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Cancer-related genes transcriptionally induced by the fungicide penconazole

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ABSTRACT

Penconazole is a systemic triazole fungicide mainly used on grapes. The UE Maximum Residue Level (MRL) for penconazole is set at 0.2 ppm in wine and grapes. In the aim of identifying potential biomarkers of exposure to penconazole and possibly highlighting its endocrine disrupting mode of action, we used a transcriptomics-based approach to detect genes, that are transcriptionally modulated by penconazole, by using an appropriate in vitro model. T-47D cells were treated with commercial penconazole or penconazole contaminated grape extracts for 4 h at doses close to the MRL. The whole-genome transcriptomic profile was assessed by using genome 44 K oligo-microarray slides. The list of common genes generated by the two treatments could be representative of potential markers of exposure. In order to understand the role of these genes in key events related to adversity, a pathway analysis was performed on a list of genes with the same modulation trend (up or down). The analysis returned a set of genes involved in Thyroid Cancer Pathway, thus confirming a role of penconazole in endocrine disrupting mediated effects and strongly suggesting a possible mode of action in thyroid carcinogenesis.

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1. Introduction

Fungicides are worldwide used in agriculture for pest control and to increase agricultural productivity. Among fungicides, many conazoles are used for the treatment of topical and systemic fungal infection and for fungal growth prevention on a variety of crops, leading to release into the environment and possible accumulation in living organisms. This raises serious concern about potential harmful effects on ecosystems (Johnson et al., 2009; Puglisi et al., 2012) and above all, on human health. Indeed, adverse toxicological outcomes, including hepatocarcinogenic effects (Allen et al., 2006) and thyroid tumors (Wolf et al., 2006; Hester and Nesnow, 2008) have been associated with exposure to conazoles.

Moreover, these molecules act by inhibiting steroidogenesis enzymes, as sterol 14α-demethylase (encoded by CYP51 gene) and aromatase (encoded by the CYP19 gene), showing potential endocrine disrupting properties (Zarn et al., 2003; Sanderson et al., 2002). Actually, several in vivo and in vitro evidences showed detrimental effects on reproductive development (Rockett et al., 2006; Gray et al., 1999; Kjærstad et al., 2010) and even embryonic teratogenicity and neurotoxicity after perinatal exposure (Moser et al., 2001; Menegola et al., 2005).

Penconazole is a systemic triazole fungicide mainly used in field treatments of vineyard. Recently, the Commission of European Communities adopted the Directive 2007/12/EC where the Maximum Residue Levels (MRL) for penconazole was fixed at 0.2 ppm for table and wine grapes (Commission Regulation, EC No 149/2008). Because of the importance of grapes and wine production and consumption in several Countries and the possible exposure of general population to residues of conazoles in food stuffs, we tried to improve our knowledge about the possible adverse effects induced by penconazole.

According to the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters (as revised in 2012) (OECD, 2012), we set an appropriate in vitro model represented by T-47D cells. The T-47D cell line is a hypotriploid human cell line, isolated from a pleural effusion obtained from a female patient with an infiltrating ductal carcinoma of the breast (Keydar et al., 1979). These cells express several endocrine receptors, including androgen receptor, estrogen receptor, progesterone receptor and other markers (calcitonin, prolactin). The model is very sensitive to endocrine disruptors (Holliday and Speirs, 2011). In order to

Abbreviations: MRL, Maximum Residue Level; UC, untreated cells.

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identify specific biological indicators in the T-47D cell line model, a transcriptomic-based approach was used to detect genes which are differentially expressed after acute exposure to penconazole and extracts from penconazole contaminated grapes.

The goal of this study was to retrieve the penconazole specific gene signature in T-47D cells in order to allow the identification of potential biomarkers of exposure and eventually provide indication about the molecular and pathogenic mechanisms of this molecule.

2. Materials and methods

2.1. Cell line

T-47D cells (ATCC HTB133) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in a 5% CO_2 humidified atmosphere at 37 °C in RPMI-1640 culture medium (Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS) and 0.2 Units/ml of bovine insulin (Sigma–Aldrich, St Louis, MO, USA).

2.2. Chemicals and treatments

Penconazole (CAS 66246-88-6, purity 98%) was purchased from Sigma–Aldrich and dissolved in dimethylsulfoxide (DMSO) in order to obtain a 40 mM stock solution. Concentrations tested in viability assays were obtained by diluting penconazole stock solution in RPMI-1640 culture medium supplemented with 10% FBS and 0.2 Units/ml of bovine insulin.

Extracts were prepared from contaminated grapes obtained by treating the vineyard with penconazole twice. Fifty g of grapes were homogenized, grounded, extracted in a liquid:liquid phase with ethyl acetate and then purified through a solid phase extraction. Samples were eluted with dichloromethane for the GC/MS analysis to determine the penconazole content and then diluted in DMSO to obtain a 240 μM stock solution.

For microarray experiments, both the stock solutions of penconazole and grape extracts were diluted in RPMI-1640 without phenol red, containing 10% dextran-coated charcoal-treated FBS. A 0.5% final concentration of DMSO was used for each treatment in all the experiments.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, purity 98%) was diluted in phosphate buffered saline (PBS) obtaining a 5 mg/ml stock solution.

2.3. MTT assay

5×10^3 cells/well were seeded in a 48-well plate, 4 wells for each treatment, and cells and incubated at 37 °C in a 5% CO_2 humidified atmosphere. After 24 h, cells were treated for 72 h with a wide range of concentrations (0.1–200 μM). MTT solution (final concentration 0.5 mg/ml) was added to each well performing an incubation of 4 h. The formazan crystals produced by MTT were dissolved in 100 μl of DMSO and the optical density (OD) was measured at 540/690 nm. (Multiskan Ascent, Labsystem). One 48-well plate was fixed by MTT solution 24 h after seeding (time zero of treatment, T_0). The growth percentage values at different doses of exposure were calculated according to the formula proposed by the US-National Cancer Institute (NCI):

$$(T - T_0)/(C - T_0) \times 100$$

where T and C represented the OD of treated and untreated cells, respectively (http://www.dtp.nci.nih.gov).

The concentration that induced 50% growth inhibition (GI_{50}) and the relative SE were calculated using linear regression analysis (Tallarida, 2000).

2.4. Cell treatment and total RNA isolation

Cells at a density of 6×10^5 were seeded in 60 mm plates and incubated at 37 °C in a 5% CO_2 humidified atmosphere. After 24 h, culture media was replaced with phenol red-free RPMI-1640 containing 10% dextran-coated charcoal-treated FBS. The cell cultures were incubated in estrogen-depleted conditions for 48 h. Then, treatment solutions were administered to the cells for 4 h. At the end of the incubation time, total RNA was isolated from cells by using TRIzol Reagent (Life Technologies, Carlsbad, CA) and purified on RneasyR affinity column (Qiagen, Valencia, CA). RNA quantification and quality were assessed respectively by Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA) and Agilent bioanalyzer 2100 using the RNA Nano Kit (Agilent Technologies, Santa Clara, CA, USA).

2.5. Total RNA Labeling and Hybridization

Fluorescently labeled cRNA was generated starting from 1 μg of total RNA by using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) according to the instructions from the supplier (http://www.chem.agilent.com). To compare each treatment to the reference, equal amounts of cyanine 5-CTP (Cy5) and cyanine 3-CTP (Cy3) (Perkin–Elmer, NEN Life Science, Boston, MA) labeled cRNAs were combined, purified with QIAquick spin columns (Qiagen), and then applied to the oligonucleotide slide (Whole Human 1 Oligo Microarray) according to the Agilent 60-mer oligomicroarray processing protocol (G4140-90050 version 2.1, available online at http://www.chem.agilent.com). Slides were scanned in both Cy-3 and Cy-5 channels with a dual laser microarray scanner (Agilent, G2565AA). Scanned images were analyzed by the Agilent Feature Extraction software 9.1 to derive the raw intensity data used in the next steps of analysis.

3. Results

3.1. MTT assay

The MTT assay was performed to test the sensitivity of T-47D cells to several concentrations of penconazole in the range 0.1–200 μM. Data expressed as % of survival in penconazole-treated

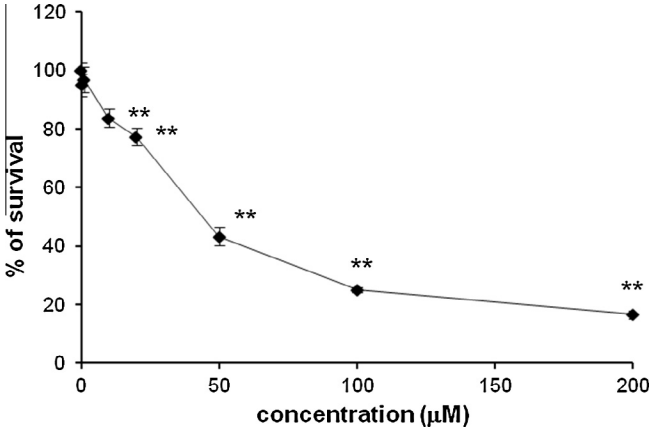

Fig. 1. % of survival of T-47D cells treated with penconazole after 72 h treatment. **Significantly different ($p < 0.01$) from controls (DMSO-treated cells) at the Student t test of at least four technical replicates.

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