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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

Table 1
Overview of the ten selected conazoles used in this study, type of conazole, method of application, and structural formula (derived from MDL ISIS™/Draw 2.5, MDL Information Systems, Inc., San Leandro, CA, USA).

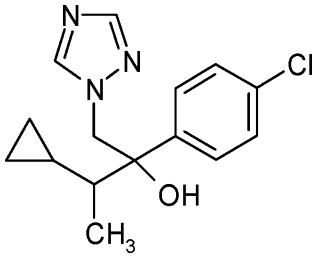
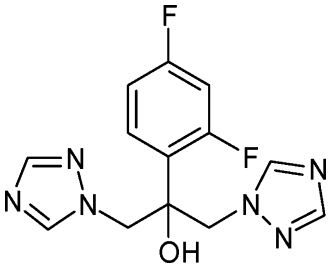
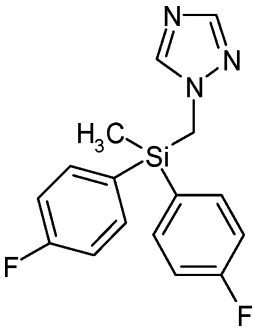
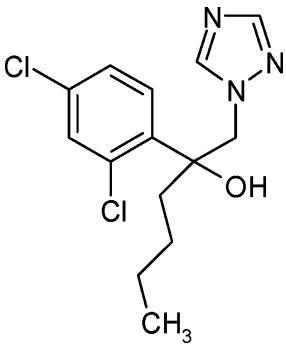
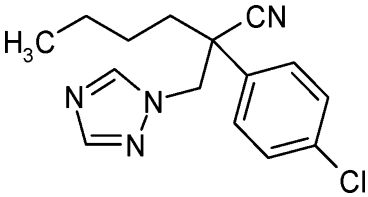
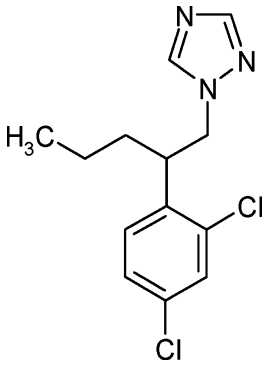
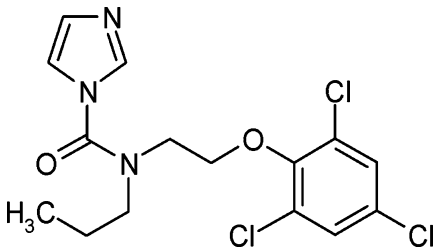
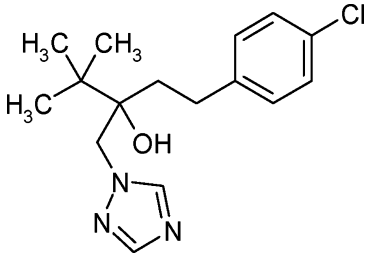
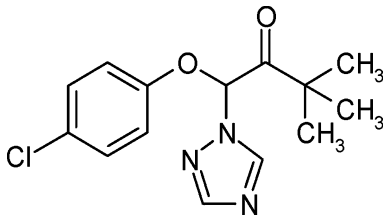
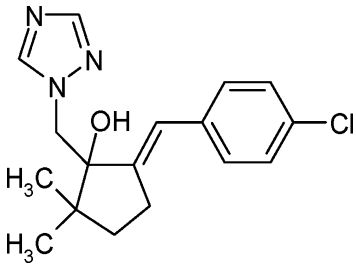
Conazole	Abbreviation	Type	Use	Structure
Cyproconazole	CYPRO	Triazole	Pesticide	
Fluconazole	FLUC	Triazole	Pharmaceutical	
Flusilazole	FLUS	Triazole	Pesticide	
Hexaconazole	HEXA	Triazole	Pesticide	
Myclobutanil	MYC	Triazole	Pesticide	

Table 1 (*Continued*)

Conazole	Abbreviation	Type	Use	Structure
Penconazole	PEN	Triazole	Pesticide	
Prochloraz	PRO	Imidazole	Pesticide	
Tebuconazole	TEBU	Triazole	Pesticide	
Triadimefon	TRIA	Triazole	Pesticide	
Triticonazole	TRIT	Triazole	Pesticide	

compared, namely testicular steroidogenesis and AR response. These conazoles were selected based on their usage as a fungicide for crop protection and included cyproconazole (CYPRO), flusilazole (FLUS), hexaconazole (HEXA), myclobutanil (MYC), penconazole (PEN), prochloraz (PRO), tebuconazole (TEBU), triadimefon (TRIA), and triticonazole (TRIT) (Table 1). For comparison, we also included one conazole used as pharmaceutical, *i.e.* fluconazole (FLUC; Table 1). Effects on basal and LH-stimulated T secretion and steroidogenic gene expression were studied in murine MA-10 Leydig cells. A number of toxicants is also known to contribute to a decrease in sperm viability and motility by increasing ROS production in the testis and epididymis [25]. Therefore, effects of conazoles on ROS formation in

MA-10 cells were determined as well. In addition, effects on AR activation were assessed in human T47D-ARE cells stably transfected with a luciferase reporter gene.

2. Materials and methods

2.1. Chemicals

The ten selected conazoles (Table 1) were purchased from Sigma–Aldrich Co. (Zwijndrecht, The Netherlands): cyproconazole (CYPRO; 99.8%, CAS# 94361-06-5), fluconazole (FLUC; ≥98%, CAS# 86386-73-4), flusilazole (FLUS; 99.8%, CAS# 85509-19-9), hexaconazole (HEXA; 99.7%, CAS# 79983-71-4), myclobutanil (MYC; 99.3%, CAS# 88671-89-0), penconazole (PEN; 99.1%, CAS# 66246-88-6), prochloraz (PRO; 99.1%, CAS# 67747-09-5), tebuconazole (TEBU; 99.6%, CAS# 107534-96-3), triadimefon (TRIA; 99.7%, CAS# 43121-43-3), and triticonazole (TRIT; 98.8%, CAS# 131983-72-7). SU10603 was a kind gift from Dr. Honora Cooper Eckhardt (Hovartis Pharmaceuticals Corporation, Summit, USA). Stock solutions were prepared in DMSO resulting in a maximal solvent concentration of 0.1% (*v*/*v*) in the exposure medium.

2.2. MA-10 Leydig cell culture

The murine Leydig tumor cell line MA-10 was kindly provided by Dr. Mario Ascoli (University of Iowa, Iowa City, IA, USA) [26]. Cells were cultured as described previously by Dankers et al. [27]. In short, cells were grown in 1:1 Dulbecco's Modified Eagle Medium/F-12 nutrient mixture (Ham) with phenol red (DMEM/F-12 1:1, #11320; Gibco, Life Technologies Europe BV, Bleiswijk, The Netherlands) supplemented with 15% HyClone (#SH30068.03; Thermo Fisher Scientific, Waltham, MA, USA), 2% HEPES [1 M] (#15630; Gibco), and 1% penicillin/streptomycin (#15140; Gibco) and maintained at 37 °C in a humidified atmosphere (95%) with 5% CO_2. Cells were cultured twice weekly and culture medium was refreshed 24 h prior to subculturing. Flasks and plates were coated at room temperature with 0.1% gelatin (Attachment Factor Protein; Gibco) 45 min prior to use.

2.3. Testosterone secretion assay

T secretion was assessed with MA-10 cells plated at a density of 2.0×10^5 cells/well in 24-well Plates 24 h prior to exposure. 8-Bromoadenosine 3′,5′-cyclic monophosphate (cAMP; [100 μM]) induces the expression of genes of steroidogenic enzymes and was used as positive control. SU10603 [1 μM] is a catalytic CYP17 enzyme inhibitor [28] and was used as a control for decreased T secretion. For basal T measurements cells were exposed to the selected conazoles [10 μM] alone. Gonadotropin LH (10 ng/mL = 8.5 IU/mL) was used to stimulate the Leydig cells to produce T. To determine the effect of selected conazoles on LH-induced T secretion, cells were exposed to a combination of LH (10 ng/mL) and SU10603 (0.05–1 μM), FLUS or TEBU (0.3–10 μM). After a 48-h exposure, medium was collected and stored at −20 °C until further use. T measurements in the media were performed with a commercially available T radioimmunoassay (T RIA) kit according to the manufacturer's instructions (#DSL-4900; analytical sensitivity = 0.18 pg/mL; Beckman Coulter GmbH, Krefeld, Germany).

2.4. Gene expression

For gene expression experiments, MA-10 cells were plated at a density of 6.0×10^5 cells/well in 12-well Plates 24 h prior to exposure. Cells were exposed for 6 h to CYPRO, FLUS, PRO, TEBU [10 μM], and the positive control cAMP [100 μM]. Total RNA was isolated from exposed MA-10 cells by chloroform-phenol extraction using RNA InstaPure according to the manufacturer's instruction (Eurogentec, Liège, Belgium). Purity and concentration of isolated RNA was determined spectrophotometrically at absorbance wavelengths of 230, 260, and 280 nm using a NanoDrop2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA samples were diluted to a concentration of 66.7 μg/mL and stored at −80 °C until further use. cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., Veenendaal, The Netherlands) and synthesized cDNA was diluted to the appropriate concentration for each primer pair (Supplementary Table 1). Real time quantitative polymerase chain reaction (RT-qPCR) was performed with a mixture containing 7.5 μL iQ SYBR green supermix (Bio-Rad Laboratories Inc., Veenendaal, The Netherlands), 0.6 μL forward primer (FP) and 0.6 μL reversed primer (RP) [each 10 μM], 0.3 μL RNAse free water, and 6 μL of diluted cDNA.

Supplementary Table 1 related to this article can be found, in the online version, at doi:10.1016/j.toxrep.2014.05.006.

The expression of five steroidogenic genes was studied: steroidogenic acute regulatory protein (StAR), cytochrome P450 enzyme 11A1 (Cyp11A1), cytochrome P450 enzyme 17 (Cyp17A1), 3β-hydroxysteroid dehydrogenase type 1 (3β-HSD1), and 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3). Also, the expression of three cholesterol biosynthesis genes was studied: cytochrome P450 enzyme 51 (Cyp51), HMG-CoA reductase (HMG-CoA red), and cytochrome P450 oxidoreductase (Por). β-Actin was used as a reference gene. Sequences of the primer pairs used are depicted in Supplementary Table 1. All primers span an exon–exon junction to ensure mRNA amplification only and were run through National Center for Biotechnology Information (NCBI) Blast (nucleotide non-redundant database) to confirm specificity. Efficiency was determined and was for all primer pairs between 90 and 110%. The mixtures were placed in the CFX Connect™ (Bio-Rad Laboratories Inc.) and firstly heated till 95 °C for 3 min, following 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 45 s. Subsequently, a melt curve was run to ensure the exclusion of primer dimers and other non-specific products formed during the RT-qPCR. Gene expression of each sample was expressed as threshold cycle (Ct), normalized to the reference gene β-actin (ΔCt), and fold induction relative to the DMSO control was calculated.

2.5. Reactive oxygen species assay

ROS production in MA-10 cells was assessed using the fluorescent dye H_2-DCFDA (#D-399; Gibco). MA-10 cells

were plated at a density of 7.5×10^4 cells/well in 96-well Plates 24 h prior to addition of the fluorescent dye. Cells were loaded with H_2-DCFDA [10 μM] for 2 h prior to exposure at 37 °C. After loading, the dye was removed and cells were washed twice with warm PBS. Subsequently, cells were exposed to the ten selected conazoles at concentrations ranging from 0.01 to 100 μM for up to 48 h. Dye and exposure solutions were prepared in serum-free assay medium (DMEM/F-12 1:1 without phenol red, #11039; Gibco). Fluorescence was measured spectrophotometrically at wavelengths of 485/530 nm (Infinite M200 microplate; Tecan Group Ltd., Männedorf, Germany) at $T = 0$ (to determine the basal background level), 1, 24, and 48 h of exposure. As a positive control for oxidative stress at the short time point ($T = 1$ h) H_2O_2 [20 mM] (hydrogen peroxide 30%, #107209; Merck KGaA, Darmstadt, Germany) was used, for the longer time points ($T = 24$ and 48 h) rotenone [100 μM] (#45656; Sigma–Aldrich Co.) was used. As non-exposed control cells show a basal ROS production over time, data are expressed as average percentage compared to the time-matched control values.

2.6. T47D-ARE cell culture

The human breast cancer cell line T47D-ARE was kindly provided by Prof. Dr. Michael Denison (University of California, Davis, CA, USA). T47D-ARE cells are transfected with an androgen responsive element with a firefly luciferase reporter gene [29]. Cells were grown in Dulbecco's Modified Eagle Medium with phenol red containing 4.5 g/L D-glucose, L-glutamine, and pyruvate with phenol red (DMEM, #41966; Gibco) supplemented with 10% fetal bovine serum (FBS, #10270; Gibco) and 1% penicillin/streptomycin (#15140; Gibco) and maintained at 37 °C in a humidified atmosphere (95%) with 5% CO_2. Cells were sub-cultured twice every week.

2.7. Androgen receptor reporter gene assay

Culture medium of T47D-ARE cell was replaced by assay medium 72 h prior to seeding. Assay medium was composed of Dulbecco's Modified Eagle Medium without phenol red containing 4.5 g/L D-glucose (DMEM, #31053; Gibco) supplemented with 10% HyClone (#SH30068.03; Thermo Fisher Scientific), 1% L-glutamine [200 mM] (#25030; Gibco), 1% sodium pyruvate [100 mM] (#11360; Gibco), and 1% penicillin/streptomycin (#15140; Gibco). Cells were seeded at a density of 4.0×10^5 cells/well in white 96-well plates with a clear flat bottom (#655098; Greiner Bio-One, Alphen aan den Rijn, The Netherlands) 48 h prior to exposure. AR activation was determined by measuring the luciferase reaction luminescence. The luminescent signal evoked by the luciferase reaction was measured as relative luminescence units (RLU) of T47D-ARE cells exposed to concentration curves ranging from 100 pM to 100 μM of the ten selected conazoles. Exposures were performed in the presence or absence of EC_{50} of T [20 nM]. The known AR antagonist flutamide (FLUT) was used as positive control for AR antagonism [30]. After a 24-h exposure, cells were washed with warm phosphate-buffered saline solution (PBS, diluted 1:10 with sterile water, #14200; Gibco) and incubated with 1x luciferase cell culture lysis reagent (pH = 7.8; #E1531; Promega, Madison, WI, USA) for 30 min. Subsequently, luciferase activity was measured by addition of reagent mix (LUMIstar Galaxy luminometer, BMG Labtech GmbH, Ortenberg, Germany). The reagent mix was composed of tricine [20 mM] (#T5816; Sigma–Aldrich Co.), $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$ [1.07 mM] (#227668; Sigma–Aldrich Co.), $MgSO_4 \cdot 7H_2O$ [2.67 mM] (#63138; Sigma–Aldrich Co.), EDTA [0.1 mM] (#ED2SS; Sigma–Aldrich Co.), DTT [33.3 mM] (#D9779; Sigma–Aldrich Co.), coenzyme A [261 μM] (#A2181; Sigma–Aldrich Co.), luciferin [470 μM] (#1605; Promega), and ATP [530 μM] (#10127531001; Roche Diagnostics Corporation, Indianapolis, IN, USA) dissolved in Milli-Q water (pH = 7.8).

For FLUT and each conazole exposure the half maximal inhibitory concentration (IC_{50}) of AR activation was derived from concentration–response curves using a sigmoidal dose–response nonlinear regression curve fit with variable slope following the formula (1):

$$y = E_0 + \left(\frac{(E_{\max} \times X^n)}{b^n + X^n} \right). \quad (1)$$

In the above Hill equation, y is the dependent variable (AR response), X the independent variable (exposure concentration), E_0 the estimated background response level, $E_{\max}$ the maximum response, b the computed half maximal inhibitory concentration of flutamide ($IC_{50;FLUT}$), and n the shaping parameter of the Hill curve.

For each conazole the concentration, *i.e.* benchmark response (BMR), needed to elicit 25% of the inhibitory effect on AR activation response caused by flutamide ($BMR_{25;FLUT}$) was calculated by using the formula (2) below:

$$BMR_{25\%FLUT}\text{ “conazole X”} = 10^{\{-[(\log((E_{\max}/(y-E_0))-1))/n]+\log(IC_{50}\text{“conazole X”})\}} \quad (2)$$

Subsequently, relative effect potencies were calculated for each conazole relative to flutamide (REP_{FLUT}) using the respective BMRs in the following formula (3):

$$REP\text{ “conazole X”} = \left(\frac{BMR_{25\%\,FLUT}}{BMR_{25\%\,FLUT}\text{ “conazole X”}} \right) \quad (3)$$

2.8. Cytotoxicity

Cell viability of MA-10 and T47D-ARE cells after exposures was determined by measuring the capacity of cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan by the mitochondrial enzyme succinate dehydrogenase. After exposure, remaining medium was removed and cells were incubated with MTT (1 mg/mL) for 30 min at 37 °C in a humidified atmosphere (95%) with 5% CO_2. After aspiration, 1 mL isopropanol was added at room temperature in order to extract the formed blue colored formazan [31]. Absorbance was measured spectrophotometrically at a wavelength of 595 nm (POLARstar Galaxy, BMG Labtech GmbH).

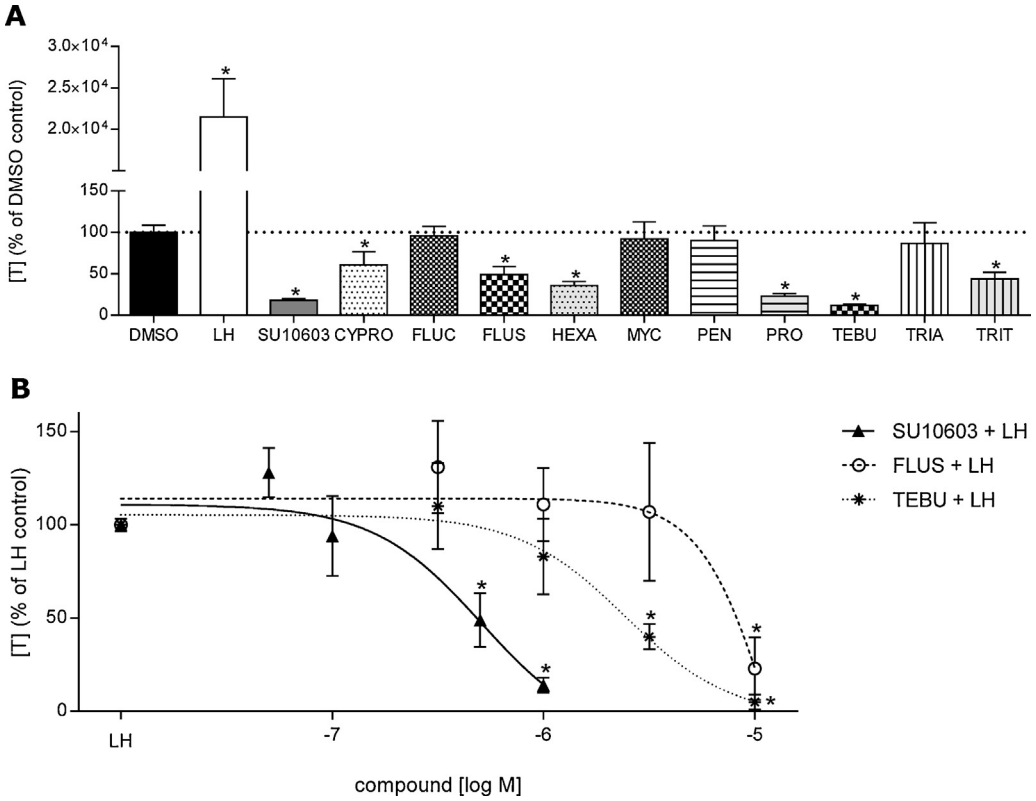


Fig. 1. Testosterone secretion. Testosterone secretion by MA-10 cells after a 48-h exposure to (1A) the DMSO control, the gonadotropin LH (10 ng/mL = 8.5 IU/mL), the Cyp17 inhibitor SU10603 (1 μM), or one of the ten selected conazoles (10 μM); and (1B) concentration curves of SU10603 (0.05–1 μM), FLUS or TEBU (0.3–10 μM) in combination with LH (10 ng/mL = 8.5 IU/mL). Testosterone was measured using a commercially available RIA. Data are represented as means ± SEM with $N = 3$ and $n = 2$. Significance was assessed using a Student's *t*-test (1A) or a one-way ANOVA test followed by Dunnett's *post hoc* test (1B). * Significantly different from vehicle-treated cells ($P < 0.05$). Dotted line (1A) indicates the reference level of the DMSO control.

2.9. Data analysis

All experiments were performed in triplo and within each independent experiment each concentration was tested in duplicate (T secretion assays), triplicate (gene expression experiments and AR reporter gene assays), or quadruplicate (ROS assays). The results are depicted as the mean of replicates of each experiment with standard error (SEM). Data calculations were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance of differences of the mean as compared to the control was calculated using a two-tailed unpaired Students' *t*-test (for single concentrations) or a one-way ANOVA and *post hoc* Dunnett's test (for concentration curves). Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Testosterone secretion inhibition

In order to study the possible effects of conazoles on male sex steroid production in Leydig cells, basal T secretion by MA-10 cells was assessed after a 48-h exposure to the selected conazoles (10 μM). This concentration did not significantly affect MA-10 cell viability (data not shown). Basal T secretion by vehicle-treated control (0.1%, *v/v* DMSO) MA-10 cells was 0.24 ± 0.09 pg/mL. Cells exposed to the positive control for increased T secretion (100 μM cAMP) showed a significant increase of 875-fold in secreted T levels in the medium compared to medium of vehicle-treated cells (data not shown). Exposure to the gonadotropin LH (10 ng/mL = 8.5 IU/mL) caused a statistically significant increase of 215-fold in T secretion compared to vehicle-control cells (Fig. 1A). Exposure to the CYP17 inhibitor SU10603 (1 μM) statistically significantly decreased T secretion by 82% compared to vehicle-treated cells (Fig. 1A). Of the ten selected conazoles (10 μM), CYPRO (61%), FLUS (49%), HEXA (36%), PRO (23%), TEBU (12%), or TRIT (44%) statistically significantly inhibited T secretion by MA-10 cells compared with vehicle-control (Fig. 1A).

To evaluate the effect of conazoles on LH-induced T secretion, MA-10 cells were exposed to various concentrations of two widely used conazoles that also showed marked basal T secretion inhibition, *i.e.* FLUS and TEBU, alone or in combination with LH (10 ng/mL). We selected both FLUS and TEBU as representatives of the group of conazoles tested because of the extensive literature on developmental and reproductive toxic effects available, their high usage, as well as their ability to decrease basal T secretion. SU10603, FLUS, and TEBU all concentration-dependently inhibited T secretion (Supplementary Fig. 1). In combination with LH, SU10603, FLUS, and TEBU inhibited T secretion with IC_{50} values of 0.5, 12.4, and

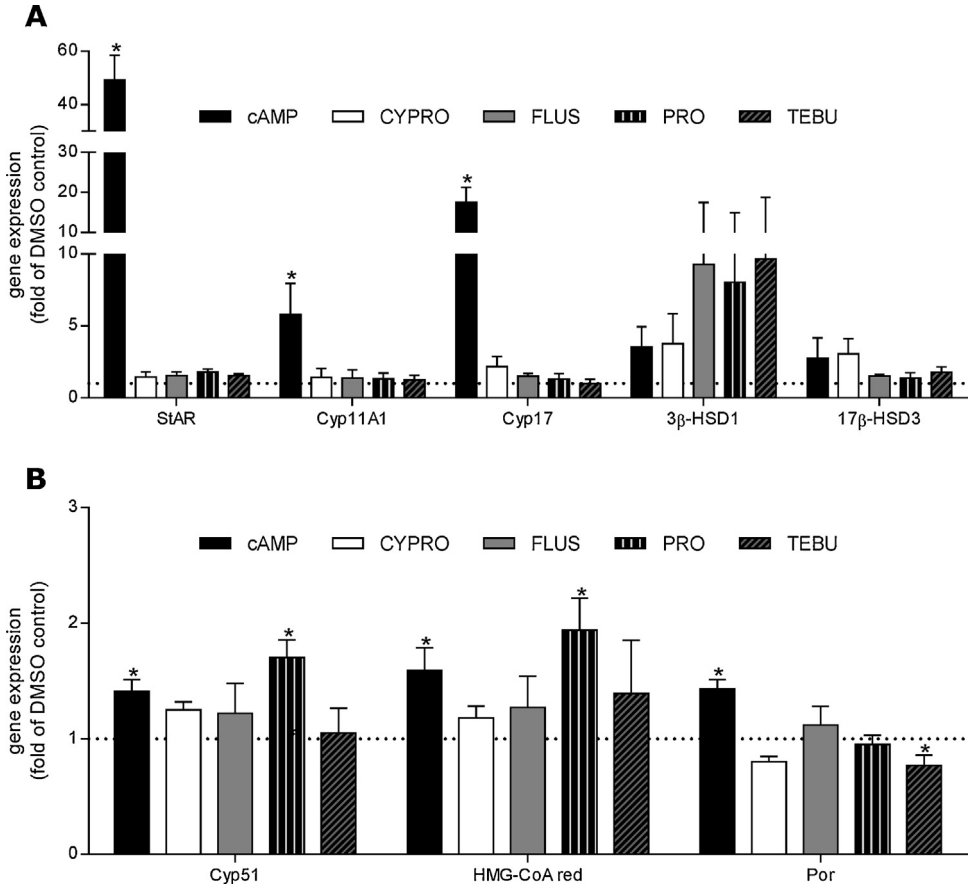


Fig. 2. Expression of (A) steroidogenic and (B) cholesterol biosynthesis genes. Expression of genes involved in (2A) steroidogenesis or (2B) cholesterol biosynthesis in MA-10 cells after a 6-h exposure to cAMP (100 μM) or the selected conazoles CYPRO, FLUS, PRO, or TEBU (10 μM). Data are represented as means ± SEM with $N = 3$ and $n = 3$. Significance was assessed by means of a Student's *t*-test. * Significantly different from DMSO control-treated cells ($P < 0.05$). Dotted lines indicate the reference levels of the DMSO controls.

2.4 μM, respectively (Fig. 1B). To determine the extent of inhibition by FLUS and TEBU, the relative effect potency (REP) of these conazoles was calculated relative to the known CYP17 inhibitor SU10603 ($REP_{SU10603}$) according to the formula (4):

$$REP_{SU10603}\text{ “conazole X”} = \left(\frac{IC_{50;SU10603}}{IC_{50}\text{ “conazole X”}}\right) \quad (4)$$

This calculation resulted in a $REP_{SU10603}$ of 0.04 for FLUS and 0.21 for TEBU.

Supplementary Fig. 1 related to this article can be found, in the online version, at doi:10.1016/j.toxrep.2014.05.006.

3.2. Expression of steroidogenic genes

Next, we investigated whether inhibition of T secretion by four of the most extensively used conazoles, *e.g.* CYPRO, FLUS, PRO, and TEBU, was a result of altered steroidogenic gene expression in MA-10 cells. For that, MA-10 cells were exposed for 6 h to non-cytotoxic concentrations of the tested compounds based on preceding cytotoxicity experiments (data not shown). The five genes selected to be studied encode for the StAR protein, the main cholesterol transport carrier, as well as for the major enzymes involved in the testis steroidogenesis route, *i.e.* Cyp11A1, Cyp17A1, 3β-HSD1, and 17β-HSD3. Exposure to cAMP (100 μM) resulted in an increase in gene expression of StAR (49-fold), Cyp11A1 (6-fold), and Cyp17A1 (18-fold) in comparison with vehicle-treated cells (Fig. 2A). Gene expression of 3β-HSD1 and 17β-HSD3 did not significantly change upon cAMP treatment. Exposure to CYPRO, FLUS, PRO, or TEBU did not significantly affect gene expression of these five steroidogenic genes (Fig. 2A).

3.3. Expression of cholesterol biosynthesis genes

Effects of conazoles on cholesterol biosynthesis were studied since genes involved in “late” steroidogenesis (following pregnenolone) could not explain the decrease in T synthesis. For proper T production, sufficient cholesterol is needed as steroid precursor. Gene expression of three enzymes involved in cholesterol biosynthesis was determined, *i.e.* cytochrome P450 enzyme 51 (Cyp51), HMG-CoA reductase (HMG-CoA red), and cytochrome P450 oxidoreductase (Por). Exposure to cAMP (100 μM) increased gene expression of Cyp51 (1.4-fold), HMG-CoA red (1.6-fold), and Por (1.4-fold) in comparison with vehicle-treated cells (Fig. 2B). Exposure to PRO increased the expression of

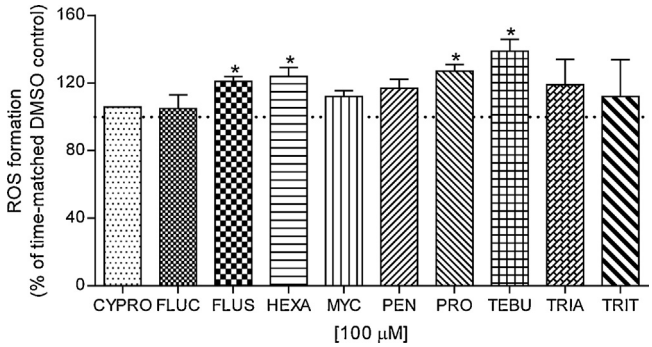


Fig. 3. ROS production. Reactive oxygen species (ROS) formation by MA-10 cells after a 48-h exposure to each of the ten selected conazoles (100 μM). Data are represented as means ± SEM with $N = 3$ and $n = 4$. Significance was assessed using a Student's *t*-test. * Significantly different from DMSO control-treated cells ($P < 0.05$). Dotted line indicates the reference level of the DMSO control.

Cyp51 (1.7-fold) and HMG-CoA red (1.9-fold) (Fig. 2B). Exposure to TEBU slightly decreased the expression of the Por gene (0.8-fold) (Fig. 2B). Other exposures did not change expression of the three cholesterol biosynthesis genes assessed (Fig. 2B).

3.4. Reactive oxygen species production

To further explore the nature of T secretion inhibition by MA-10 cells after exposure to certain conazole fungicides, we considered ROS formation as a possible cause for deterioration of Leydig cell function resulting in decreased T secretion. MA-10 cells were exposed to non-cytotoxic concentrations (10 nM–100 μM) of the tested compounds. The control, rotenone, showed a ROS production of 158 ± 18% of the control at 48 h, indicating that the cells were able to produce ROS. Only ROS levels in MA-10 cells exposed for 48 h to the highest concentration (100 μM) of FLUS, HEXA, PRO, and TEBU were statistically significantly increased compared to time-matched DMSO-treated control cells (Fig. 3). Increase in ROS formation was 1.2, 1.2, 1.3, and 1.4-fold compared with vehicle-treated control cells by FLUS, HEXA, PRO, and TEBU, respectively. The other tested conazoles (CYPRO, FLUC, MYC, PEN, TRIA, and TRIT) did not significantly change ROS production compared to vehicle-treated cells (Fig. 3). At lower concentrations (<100 μM) or at earlier time points (1 and 24 h) no differences in ROS formation between vehicle-treated and conazole-treated cells were observed (data not shown).

3.5. Inhibition of androgen receptor activation

Because AR activation is a prerequisite for proper spermatogenesis, possible effects of conazole exposure on AR activation were determined using an AR reporter gene assay. T47D-ARE cells were exposed to non-cytotoxic concentrations (10 pM to 100 μM) of the tested compounds. Testosterone (T) activated the AR in a concentration-dependent manner with an EC_{50} of 13.6 nM (Fig. 4A and Table 2). Exposure to conazoles alone did not significantly affect AR activation (data not shown). Next, cells were exposed to 20 nM T in combination with concentration ranges of the selected conazoles (10 pM to 100 μM) or the AR antagonist flutamide (FLUT; 10 nM to 100 μM). FLUT concentration-dependently decreased AR activation with an IC_{50} value of 7.0 μM (Fig. 4B and Table 2). All conazoles tested, except for FLUC, concentration-dependently inhibited T-induced AR activation with IC_{50}s ranging from 10.7 to 71.5 μM (Fig. 4C–L and Table 2). All of the tested compounds inhibited T-induced AR activation by maximally 82%.

Table 2
Androgen receptor reporter gene assays outcome expressed as half maximal effective or inhibitory concentrations (EC/IC_{50} [M]), benchmark response relative to 25% response of flutamide ($BMR_{25\%FLUT}$ [M]), and the relative effect potency (REP) as compared to flutamide of the selected conazoles. n.a. = not applicable.

Compound	EC/IC_{50} [M]	$BMR_{25\%FLUT}$ [M]	REP
T	1.36E−08	n.a.	n.a.
FLUT	7.02E−06	1.98E−06	1.00
CYPRO	1.36E−05	5.25E−06	0.38
FLUC	n.a.	n.a.	n.a.
FLUS	1.19E−05	5.49E−07	3.61
HEXA	2.32E−05	7.64E−06	0.26
MYC	7.15E−05	7.06E−05	0.03
PEN	1.71E−05	5.54E−06	0.36
PRO	1.17E−05	9.43E−06	0.21
TEBU	2.55E−05	9.01E−06	0.22
TRIA	3.21E−05	1.60E−05	0.12
TRIT	1.07E−05	7.80E−06	0.25

In order to compare the potencies of the tested conazoles to inhibit AR activation, relative effect potencies (REPs) were calculated using the concentrations where inhibition of AR activation was similar to 25% inhibition by FLUT ($BMR_{25\%FLUT}$).

This leads to the following potency ranking based on the REP: FLUS > FLUT > CYPRO > PEN > HEXA > TRIT > TEBU > PRO > TRIA > MYC (Table 2).

4. Discussion

We show here that six of the ten tested conazole fungicides cause a decrease in basal T secretion in murine MA-10 Leydig cells. In addition, we demonstrated for two selected conazoles a concentration-dependent inhibition of LH-stimulated T secretion. These effects cannot be adequately explained by changes in steroidogenic and cholesterol biosynthesis gene expression nor by increased ROS

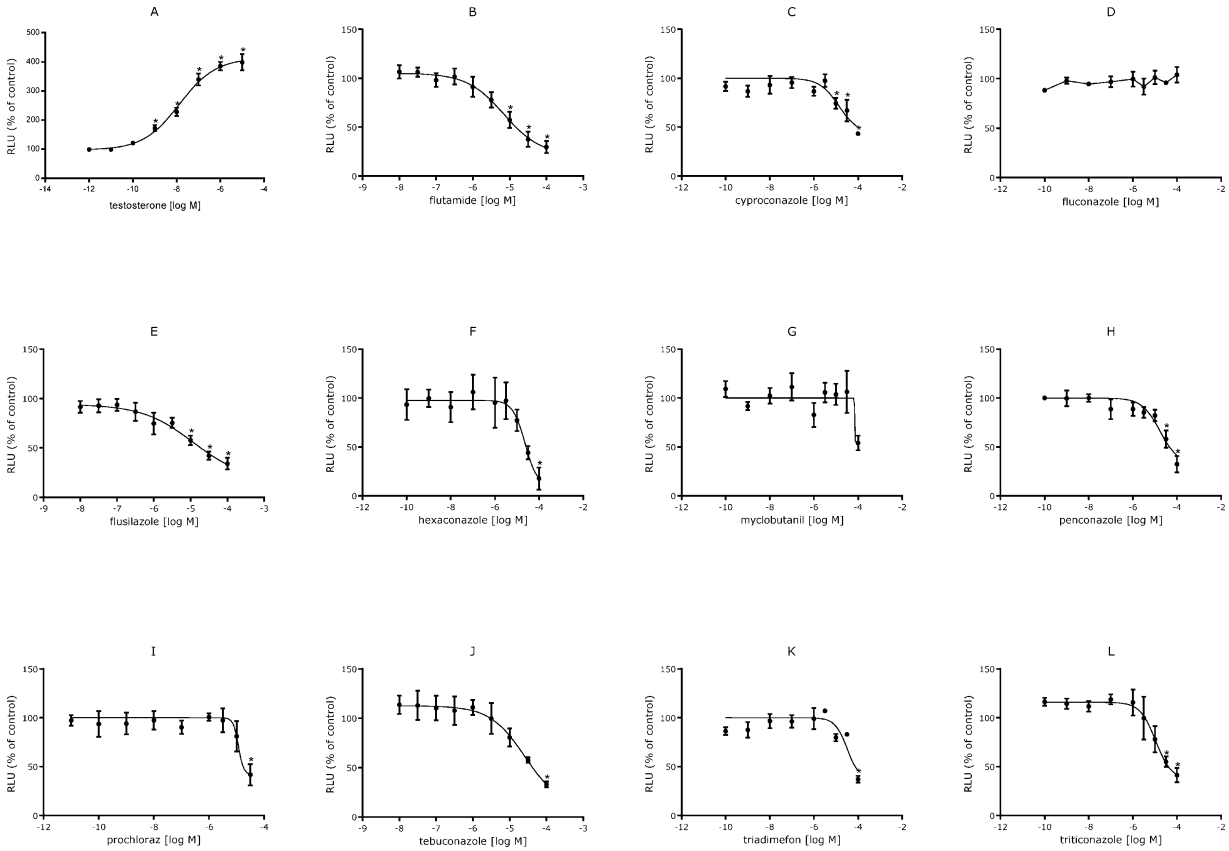


Fig. 4. AR response. AR response measured as relative luminescence units (RLU) as compared to vehicle-treated cells by T47D-ARE cells after a 24-h exposure to a concentration curve of (A) testosterone (1 pM to 10 μM), (B) flutamide (10 nM to 100 μM) or (C–L) each of the ten selected conazoles (100 pM to μM) in combination with the EC_{50} of testosterone (20 nM). Data are represented as means ± SEM with $N = 3$ and $n = 3$. Significance was assessed using a one-way ANOVA test followed by Dunnett's *post hoc* test. * Significantly different from vehicle-treated cells ($P < 0.05$).

production. Further, nine of the ten tested conazoles inhibited T-induced AR activation in a reporter gene assay. These data show that some conazoles can act as anti-androgens *via* two modes of action. Anti-androgenic effects have been shown to cause adverse effects in both the adult as well as the developing male. Without adequate steroidogenesis leading to sufficient T secretion or by blockage of the AR response, spermatogenesis cannot be properly realized, resulting in abnormal or even absent sperm production and consequently sub- and/or infertility of the adult male. In the fetus, proper T production and responsiveness are especially important during the masculinization programming window to ensure correct development into a phenotypical male [32].

In our study, five of the tested conazoles, *i.e.* FLUS, HEXA, PRO, TEBU, and TRIT, decreased basal T secretion by MA-10 cells by more than 50% compared with vehicle-treated control cells (Fig. 1A). FLUS and TEBU also concentration-dependently inhibited T secretion by MA-10 cells stimulated with LH (Fig. 1B; $REP_{SU10603} = 0.04$ and 0.21, respectively), indicating that these compounds can act as (*in vitro*) T secretion inhibitors.

To mechanistically investigate the inhibition of T secretion by conazoles, we assessed gene expression of several enzymes involved in the "late" steroidogenic and cholesterol biosynthesis pathways. However, the conazoles only inflicted minor effects on expression levels of the selected genes (Fig. 2), which cannot explain the inhibitory effects of these conazoles on T secretion in MA-10 Leydig cells. Makker et al. proposed a role for oxidative stress in the occurrence of male infertility [33]. However, levels of ROS resulting from exposure to conazoles were only moderately increased in our study (Fig. 3). Therefore, changes in ROS formation were also most likely not the cause of the decreased T secretion by MA-10 Leydig cells by the conazoles used in this study.

Our results are in accordance with the suggestion from earlier studies that conazoles show catalytic inhibition in the steroidogenic pathway [10,19,21]. Previous studies indicate that conazoles have the potential to inhibit mammalian CYP51 enzyme activity *via* catalytic inhibition [9,11]. CYP51 is highly expressed in the testis and has an important role in the cholesterol biosynthesis and ultimately in testosterone production [34,35]. T levels could also be affected by conazoles *via* catalytic inhibition of other steroidogenic cytochrome P450 enzymes. Several studies have suggested that conazoles can target cytochrome P450 enzymes, *e.g.* imidazoles also inhibit cytochrome P450 enzymes in human liver, thereby inhibiting hepatic T metabolism and lowering circulating levels,

and lymphoblast cells [20,36,37], This illustrates that conazoles are able to affect the function of multiple CYP enzymes, also the ones not specifically within the steroidogenic pathway, indicating that this type of enzymes could be one of the main targets for endocrine disruption by conazoles. We have previously shown that FLUS, ketoconazole (an imidazole), and TEBU inhibit CYP17 activity in porcine adrenal cortex microsomes [20]. Furthermore, rat studies showed that hepatic cytochrome P450 activity is inhibited *in vivo* by FLUC and ketoconazole [36]. Previously, Kjaerstad et al. stated that the imidazoles are more potent inhibitors of T secretion by H295R cells than the triazoles [10]. This phenomenon was also seen for decreased T levels in fetal rat testes, where PRO showed higher potency than TEBU [38]. In contrast, our study did not show a stronger inhibition of T secretion *in vitro* by the imidazole PRO compared to the triazole TEBU in murine MA-10 cells (Fig. 1A). In fact, TEBU is a quite potent inhibitor of T secretion in comparison with the CYP17 inhibitor SU10603 ($REP_{SU10603} = 0.21$; Fig. 1B). Steroidogenesis in adrenal tissue occurs predominantly *via* DHEA whereas in the testes the main route for steroidogenesis includes production of androstenedione [39]. Since H295R cells are fetal-like adrenal cells and MA-10 cells are of testicular origin this might explain the differences in magnitude of response for conazoles to affect T secretion found between both cell systems. The discordance seen between our *in vitro* results and the *in vivo* results might be explained by differential effects on aromatase (*Cyp19*) enzyme activity in the two systems used and a species difference of mouse Leydig cells *versus* rat fetal testes. It has been shown that MA-10 cells do not express the Cyp19 gene and estradiol does not repress Cyp17 and 3βHSD gene expression [40]. In primary cultures of Leydig cells from C57BL/6j mice a significant upregulation of Cyp19 gene expression but coordinated suppression of the LHR, StAR, 3βHSD, and Cyp17A1 genes was found, which was associated with attenuated androgen production compared to CBA/Lac mice [41]. Further, estradiol has been found to interfere with *in vivo* Leydig cell function in the rat, thereby lowering CYP17 activity leading to reduced T biosynthesis [42]. *In vitro* assays can be effectively used to study the effect of chemicals on a certain specific mechanism. The shortcoming of these assays is that they do not involve an intact organism, therefore lacking certain physiological feedback mechanisms within the body. A holistic evaluation of data from a panel of cell-based assays has shown to give a better prediction for the ranking of conazoles fungicides for *in vivo* toxicity data [38]. In addition, it has been shown previously that exposure to endocrine disruptors exerts differential effects on steroidogenesis in human, mouse, and rat testes, raising concern about the use of rodent models and extrapolation of results for human risk assessment [43]. Possibly, the species-difference in testicular responsiveness plays a role in the different potencies of prochloraz and TEBU in the rat developmental *in vivo* model described by Dreisig et al. and our *in vitro* mouse MA-10 study [38].

None of the conazoles showed AR agonistic activity but nine out of ten selected conazoles inhibited T-induced AR activation concentration-dependently in our reporter gene assay (Fig. 4). The AR antagonistic activity of PRO and TEBU is in agreement with a previous study that used AR-transfected Chinese hamster ovary cells (CHO) [10]. In our present study with conazoles, FLUS is the most potent AR antagonist with an even higher potency (REP = 3.61) than FLUT (REP = 1.00), a well-studied pharmacological AR antagonist. All of the selected conazoles contain at least one hexacyclic moiety (Table 1), which may have a function comparable to the hexacyclic moiety of T, and thus may cause a competitive receptor binding between these fungicides and androgens [44]. Comparison of the chemical structures of these selected conazoles (Table 1) shows that FLUS is the only conazole containing an additional hexacyclic moiety. Possibly, this extra moiety plays a role in the AR antagonistic properties of FLUS. Further studies on receptor–ligand kinetics are needed to determine the nature of these antagonistic interactions.

Besides environmental exposure, individuals can also be exposed to conazoles *via* pharmaceutical application. FLUC has a pharmacotherapeutic application and treated patients showed serum levels ranging from 16.3 to 25.8 μM after oral administration of 200 mg FLUC per day [45]. It should be noted that these blood levels are higher than the maximum medium concentration of 10 μM that caused an inhibition of T secretion by MA-10 cells in this study. Moreover, the fast uptake rate with a T_{max} of approximately 2 h together with the plasma half-life of approximately 30 h in humans suggests that significant internal exposure to conazoles can occur, possibly even resulting in accumulation of these compounds leaving more opportunity for causing (adverse) effects, *e.g.* inhibition of CYP enzymes [46].

In addition, because different crops are treated with different fungicides and conazole mixtures are commercially available, the possibility exists that people may be frequently exposed to several conazoles simultaneously. Mixtures of individual endocrine active compounds, including conazoles, have been shown to cause additive effects and antagonism has also been observed [47]. Also combinations of low doses of multiple conazoles have been shown to cause additive effects [48]. An earlier study by Kjaerstad et al. showed additivity of a mixture containing two triazoles (propiconazole and TEBU) and one imidazole (epoxiconazole) on AR antagonism in AR-transfected CHO cells as well as T synthesis in H295R cells [49]. Likewise, the triazole propiconazole in combination with other pesticides showed additive AR activity antagonism [50]. Hence, low effect concentrations of conazoles, to which humans are most likely frequently exposed and in mixtures, may potentially pose a risk for endocrine disruptive effects. Unfortunately, a proper risk assessment is hampered by the lack of (systemic) human exposure data.

5. Conclusion

In summary, this *in vitro* study shows clear anti-androgenic effects of several conazole fungicides. These anti-androgenic effects suggest that potential testicular toxicity can arise from two mechanisms: inhibition of T secretion and AR antagonism. In view of the dual anti-androgenic effects of the conazoles described here, further

studies on the male reprotoxic effects of conazole fungicides in combination with accurate exposure data are highly recommended.

Transparency document

The Transparency document associated with this article can be found in the online version.

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References

[1] A.M. Andersson, N. Jorgensen, K.M. Main, J. Toppari, E. Rajpert-De Meyts, H. Leffers, et al., Adverse trends in male reproductive health: we may have reached a crucial 'tipping point', Int. J. Androl. 31 (2008) 74–80.
[2] N. Jorgensen, U.N. Joensen, T.K. Jensen, M.B. Jensen, K. Almstrup, I.A. Olesen, et al., Human semen quality in the new millennium: a prospective cross-sectional population-based study of 4867 men, BMJ Open 2 (2012), http://dx.doi.org/10.1136/bmjopen-2012-000990 (Print 2012).
[3] T.G. Travison, A.B. Araujo, A.B. O'Donnell, V. Kupelian, J.B. McKinlay, A population-level decline in serum testosterone levels in American men, J. Clin. Endocrinol. Metab. 92 (2007) 196–202.
[4] A. Nyboe Andersen, K. Erb, Register data on Assisted Reproductive Technology (ART) in Europe including a detailed description of ART in Denmark, Int. J. Androl. 29 (2006) 12–16.
[5] I.D. Sharlip, J.P. Jarow, A.M. Belker, L.I. Lipshultz, M. Sigman, A.J. Thomas, et al., Best practice policies for male infertility, Fertil. Steril. 77 (2002) 873–882.
[6] J. Jurewicz, W. Hanke, M. Radwan, J.P. Bonde, Environmental factors and semen quality, Int. J. Occup. Med. Environ. Health 22 (2009) 305–329.
[7] E.W. Wong, C.Y. Cheng, Impacts of environmental toxicants on male reproductive dysfunction, Trends Pharmacol. Sci. 32 (2011) 290–299.
[8] N. Roeleveld, R. Bretveld, The impact of pesticides on male fertility, Curr. Opin. Obstet. Gynecol. 20 (2008) 229–233.
[9] J.A. Zarn, B.J. Bruschweiler, J.R. Schlatter, Azole fungicides affect mammalian steroidogenesis by inhibiting sterol 14 alpha-demethylase and aromatase, Environ. Health Perspect. 111 (2003) 255–261.
[10] M.B. Kjaerstad, C. Taxvig, C. Nellemann, A.M. Vinggaard, H.R. Andersen, Endocrine disrupting effects in vitro of conazole antifungals used as pesticides and pharmaceuticals, Reprod. Toxicol. 30 (2010) 573–582.
[11] N. Strushkevich, S.A. Usanov, H.W. Park, Structural basis of human CYP51 inhibition by antifungal azoles, J. Mol. Biol. 397 (2010) 1067–1078.
[12] S.A. Hermsen, T.E. Pronk, E.J. van den Brandhof, L.T. van der Ven, A.H. Piersma, Triazole-induced gene expression changes in the zebrafish embryo, Reprod. Toxicol. 34 (2012) 216–224.
[13] C. Taxvig, A.M. Vinggaard, U. Hass, M. Axelstad, S. Metzdorff, C. Nellemann, Endocrine-disrupting properties in vivo of widely used azole fungicides, Int. J. Androl. 31 (2008) 170–177.
[14] S.T. Page, Physiologic role and regulation of intratesticular sex steroids, Curr. Opin. Endocrinol. Diabetes Obes. 18 (2011) 217–223.
[15] R.S. Ge, Q. Dong, C.M. Sottas, V. Papadopoulos, B.R. Zirkin, M.P. Hardy, In search of rat stem Leydig cells: identification, isolation, and lineage-specific development, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 2719–2724.
[16] R.S. Wang, S. Yeh, C.R. Tzeng, C. Chang, Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice, Endocr. Rev. 30 (2009) 119–132.
[17] J. Odum, J. Ashby, Detection of aromatase inhibitors in vitro using rat ovary microsomes, Toxicol. Lett. 129 (2002) 119–122.
[18] J.T. Sanderson, J. Boerma, G.W. Lansbergen, M. van den Berg, Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells, Toxicol. Appl. Pharmacol. 182 (2002) 44–54.
[19] A.M. Vinggaard, C. Hnida, V. Breinholt, J.C. Larsen, Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro, Toxicol. In Vitro 14 (2000) 227–234.
[20] M.J. Roelofs, A.H. Piersma, M. van den Berg, M.B. van Duursen, The relevance of chemical interactions with CYP17 enzyme activity: assessment using a novel in vitro assay, Toxicol. Appl. Pharmacol. 268 (2013) 309–317.
[21] P. Laier, S.B. Metzdorff, J. Borch, M.L. Hagen, U. Hass, S. Christiansen, et al., Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz, Toxicol. Appl. Pharmacol. 213 (2006) 160–171.
[22] M. Kahle, I.J. Buerge, A. Hauser, M.D. Muller, T. Poiger, Azole fungicides: occurrence and fate in wastewater and surface waters, Environ. Sci. Technol. 42 (2008) 7193–7200.
[23] Tebuconazole risk assessment reports, US Environmental Protection Agency. http://www.epa.gov/ (accessed 22.04.14).
[24] Pesticides Usages Statistics, Department for Environment, Food & Rural Affairs, UK. https://secure.fera.defra.gov.uk/pusstats/ (accessed 12.11.13).
[25] G. Lavranos, M. Balla, A. Tzortzopoulou, V. Syriou, R. Angelopoulou, Investigating ROS sources in male infertility: a common end for numerous pathways, Reprod. Toxicol. 34 (2012) 298–307.
[26] M. Ascoli, Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses, Endocrinology 108 (1981) 88–95.
[27] A.C. Dankers, M.J. Roelofs, A.H. Piersma, F.C. Sweep, F.G. Russel, M. van den Berg, et al., Endocrine disruptors differentially target ATP-binding cassette transporters in the blood-testis barrier and affect Leydig cell testosterone secretion in vitro, Toxicol. Sci. (2013).
[28] L.B. LaCagnin, M. Levitt, J.M. Bergstrom, H.D. Colby, Inhibition of adrenocortical, mitochondrial and microsomal monooxygenases by SU-10'603, a steroid 17 alpha-hydroxylase inhibitor, J. Steroid Biochem. 33 (1989) 599–604.
[29] K.C. Ahn, B. Zhao, J. Chen, G. Cherednichenko, E. Sanmarti, M.S. Denison, et al., In vitro biologic activities of the antimicrobials triclocarban, its analogs, and triclosan in bioassay screens: receptor-based bioassay screens, Environ. Health Perspect. 116 (2008) 1203–1210.
[30] J. Simard, I. Luthy, J. Guay, A. Belanger, F. Labrie, Characteristics of interaction of the antiandrogen flutamide with the androgen receptor in various target tissues, Mol. Cell. Endocrinol. 44 (1986) 261–270.
[31] F. Denizot, R. Lang, Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability, J. Immunol. Methods 89 (1986) 271–277.
[32] H.M. Scott, J.I. Mason, R.M. Sharpe, Steroidogenesis in the fetal testis and its susceptibility to disruption by exogenous compounds, Endocr. Rev. 30 (2009) 883–925.
[33] K. Makker, A. Agarwal, R. Sharma, Oxidative stress & male infertility, Indian J. Med. Res. 129 (2009) 357–367.
[34] T. Budefeld, D. Jezek, D. Rozman, G. Majdic, Initiation of steroidogenesis precedes expression of cholesterologenic enzymes in the fetal mouse testes, Anat. Histol. Embryol. 38 (2009) 461–466.
[35] N. Debeljak, M. Fink, D. Rozman, Many facets of mammalian lanosterol 14alpha-demethylase from the evolutionarily conserved cytochrome P450 family CYP51, Arch. Biochem. Biophys. 409 (2003) 159–171.
[36] C.M. Ervine, D.E. Matthew, B. Brennan, J.B. Houston, Comparison of ketoconazole and fluconazole as cytochrome P450 inhibitors. Use of steady-state infusion approach to achieve plasma concentration–response relationships, Drug Metab. Dispos. 24 (1996) 211–215.
[37] W. Zhang, Y. Ramamoorthy, T. Kilicarslan, H. Nolte, R.F. Tyndale, E.M. Sellers, Inhibition of cytochromes P450 by antifungal imidazole derivatives, Drug Metab. Dispos. 30 (2002) 314–318.
[38] K. Dreisig, C. Taxvig, M. Birkhoj Kjaerstad, C. Nellemann, U. Hass, A.M. Vinggaard, Predictive value of cell assays for developmental toxicity and embryotoxicity of conazole fungicides, ALTEX 30 (2013) 319–330.
[39] J.T. Sanderson, The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals, Toxicol. Sci. 94 (2006) 3–21.
[40] A.H. Payne, G.L. Youngblood, L. Sha, M. Burgos-Trinidad, S.H. Hammond, Hormonal regulation of steroidogenic enzyme gene

expression in Leydig cells, J. Steroid Biochem. Mol. Biol. 43 (1992) 895–906.
[41] I. Savchuk, O. Soder, K. Svechnikov, Mouse Leydig cells with different androgen production potential are resistant to estrogenic stimuli but responsive to bisphenol a which attenuates testosterone metabolism, PLOS ONE 8 (2013) e71722.
[42] N.R. Kalla, B.C. Nisula, R. Menard, D.L. Loriaux, The effect of estradiol on testicular testosterone biosynthesis, Endocrinology 106 (1980) 35–39.
[43] K.J. Johnson, N.E. Heger, K. Boekelheide, Of mice and men (and rats): phthalate-induced fetal testis endocrine disruption is species-dependent, Toxicol. Sci. 129 (2012) 235–248.
[44] N.M. DeVore, E.E. Scott, Structures of cytochrome P450 17A1 with prostate cancer drugs abiraterone and TOK-001, Nature 482 (2012) 116–119.
[45] M. Kami, Y. Sawada, S. Mori, J. Hirate, N. Kojima, Y. Kanda, et al., Serum levels of fluconazole in patients after cytotoxic chemotherapy for hematological malignancy, Am. J. Hematol. 66 (2001) 85–91.
[46] K.W. Brammer, P.R. Farrow, J.K. Faulkner, Pharmacokinetics and tissue penetration of fluconazole in humans, Rev. Infect. Dis. 12 (Suppl. 3) (1990) S318–S326.
[47] A. Kortenkamp, Low dose mixture effects of endocrine disrupters: implications for risk assessment and epidemiology, Int. J. Androl. 31 (2008) 233–240.
[48] H.J. Heusinkveld, J. Molendijk, M. van den Berg, R.H. Westerink, Azole fungicides disturb intracellular Ca^{2+} in an additive manner in dopaminergic PC12 cells, Toxicol. Sci. 134 (2013) 374–381.
[49] M.B. Kjaerstad, C. Taxvig, H.R. Andersen, C. Nellemann, Mixture effects of endocrine disrupting compounds in vitro, Int. J. Androl. 33 (2010) 425–433.
[50] L.S. Kjeldsen, M. Ghisari, E.C. Bonefeld-Jorgensen, Currently used pesticides and their mixtures affect the function of sex hormone receptors and aromatase enzyme activity, Toxicol. Appl. Pharmacol. 272 (2013) 453–464.