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Conazole fungicides inhibit Leydig cell testosterone secretion and androgen receptor activation *in vitro*

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ABSTRACT

Conazole fungicides are widely used in agriculture despite their suspected endocrine disrupting properties. In this study, the potential (anti-)androgenic effects of ten conazoles were assessed and mutually compared with existing data. Effects of cyproconazole (CYPRO), fluconazole (FLUC), flusilazole (FLUS), hexaconazole (HEXA), myconazole (MYC), penconazole (PEN), prochloraz (PRO), tebuconazole (TEBU), triadimefon (TRIA), and triticonazole (TRIT) were examined using murine Leydig (MA-10) cells and human T47D-ARE cells stably transfected with an androgen responsive element and a firefly luciferase reporter gene. Six conazoles caused a decrease in basal testosterone (T) secretion by MA-10 cells varying from 61% up to 12% compared to vehicle-treated control. T secretion was concentration-dependently inhibited after exposure of MA-10 cells to several concentrations of FLUS (IC_{50} = 12.4 μM) or TEBU (IC_{50} = 2.4 μM) in combination with LH. The expression of steroidogenic and cholesterol biosynthesis genes was not changed by conazole exposure. Also, there were no changes in reactive oxygen species (ROS) formation that could explain the altered T secretion after exposure to conazoles. Nine conazoles decreased T-induced AR activation (IC_{50}s ranging from 10.7 to 71.5 μM) and effect potencies (REPs) were calculated relative to the known AR antagonist flutamide (FLUT). FLUC had no effect on AR activation by T. FLUS was the most potent (REP = 3.61) and MYC the least potent (REP = 0.03) AR antagonist. All other conazoles had a comparable REP from 0.12 to 0.38. Our results show distinct *in vitro* anti-androgenic effects of several conazole fungicides arising from two mechanisms: inhibition of T secretion and AR antagonism, suggesting potential testicular toxic

Abbreviations: 3β-HSD1, 3β-hydroxysteroid dehydrogenase type 1; 17β-HSD3, 17β-hydroxysteroid dehydrogenase type 3; AR, androgen receptor; BMR, benchmark response; cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; CHO cells, Chinese hamster ovary cells; Cyp11A1, cytochrome P450 enzyme 11A; Cyp17, cytochrome P450 enzyme 17; CYP19, cytochrome P450 enzyme 19 (aromatase); CYP51, cytochrome P450 enzyme 51/lanosterol 14α-demethylase; CYPRO, cyproconazole; DMEM, Dulbecco's Modified Eagle Medium; EC_{50}, half maximal effective concentration; EDCs, endocrine disrupting chemicals; FLUC, fluconazole; FLUS, flusilazole; FLUT, flutamide; FP, forward primer; FSH(R), follicle-stimulating hormone (receptor); H295R, human adrenocortical carcinoma cells; HEXA, hexaconazole; HMG-CoA red, HMG-CoA reductase; HSD(s), hydroxysteroid dehydrogenase(s); IC_{50}, half maximal inhibitory concentration; LH(R), luteinizing hormone (receptor); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MYC, myclobutanil; NCBI, National Center for Biotechnology Information; PBS, phosphate-buffered saline; PEN, penconazole; Por, cytochrome P450 oxidoreductase; PRO, prochloraz; REP, relative effect potency; RIA, radioimmunoassay; ROS, reactive oxygen species; RP, reverse primer; RT-qPCR, real time quantitative polymerase chain reaction; StAR, steroidogenic acute regulatory protein; T, testosterone; TEBU, tebuconazole; TRIA, triadimefon; TRIT, triticonazole.

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Endocrine disrupting chemicals (EDCs)
MA-10 Leydig cells
Spermatogenesis
Testosterone (T)

effects. These effects warrant further mechanistic investigation and clearly show the need for accurate exposure data in order to perform proper (human) risk assessment of this class of compounds.

1. Introduction

Several studies indicate a global decline in human male fertility over the past decades due to poor semen quality, a suggested decline in sperm count, and lowered testosterone levels in men [1–3]. Furthermore, an overall increase up to 12% in assisted reproductive treatments is observed in Scandinavian countries as well as Switzerland, The Netherlands, and United Kingdom over the past years [4]. In 20% of infertile couples this infertility was attributed to male factors solely and in another 30–40% male factors are conducive [5]. Exposure to environmental chemicals, including endocrine disrupting chemicals (EDCs), is often suggested to be an important contributing factor to these trends in male infertility [6,7].

Among the list of suggested EDCs pesticides are strongly represented [8]. Conazoles are a class of azole-based fungicides that are widely used as pesticides in the cultivation of crops [9] but also as human and veterinary pharmaceuticals for the treatment of oropharyngeal, vaginal, as well as systemic candida and mycosis infections [10]. These compounds decrease fungal membrane integrity by inhibiting the cytochrome P450 enzyme lanosterol 14α-demethylase (CYP51), which is essential for ergosterol biosynthesis and maintaining proper membrane fluidity and permeability in fungi [9]. Besides fungal CYP51, conazoles also target CYP51 of mammals and other vertebrates, which catalyzes the formation of the cholesterol precursor zymosterol [11,12]. Conazoles are known to have *in vivo* endocrine disruptive effects in mammals. For instance, demasculinization of male rat fetuses occurred upon *in utero* exposure to several conazoles [13]. Yet, it remains to be investigated to what extent the known effects of a few tested conazoles are reminiscent for the whole group of conazoles.

The testicular microenvironment is pivotal for mammalian steroidogenesis and intratesticular androgens are required for normal spermatogenesis [14]. In adult males, spermatogenesis is driven by the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). *Via* activation of the LH receptor (LHR), LH stimulates testosterone (T) production in the Leydig cells. Testicular production of T in interstitial Leydig cells is prerequisite for proper spermatogenesis and involves multiple steroidogenic enzymes, *e.g.* steroidogenic acute regulatory protein (StAR), cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1), 17α-hydroxylase/20-lyase (CYP17A1), 3β- and 17β-hydroxysteroid dehydrogenase (3β-HSD and 17β-HSD, respectively) [15]. Subsequently, testosterone binds to the androgen receptor (AR) present in Sertoli cells, which, in combination with FSH binding to the FSH receptor (FSHR), stimulates the progression of spermatogenesis [16].

Conazoles are known to inhibit the steroidogenic enzyme aromatase (CYP19) in several tissues and cell lines, which is involved in the conversion of androgens to estrogens [9,10,17–19]. Conazoles also cause catalytic inhibition of the CYP17 enzyme, responsible for the conversion of pregnenolone and progesterone to androgen precursors, in the human adrenocortical carcinoma H295R cell line and porcine adrenal cortex microsomes [20]. Previous work in H295R cells showed a decrease in T secretion after exposure to econazole, epoxiconazole, ketoconazole, miconazole, prochloraz, propiconazole, and tebuconazole [10]. In combination with the drop in T secretion, an increase in progesterone biosynthesis was seen after exposure to prochloraz, indicating that the role of the CYP17 enzyme is very important in this matter [21]. Furthermore, Cyp26A1, a crucial enzyme within in the retinol metabolism pathway, seems to be a target for conazoles in the zebrafish embryo [12], an underlying mechanism for developmental toxicity. Spermatogenesis is tightly regulated by several steroidogenic processes involving multiple enzymatic conversions. The production of steroids by conversion of cholesterol *via* a cascade of several (CYP) enzymes is the first and crucial step to initiate sperm production, which makes it a vulnerable target for EDCs interference.

In spite of the large production and extensive usage of many conazoles, accurate data on human exposure levels are scarce. Besides occupational and pharmaceutical exposure, individuals can also be exposed to conazoles by environmental, food, resident, or bystander exposure. This is supported by increasing concentrations of conazole pesticides found in surface and waste waters [22]. According to case reports on the risk assessment of tebuconazole, conazoles are moderately and chronically toxic to aquatic species. The environmental fate route is mainly *via* the soil, where it is persistent due to its elimination half-life of approximately 800 days [23]. Pesticide usage surveys performed in the UK show that triazole usage has increased from 6.1 in 1990 to approximately 16.4 million ha treated in 2011 [24]. Among the conazoles, tebuconazole (2.5 million ha) is the most frequently used conazole fungicide, followed by prochloraz and cyproconazole (both 1.3 million ha), and then flusilazole and triticonazole (0.6 and 0.5 million ha, respectively). Because of this extensive usage of conazoles, there is a potential risk that humans and wildlife are frequently, possibly chronically exposed to these compounds *via* their environment. The potential to affect steroid hormone synthesis in combination with the likelihood of frequent exposure make conazoles an important and relevant group of compounds to consider for effects on male fertility.

In this *in vitro* study, the effects of ten conazoles on two key male reproductive factors were assessed and