Difco™ Dextrose) for use in preparing microbiological culture media were obtained from Becton Dickinson (France). Ammonium sulfate (AMS) for molecular biology (99.2% purity), and L-leucine from non-animal source, suitable for cell culture (99.5% purity) were provided by Sigma-Aldrich (Poland). The minimal medium with L-leucine (MM/*L*) consisted of YNB (1.7 g/L), D-(+)-glucose (20 g/L), AMS (5 g/L) and L-leucine (30 mg/L: MM/L_1 or 60 mg/L: MM/L_2).

Alternariol (3,7,9–trihydroxy −1-methyl-6H-dibenzo[b,d]pyran-6-one; Mw = 258.229 g/mol, CAS No 641-38-3), crystalline solid of 98.08% purity (HPLC), soluble in organic solvents such as DMSO (30 mg/ml) was purchased from Apollo Scientific (Code No: BIA2301, Batch No: AS451526). Reference estrogen 17β-estradiol (E2, 100%

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purity, CAS No 50-28-2) and reference androgen 17β-testosterone (T, 99% purity, CAS No 58-22-0) were obtained from Sigma-Aldrich (Poland). Stock and working solutions of test compounds were made in DMSO and were kept in amber glass tubes at −20 °C and + 4 °C, respectively. Working solutions were prepared at concentrations ranging from 10 pM to 10 mM and were stored for maximum 3 months. The final concentration of DMSO in the culture medium was less than 1%.

2.2. Yeast strains and culturing conditions

Two recombinant yeast (*Saccharomyces cerevisiae*) strains were kindly provided by dr T.F.H. Bovee (RIKILT, Institute of Food Safety, Wageningen, The Netherlands). The yeast cells were stably transformed with either the human estrogen receptor (hER) or human androgen receptor (hAR) gene and the yeast-enhanced green fluorescent protein (*yEGFP*) reporter gene under control of consensus ERE or ARE sequences. Details of the estrogen and androgen-inducible expression systems have been described previously (Bovee et al., 2004, 2007).

The yeasts were cultured as described previously (Stypuła-Trębas et al., 2016). Two days before running the assay, five yeast colonies were used to inoculate 7 ml of the selective MM/L_1 medium. The culture was grown overnight in 25 cm^2 cell culture flasks (Nunc, Denmark), at 30 °C with vigorous orbital shaking.

2.3. Yeast estrogen and androgen bioassays

The bioassays were performed as described previously (Stypuła-Trębas et al., 2016). In brief, cultures of yeasts were grown in MM/L_1 until late log phase growth (~18 h) at 30 °C with constant shaking and then diluted with MM/L_2 to an optical density OD_{620nm} = 0.06 ± 0.01 with Multiscan RC microplate reader (Labsystems). The diluted suspension (250 µl/well) was added to sterile, clear 96-well plates (Nunc) that were previously treated with either vehicle (DMSO, 1%) alone or increasing concentrations of test compounds (AOH, 17βE2, T) dissolved in DMSO. After 24 h post-treatment, OD_{620nm} and fluorescence ($\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 528 nm) were directly measured using a Synergy 2 microplate reader (BioTek® Instruments Inc., USA). Test substances were assayed in nine concentrations and six replicates, in at least two independent experiments. Alternariol was assayed in the concentrations from 10 nM to 400 µM. Differences of the mean raw fluorescence signals at 24 h and 0 h of yeast exposed to tested chemicals were calculated and corrected for the DMSO data. The absorbance of the yeast culture at 0 and 24 h was measured to check whether a substance was not toxic for yeast.

2.4. Data analysis

Dose–response curves were fitted based on the four-parameter logistic equation (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California, USA). The curves were used to determine half effective concentrations (EC50s). Based on the EC50 values, the relative estrogenic (REPs) and androgenic (RAPs) potencies were calculated for AOH in both bioassays as EC50 of reference compound (17βE2 or T) dividing by EC50 of the AOH and multiplied by 100%.

3. Results

3.1. Estrogenic activity of AOH in the yeast estrogen bioassay

The dose-response curve of E2 and AOH in the hER yeast bioassay is shown in Fig. 1. The half-maximal effective concentration (EC50) for AOH was 200.0 µM. Comparing with the 938.7 pM potency of the reference estrogen (E2), the calculated%REP of AOH was 0.0005%.

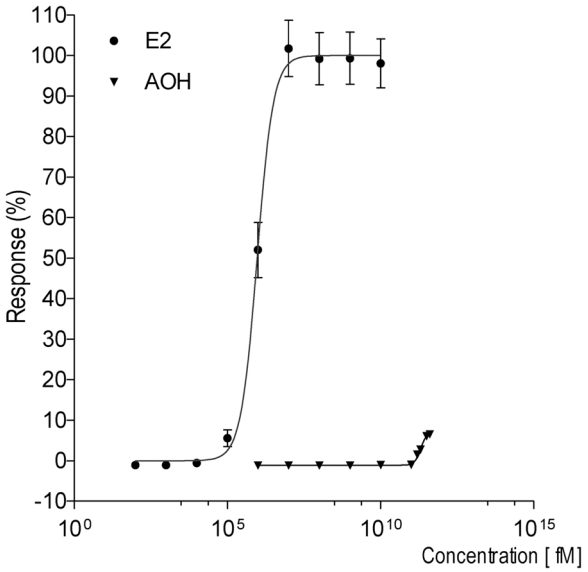

Fig. 1. Concentration–response curves for the 17β-estradiol (E2) and alternariol (AOH) in the yeast estrogen bioassay. Error bars represent standard error of the mean (S.E.M.), to provide an illustration of the reproducibility of the data.

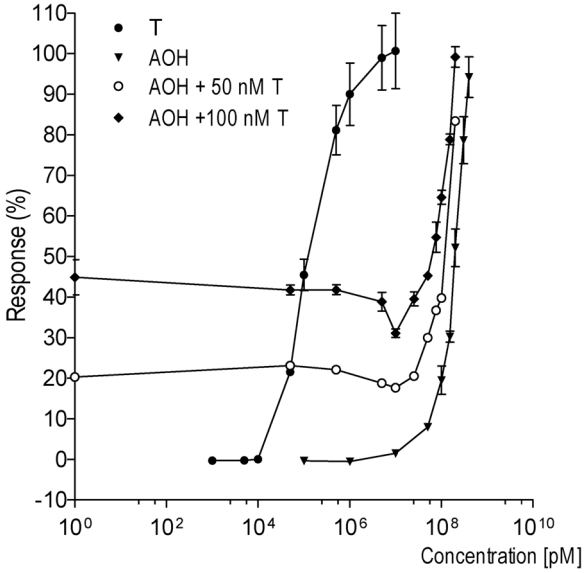

Fig. 2. The response of the yeast androgen bioassay to reference androgen, 17β-testosterone (T, ●), alternariol (AOH, ▼), AOH in the presence of 50 nM T (○) and 100 nM T (♦). Error bars represent standard error of the mean (S.E.M.), to provide an illustration of the reproducibility of the data.

3.2. Activity of AOH in the yeast androgen bioassay

AOH induces the full androgenic response of the yeast androgen bioassay with the EC50 of 269.4 µM (Fig. 2) without significant influence on yeast viability or visible cell pellet formation at the bottom of the 96 well plate. The cytotoxicity of the highest applied AOH concentration was below 30%. Comparing with the 124.85 nM potency of the reference androgen (T), the calculated%RAP of AOH is 0.046%, indicating its significant androgenic potency.

The interaction of AOH with testosterone (Fig. 2) differs, depending on the AOH and T concentrations. At concentration of 5 µM, AOH diminishes the response of the bioassay for 100 nM T by 13.8%. Similar, but less pronounced antiandrogenic effect with 2.3% decrease of the bioassay response was observed for the mixture of 5 µM AOH and 50 nM T. In contrast, at and above the concentration of 50 µM, AOH