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Predictive Value of Cell Assays for Developmental Toxicity and Embryotoxicity of Conazole Fungicides

Karin Dreisig, Camilla Taxvig, Mia Birkhøj Kjaerstad, Christine Nellemann, Ulla Hass, and Anne Marie Vinggaard

National Food Institute, Technical University of Denmark, Søborg, Denmark

Summary

This paper evaluates *in vivo* predictability of a battery of *in vitro* tests covering developmental toxicity and embryotoxicity of five widely used conazole fungicides. The conazoles were investigated in the embryonic stem cell test, and data were compared to *in vivo* embryotoxicity data. The same conazoles were evaluated on the basis of data from a battery of cell assays for endocrine activity, including assays for AR, ER, AhR, and sex hormone synthesis, and data were compared to *in vivo* developmental toxicity data. Overall, the ranking of the five conazole fungicides based on *in vitro* data were in reasonably good agreement with available *in vivo* effects. Ketoconazole and epoxiconazole are the most potent embryotoxic compounds, whereas prochloraz belongs to the most potent developmental toxicants. In conclusion, a rough prediction of the ranking of these conazole fungicides for *in vivo* toxicity data was possible by a holistic evaluation of data from a panel of cell-based assays.

Keywords: conazole fungicide, developmental toxicity, embryotoxicity

1 Introduction

Toxicology faces a great challenge as humans are exposed to large numbers of chemicals for which we lack toxicological information. The lack of toxicity data calls for new approaches. Some see the future in the strength of *in vitro* and *in silico* methodologies based on human material in which pathways of toxicity can be elucidated and evaluated by a holistic approach. Apart from convincing scientific visions and arguments, a variety of other good reasons exist for promoting the development of cell-based alternatives to animal testing. These include important political, ethical, as well as economic reasons. In 2013 the final ban on using animals to test ingredients for cosmetics in the EU came into force although alternative methods are still lacking for various endpoints. REACH – the EU legislation on chemicals – requires comprehensive testing of many “old” substances. Performing this testing in animals would be time-consuming and costly and therefore alternative methods are needed. Pharmaceutical companies have a great interest to develop early, predictive toxicity tests for termination of problematic drug candidates early on.

Good case studies are needed to assess the predictability and value of *in vitro* tests. We have studied conazole fungicides *in vitro* and *in vivo* for a number of years and have compiled the data for the purpose of retrospectively evaluating the predictive potential of *in vitro* tests in terms of developmental toxicity and

embryotoxicity. In this paper we have assessed the embryotoxic potential *in vitro* of five conazole fungicides and compared these and other *in vitro* results to our own and other published data from *in vivo* developmental animal studies.

Imidazoles and triazoles currently are used worldwide as fungicides for grain, vegetables, fruit, and flower production and as pharmaceuticals for treatment of human mycoses (Kjaerstad et al., 2010b). Their pharmacological action is based on the binding and inhibition of the enzyme lanosterol-14- α -demethylase (CYP51) that converts lanosterol to ergosterol in fungal cell membranes (Giavini and Menegola, 2010). However, CYP inhibitory ability of these compounds is not limited to this enzyme or to fungi. Some conazole fungicides have been shown to influence the activity of other members of the CYP450 family, including key enzymes involved in the biosynthesis and metabolism of steroids in humans and animals. Two examples are 17 α -hydroxylase/17,20-lyase (CYP17) and aromatase enzyme (CYP19) that are responsible for the conversion of pregnenolone and progesterone to androgens and the conversion of androgens to estrogens, respectively (Kjaerstad et al., 2010a; Taxvig et al., 2007; Vinggaard et al., 2000).

Beside interference with the CYP450 monooxygenases, conazole fungicides have been found to antagonize steroid hormone receptors such as the androgen (AR) and estrogen receptor (ER), along with an ability to activate the aryl hydrocarbon receptor (AhR) in cell assays (Vinggaard et al., 2000; Andersen

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et al., 2002; Kjaerstad et al., 2007). Additionally, epoxiconazole and propiconazole gave rise to a weak estrogenic response in the MCF-7 cell proliferation assay (Kjaerstad et al., 2007). Taken together, this indicates that the conazoles might have multiple modes of action in regard to affecting reproductive development. Evidence for their adverse effects on development has been presented by rodent studies. Prochloraz, e.g., caused feminization and sexual malformations in male offspring after gestational and lactational exposure (Blystone et al., 2007; Vinggaard et al., 2005). Despite these known adverse effects, prochloraz was re-approved by the EU Commission in late 2011 after a short withdrawal, and it currently is approved for a wide variety of crops in the European Union (Muilerman et al., 2012).

Beside their effect on reproductive development, conazoles, including ketoconazole, epoxiconazole, and tebuconazole, have shown another toxicological potential in animal studies as they cause, e.g., increased embryoletality, cleft palate, reduced fetal weight, and axial skeleton malformations (Giavini and Menegola, 2010; Marotta and Tiboni, 2010). Human epidemiological studies on various azole antifungals found no associations between exposure to either triazole or imidazole substances during pregnancy (Marotta and Tiboni, 2010). However, Marotta and Tiboni (2010) state that these findings cannot be regarded as conclusive, given the methodological problems and limitations of the majority of the available human studies. Consequently, several triazoles and imidazoles, e.g., ketoconazole used for treatment of fungal infections were classified by the U.S. Food and Drug Administration, according to the risk in pregnancy, as category C (teratogenic or embryotoxic in animal studies without available reports or studies on humans). For this reason, the conazoles, used as drugs for systemic therapy against deep-seated infections, extensive cutaneous diseases, and/or hair or nail mycosis are not recommended during the first trimester or during the pregnancy period in general, depending on the type of conazole fungicide (Menegola et al., 2006).

The cause of the teratogenic effect has been suggested to be another important group of enzymes believed to be inhibited by the conazoles, i.e., the CYP26 family. This family plays a crucial role in controlling endogenous retinoic acid (RA) levels in embryonic tissue. Imbalance in RA metabolism caused by triazoles has been demonstrated both *in vitro* and *in vivo* (Marotta and Tiboni, 2010). An increased level of endogenous RA was the suggested mechanism behind altered craniofacial morphogenesis such as cleft palate and reduced ossification of skull bones after *in utero* exposure to ketoconazole (Amaral and Nunes, 2008). Another possible mechanism for the developmental toxicity of conazole compounds might be via blocking of cardiac potassium channels. Chemicals blocking these channels cause malformations by a common embryonic cardiac arrhythmia/hypoxia mechanism (Danielsson et al., 2007). Recently, ketoconazole and other conazole compounds have been found to strongly inhibit cardiac potassium channels in neonatal rat ventricular myocytes (Sung et al., 2012) suggesting an alternative mode of action for the teratogenic effects of conazoles.

Overall, literature provides relatively comprehensive information on the *in vivo* effects of conazole fungicides in rodents.

In addition, there is fairly extensive *in vitro* information on the potential endocrine activity of these compounds. However, knowledge of embryotoxicity *in vitro* was lacking in order to perform a holistic evaluation of the predictability of *in vitro* assays for embryotoxicity as well as for developmental toxicity. Hence, in this paper we first investigated the embryotoxic potential of five conazole fungicides, i.e., epoxiconazole, ketoconazole, prochloraz, propiconazole, and tebuconazole *in vitro* using the embryonic stem cell test (EST). This assay currently is a promising approach to assess the embryotoxic potency of chemicals *in vitro* (Adler et al., 2008). It was developed to reduce animal use and represents an alternative test method for hazard assessment (Riebeling et al., 2011). A scientific validation by the European Center for the Validation of Alternative Methods (ECVAM) demonstrated that this test may be a reliable alternative or supplementary method to animal experiments testing for embryotoxicity. However, there is a need for further testing and evaluation of more chemicals by this method before it can be considered for regulatory acceptance and application (Genschow et al., 2002, 2004; Kuske et al., 2012; Marx-Stoelting et al., 2009). We gathered the available *in vitro* and *in vivo* information from literature with the aim of elucidating, retrospectively, whether we could have predicted developmental *in vivo* outcomes in animals from the selected battery of *in vitro* tests. Such information is important to learn from, as a future target is to design a panel of *in vitro* tests that can be used for predicting human toxic effects.

2 Materials and methods

Principle of EST

The principle and establishment of the EST has been described previously (Seiler et al., 2006). Briefly, two stable mouse cell lines were used: 1) an embryonic stem (ES) cell clone D3 to represent undifferentiated embryonic tissue and 2) 3T3 fibroblasts to represent differentiated adult tissue. Suspension cultures of stem cells that spontaneously differentiate and form multicellular aggregates are referred to as embryonic bodies (EBs). Plating of the EBs allowed further differentiation and outgrowth, and the morphological assessment of contracting cardiomyocytes was used as an endpoint for differentiation (Seiler et al., 2006; Seiler and Spielmann, 2011). In order to predict the *in vivo* embryotoxic potential of a test compound a biostatistical model was applied (Seiler et al., 2006).

Chemicals

The following test compounds – all from Dr Ehrendorfer – were used: epoxiconazole 99% pure (CAS 106325-08-0, cat. EHRC 13185000), ketoconazole 99% pure (CAS 65277-42-1, cat. EHRC14532000), prochloraz 99.6% pure (CAS 67747-09-5, cat. W16290000), propiconazole 97.5% pure (CAS 60207-90-1, cat. EHRC16480000), and tebuconazole 98% pure (CAS 107534-96-3, cat. EHRC17178700). All test compounds were dissolved in dimethylsulfoxide (DMSO, Sigma, cat. D4540). Positive control was 5-fluorouracil (5-FU, CAS 51-21-8, Sigma, cat. F-6627) in DMSO. Negative control was penicillin G



(PenG, CAS 69-57-8, Sigma, cat. A2058) in water. Cell medium solutions were constantly shaken during addition to plates in order to avoid precipitation.

Cell culture

Undifferentiated murine ES cells clone D3 (ATCC, cat. CRL-1934) were cultured in humidified atmosphere at 37°C and 5% CO₂ in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco, cat. 41965-039) containing 20% heat-inactivated fetal calf serum (FCS, Hyclone, cat. A-1111-L), antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin, Invitrogen, cat. 15070-063), 1% non-essential amino acids (NEAA, Gibco, cat. 11140-035), 2 mM L-glutamine (Gibco, cat. 25030-024), 0.1 mM β-mercaptoethanol (Sigma, cat. M7522). The cells were maintained in the presence of murine leukemia inhibitory factor (LIF, Sigma, cat. L5158-5UG) to inhibit spontaneous differentiation.

Balb/c 3T3 fibroblasts of type A31 (ATCC, cat. CCL-163) were cultured under similar conditions in high-glucose DMEM with 50 U/ml penicillin, 50 µg/ml streptomycin, 10% heat-inactivated FCS, and 4 mM L-glutamine.

EST differentiation assay

The scientifically validated EST assay has been described in detail (Seiler et al., 2006). Briefly, suspensions of D3 cells in supplemented DMEM without LIF (3.75 × 10⁴ cells/ml) were prepared for each concentration of the test chemical. 20 µl cell suspension was placed as hanging drops on the lid of a bacterial Petri dish filled with 5 ml phosphate buffered saline (PBS, OXOID, cat. BR0014g) and incubated for three days. During this period the cells aggregate to form EBs. After three days the EBs were transferred to a Petri dish with 10 ml medium containing test concentration of each chemical and further cultivated as suspension culture for two days. On day five, separate EBs were plated into a 24-multiwell culture plate (Sarstedt, cat. 83.1835.300) containing 1 ml medium/well with test compound. Differentiation was evaluated by microscopic inspection on day ten by determining the number of contracting cardiomyocytes in each well on each plate and comparing the number to the number of contracting EBs in the solvent control plate. Experiments were considered valid when 21 of 24 wells in the solvent control plate contained spontaneously beating cardiomyocytes. Interference with differentiation was evaluated at six test concentrations for each test compound between 4–128 µM in three independent experiments, except for tebuconazole, which was tested four times.

Cytotoxicity assay

Roughly, the cytotoxicity data is used to assess if stem cells are more sensitive to toxic agents than adult cells by determining the inhibition of growth of D3 and 3T3 cells. In the current study, cytotoxic effects on D3 and 3T3 were determined by applying the resazurin assay. 500 D3 cells/well and 2,000 3T3 cells/well were allowed to attach during incubation for two hours in a black clear-bottomed 96-well plate (Biotech Line, cat. 3603) before adding medium with test compounds, the positive control, 5-FU, or the negative control, PenG. Cells were incubated until

day ten with changes of medium containing test compound on days three and five. On day ten, a resazurin (Sigma, cat. 1770) solution in PBS was added directly to the wells for a final concentration of 2.5 µg resazurin/ml and incubated for three hours. Fluorescent signal was monitored using 560 nm excitation and 590 nm emission wavelength. Cytotoxicity of ketoconazole was tested in the concentration range 1.6–100 µM (n=3) and for propiconazole, epoxiconazole, prochloraz, and tebuconazole in the concentration range 7.8–500 µM (n=4).

Statistics

Three single endpoint values (50% inhibition of cardiac cell differentiation (ID₅₀), 50% viability of D3 cells (IC₅₀D3), and 50% viability of 3T3 cells (IC₅₀3T3)) for differentiation and cytotoxicity were determined from dose-response-curves (x = log(x)) using non-linear “Sigmoidal dose-response (variable slope)” fitted in GraphPad Prism 5. Constraints for bottom were between 0 and 100, and top was constant equal to 100. A biostatistically-based prediction model (PM) that takes the three variables into account was applied to discriminate between three classes of embryotoxicity (non-embryotoxic, weakly embryotoxic, and strongly embryotoxic) as shown below. The PM for the EST has been described previously (Genschow et al., 2002, 2004).

$$\text{Function I: } 5.92 * \log(IC_{50}3T3) + 3.50 * \log(IC_{50}D3) - 5.31 * \left(\frac{IC_{50}3T3 - ID_{50}}{IC_{50}3T3} \right) - 15.7$$

$$\text{Function II: } 3.65 * \log(IC_{50}3T3) + 2.39 * \log(IC_{50}D3) - 2.03 * \left(\frac{IC_{50}3T3 - ID_{50}}{IC_{50}3T3} \right) - 6.85$$

$$\text{Function III: } -0.125 * \log(IC_{50}3T3) - 1.92 * \log(IC_{50}D3) + 1.50 * \left(\frac{IC_{50}3T3 - ID_{50}}{IC_{50}3T3} \right) - 2.67$$

Classification criteria

Class I: Non-embryotoxic if I>II and I>III

Class II: Weakly embryotoxic if II>I and II>III

Class III: Strongly embryotoxic if III>I and III>II

3 Results and discussion

Five conazole fungicides, three triazoles and two imidazoles, were tested for embryotoxic effects in the *in vitro* EST assay. The purpose was to evaluate how well the EST assay predicts the embryotoxic potential of conazole fungicides. The results, therefore, were compared to embryotoxicity in animals. Furthermore, we wanted to compare previously published *in vitro* data for endocrine activity with developmental toxicity outcomes from animal studies for the same fungicides.

EST endpoint values (ID₅₀, IC₅₀D3, and IC₅₀3T3) and their 95% confidence intervals (95% C.I.) were derived from the non-linear regression lines in Figure 1 and shown in Table 1. IC₅₀ values for resazurin reduction were in the range of approximately 10–76 µM and 25–132 µM for D3 and 3T3 cells, respectively, and 50% inhibition of cardiac beating was found in the range of approximately 15–69 µM (Tab. 1). All five conazoles were classified as “weakly embryotoxic” when using the prediction model developed for the EST assay (Genschow et al., 2002, 2004).

The original EST has been expanded using, e.g., transcriptomics in ES cells or metabolomics of pluripotent human ES cells

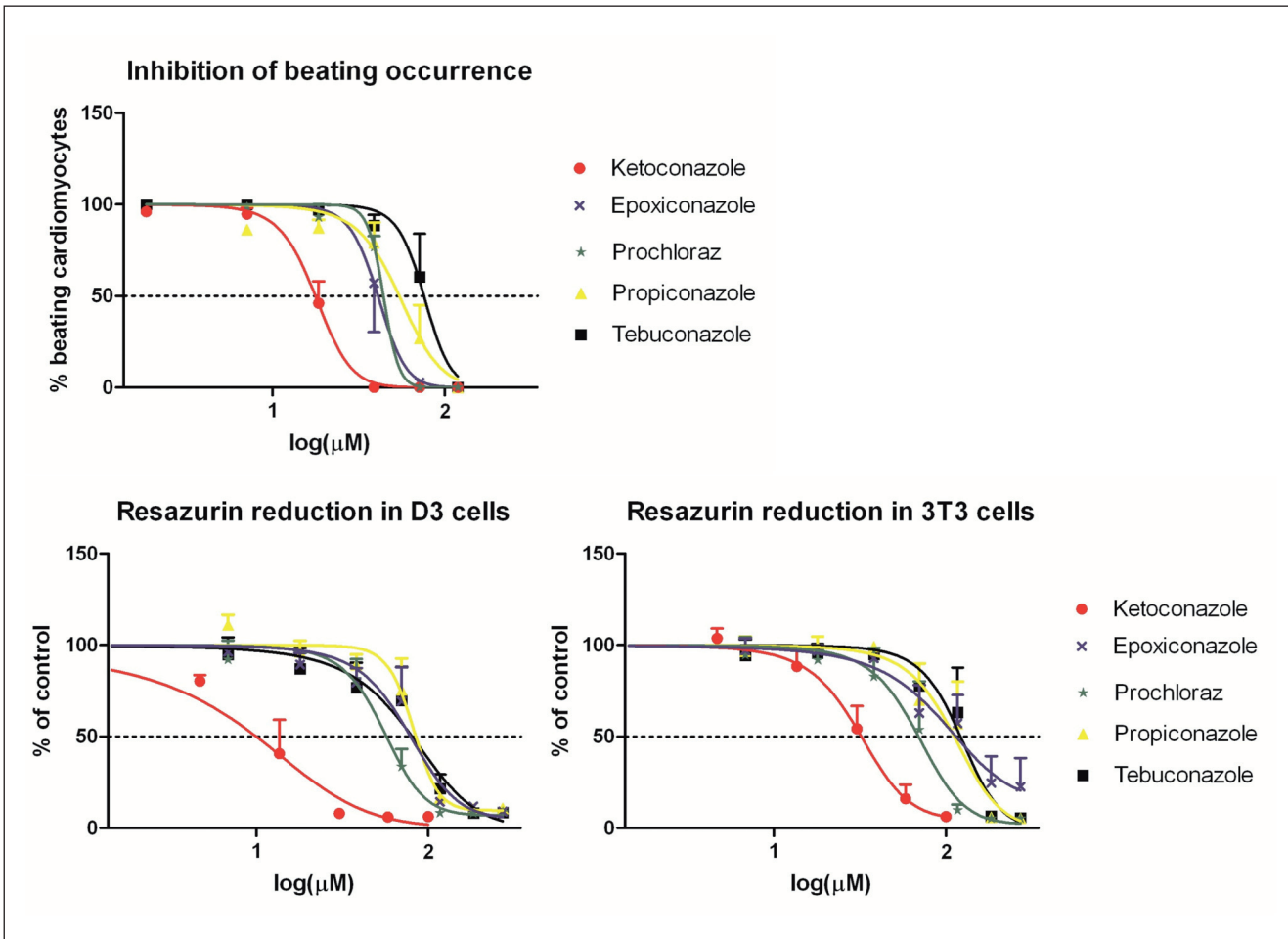


Fig. 1: Dose-response curves for inhibition of beating occurrence of embryonic stem cells and resazurin reduction in D3 and 3T3 cells for five selected conazole fungicides

Beating occurrence was evaluated in three independent experiments (except for tebuconazole, which was tested four times) and the resazurin reduction experiments were conducted four times (except for ketoconazole, which was tested three times). Data represent mean \pm SEM. The horizontal dashed lines represent 50% of control level.

Tab. 1: Embryonic Stem Cell Test endpoints for five conazole compounds

EST endpoints and their 95% confidence intervals (95% C.I.) were derived from the non-linear regressions to the plots in Fig. 1; ^a = ID_{50} and/or IC_{50} values were considered different as their 95% C.I. were not overlapping.

Conazole fungicide	<i>In vitro</i> EST assay endpoints					
	ID_{50} , μ M (95% C.I.)	IC_{50D3} , μ M (95% C.I.)	IC_{503T3} , μ M (95% C.I.)	EST classification	ID_{50}/IC_{50D3} ratio	IC_{50D3}/IC_{503T3} ratio
Epoxiconazole	33.8 (25.6-44.5)	69.4 (51.3-94.0)	97.0 (40.1-234.6)	Weakly embryotoxic	0.5 ^a	0.7
Ketoconazole	15.4 (14.1-16.9)	9.9 (7.0-14.1)	25.6 (20.3-32.2)	Weakly embryotoxic	1.6	0.4 ^a
Prochloraz	36.5 (24.6-54.2)	46.7 (38.7-56.5)	60.1 (42.4-87.2)	Weakly embryotoxic	0.8	0.8
Propiconazole	46.3 (31.8-67.3)	76.1 (62.0-93.4)	116.8 (69.7-195.5)	Weakly embryotoxic	0.6	0.7
Tebuconazole	69.1 (44.4-107.4)	74.7 (44.0-126.6)	131.9 (87.1-199.9)	Weakly embryotoxic	0.9	0.6

to identify embryotoxic compounds (van Dartel et al., 2011; West et al., 2010). Chandler et al. (2011) modified to deliver a high-throughput, sensitive method for quantitatively measuring cardiomyocyte differentiation using primary mouse antibody that recognizes α and β cardiac myosin heavy chain. Two of the present fungicides, i.e., prochloraz and propiconazole, were tested along with other related conazole compounds. Half-maximal activity concentration (AC_{50}) values for cardiomyocyte differentiation were determined to be 12 and 9, respectively. These values are lower than the ID_{50} values found in this study and indicate that expanding the endpoints in the EST might heighten the sensitivity of this assay.

The $ID_{50}/IC_{50}D3$ ratio was used as a measure for specific inhibition of the differentiation process versus cytotoxicity (Stummann et al., 2008). The 95% confidence intervals for the ID_{50} and $IC_{50}D3$ were not substantially different for prochloraz, ketoconazole, tebuconazole, and propiconazole implying no specific interference with cardiac differentiation, i.e., their $ID_{50}/IC_{50}D3$ ratios are not significantly different from 1. In contrast, epoxiconazole had non-overlapping 95% C.I. and an $ID_{50}/IC_{50}D3$ ratio of 0.5 (Tab. 1) indicating that this conazole fungicide may induce specific inhibition of cardiac differentiation.

Similarly, $IC_{50}D3/IC_{50}3T3$ ratios were estimated to compare the D3 cell toxicity versus 3T3 fibroblast cytotoxicity. Ketoconazole exhibited an $IC_{50}D3/IC_{50}3T3$ ratio below 0.5 with non-overlapping 95% confidence intervals between the $IC_{50}D3$ and $IC_{50}3T3$ values indicating a greater sensitivity of embryonic cells towards the cytotoxic effects of this fungicide. Ketoconazole inhibited the embryonic differentiation process at significantly lower concentrations than the other conazoles, since ketoconazole had the lowest ID_{50} value with non-overlapping 95% confidence intervals.

Although all five conazole compounds were classified as “weakly embryotoxic” by the PM, a ranking of the potencies according to the ID_{50} values was performed to position the compounds relative to their embryotoxicity, resulting in the following order: ketoconazole > epoxiconazole \approx prochloraz > propiconazole \approx tebuconazole.

Publicly available data from in-house experiments or others’ studies on selected *in vivo* embryotoxic effects of the conazoles were gathered and presented in Table 2. The endpoints included were direct toxic effects to the fetus such as implantation loss, fetal/birth weight, number of resorptions, number of live fetuses, malformations, postnatal death, and pup weight or

Tab. 2: Overview of embryotoxicity *in vivo* data for five conazole compounds

Ranges of doses in mg/kg bw/day for significant effects on each specific endpoint are shown. Numbers in parentheses are the reference identification numbers as listed in Supplementary materials at www.altex-edition.org. Italic numbers refer to the tested dose range in mg/kg bw/day in all included studies. NI = parameter not investigated, NS = effect not significant. The lowest lowest-observed-adverse-effect-level (LOAEL) value (in mg/kg bw/day) for each chemical and parameter was converted into colors using an arbitrary scale selected as shown in the row marked scale. Non-significant effects were assigned the highest LOAEL value on the scale.

Conazole fungicides (mg/kg bw/day)	<i>In vivo</i> embryotoxicity endpoints								
	Gestation length	Maternal weight gain	Implantation loss	Fetal/birth weight	Resorptions	Live fetuses	Malformations	Postnatal death	Pup weight
Epoxiconazole 3.75-250	↑15-50 (1-3)	↓20-180 (4-6)	↑50-180 (2,5,6,7)	↓↑15,50 (2,7)	↑45-180 (4-7)	↓≈23-180 (2,3,5)	↑45 (4)	↑≈23,50 (2,3)	↓♂≈23 (3)
Ketoconazole 50-100	NI	↓50,100 (7,8)	↑10-100 (7-10)	↓80,100 (8,9)	↑50-100 (7-10)	↓40-80 (7,11)	↑80 (9,11)	NI	NI
Prochloraz 5-500	↑30-250 (12-15)	↓30-500 (12,13,15-18)	↑250 (13)	↑♀62.5 ↓♂100 (13,18)	↑160 (19)	↑58-81 (15)	↑50-250 (13,17)	↑58-250 (13,15)	↓125-250 (13,14)
Propiconazole ≈ 7.6-414	NI	NS (7)	NS (7)	↑♂50 (7)	NS (7)	↓250 (20)	↑90,400 (21,22)	NI	↓♂≈145-414 (20,23)
Tebuconazole 6-988	↑100 (2)	↓60,100 (2,24)	↑50,100 (2,7)	↓60,100 (2,24,25)	NS (2,7)	↓100 (26)	↑30-120 (25-28)	↑60,100 (2,24,29)	↓100 (29)
Scale	LOAEL≤10	10<LOAEL≤30	30<LOAEL≤50	50<LOAEL≤70	70<LOAEL≤100	LOAEL>100			



maternal effects such as gestational length and maternal weight gain. Although maternal weight gain and gestational length may be due to other mechanisms, such as maternal toxicity or hormonal changes, respectively, they were included as embryotoxic parameters, since they both can lead to increased fetal death. In this study, ranking the conazole fungicides relative to *in vivo* embryotoxicity was based on the lowest lowest-observed-adverse-effect-levels (LOAELs) and the number of affected endpoints among the selected endpoints in the table, resulting in the following order: ketoconazole > epoxiconazole > prochloraz > propiconazole ≈ tebuconazole. This order is fairly similar for *in vivo* and *in vitro* data, and we therefore conclude that the qualitative embryotoxic potential is fairly well predicted by the EST for these five conazoles. However, *in vivo* potency of epoxiconazole compared to prochloraz could not be discriminated by the EST.

Various *in vivo* data indicate that the timing of exposure during gestation may be important for the adverse effects observed. Taxvig et al. (2007, 2008) found that epoxiconazole had several adverse embryotoxic outcomes such as increased post-implantation loss and late resorptions in Wistar rats (Tab. 2). De Castro and Maia (2012) investigated the effects of epoxiconazole in the same rat strain and did not find developmental toxicity parameters to be altered even at a dose of 150 mg/kg bw/day. Differences in the dosing period between the studies (i.e., GD1-6 or GD6-15 in the de Castro study versus from GD7-21 and throughout lactation in the Taxvig study) are likely to explain the observed difference. This would imply that dosing during the final week of pregnancy or during lactation may be the critical period of concern of exposure to epoxiconazole (de Castro and Maia, 2012). Choosing the relevant dosing period is critical regarding developmental toxicity, not only for conazoles, but for all chemicals.

Other *in vitro* models have studied the embryotoxic effects of the conazole compounds. Among these are the post-implantation whole embryo culture (WEC) teratogenicity assay that investigates the growth *in vitro* of rodent embryos during some days in the period of organogenesis (Giavini and Menegola, 2010). Several WEC studies using rat or mouse embryos showed that conazoles were able to produce specific malformations in embryos. For instance, tebuconazole induced branchial arches malformations at exposure levels of 62.5–250 μM and in a concentration-related manner in the rat WEC system (Di Renzo et al., 2011). Additionally, severe abnormalities including abnormally shaped otic vesicle and head length were concentration-related and observed in rat embryos exposed to 10–100 μM ketoconazole (Menegola et al., 2006; Bechter and Schmid, 1987; Kucera et al., 1993). Also, propiconazole interfered with embryonic development of the crustacean *Daphnia magna* (Kast-Hutcheson et al., 2001). In a study by de Jong et al. (2011) comparing EST, rat WEC, and the Zebrafish Embryotoxicity Test (ZET), six related triazole compounds inhibited cardiomyocyte differentiation concentration-dependently in EST. These data support the hypothesis regarding the suggested embryotoxicity potential of the conazoles and further underline that this may be a general effect caused by this class of chemicals. The study also concluded that ZET gave the best

correlation with the relative *in vivo* toxicities of the test compounds, closely followed by the EST.

The same group also developed a novel quantitative evaluation method to assess the development of the zebrafish embryo based on specific endpoints in time, i.e., the general morphology score (GMS) system, and found comparable ranking of the six tested triazole compounds with ranking *in vivo* (Hermesen et al., 2011). As pinpointed by this group, it is unlikely that one single alternative testing method will be able to give a prediction for the entire range of possible mechanisms. Beside their embryotoxic effects, triazoles and imidazoles share endocrine disrupting modes of actions with imidazoles being found to be more potent than triazoles (Kjaerstad et al., 2010b). Results from various *in vitro* studies on endocrine disrupting effects of the conazoles are gathered and presented in Table 3. These include receptor reporter gene assays testing for antagonism or increase in transactivational activity of the androgen (AR), estrogen (ER), and aryl hydrocarbon (AhR) receptors along with studies investigating interference with sex hormone synthesis.

The critical mechanism for both imidazoles and triazoles seems to be inhibition of sex hormone biosynthesis, as these effects occur at lower concentrations compared to effect concentrations obtained in the nuclear receptor-based assays (Tab. 3). Ranking of *in vitro* endocrine activity of the conazole compounds was therefore carried out solely based on data from the steroidogenesis assay based on H295R cells. The ranking of the five compounds in terms of *in vitro* potency on interference with steroidogenesis (Tab. 3) was found to be:

Decreased estradiol production:

prochloraz > epoxiconazole > ketoconazole ≈ propiconazole ≈ tebuconazole

Decreased testosterone production:

prochloraz > ketoconazole > tebuconazole > epoxiconazole > propiconazole

Increased progesterone production:

prochloraz > epoxiconazole = propiconazole > ketoconazole > tebuconazole

Aromatase inhibition:

prochloraz > ketoconazole ≈ propiconazole ≈ epoxiconazole > tebuconazole

The effect of the conazoles on key enzymes involved in the biosynthesis and metabolism of steroids is well known, i.e., inhibition of CYP19 (Vinggaard et al., 2000; Taxvig et al., 2007; Kjaerstad et al., 2010b) and CYP17 (Laier et al., 2006). *In vivo*, the conazole fungicides also affect hormone levels in dams, i.e., ketoconazole, tebuconazole, prochloraz, and epoxiconazole reduced estradiol levels, and epoxiconazole increased testosterone plasma concentration in dams after exposure during pregnancy (Taxvig et al., 2007, 2008; Blystone et al., 2007). This may be the result of aromatase inhibition by these substances. Furthermore, epoxiconazole and tebuconazole increased progesterone levels in dams on GD21, i.e., one or two days before expected birth (Taxvig et al., 2007, 2008). Overall these data are in line with the hormonal effects observed in the H295R assay (Tab. 3). The effects on testosterone and progesterone biosynthesis

Tab. 3: Overview of data for five conazole fungicides obtained from *in vitro* assays commonly used to evaluate endocrine disrupting properties

Range of lowest-observed-effect-concentrations (LOECs), EC₅₀ (effect concentration eliciting 50% of maximum response), IC_{25/50} (inhibitory concentration resulting in 25% or 50% of control response, respectively), or REC₂₀ (relative effective concentration eliciting 20% of positive agonist response) values in μM are shown. Numbers in parentheses are the reference identification numbers as listed in Supplementary materials at www.altex-edition.org. AhR = aryl hydrocarbon receptor, AR = androgen receptor, NI = parameter not investigated. Colors indicate the potency of each chemical in each assay. Potency in terms of exposure concentrations were converted into colors using an arbitrary scale selected as shown in the row marked scale.

Conazole fungicide	<i>In vitro</i> endpoints for endocrine disruption						
	AR antagonism	Antiestrogenicity	AhR trans-activation	Steroidogenesis			
				Estradiol production↓	Testosterone production↓	Progesterone production↑	Aromatase inhibition
Epoxiconazole	LOEC:1.6-2 (1,30,31) 3<IC ₂₅ <10 (32) IC ₅₀ :10 (30)	LOEC:25 (30) IC ₅₀ :52 (30)	LOEC:6.3 (33)	LOEC:0.1 (30) IC ₅₀ :0.2 (30)	LOEC:3 (30) IC ₅₀ :5.3 (30)	LOEC:0.3 (30)	LOEC:1-100 (30,34,35) IC ₅₀ :1.44-20 (30,34,36)
Ketoconazole	3<IC ₂₅ <10 (32)	LOEC:0.2 (30) IC ₅₀ :12 (30)	NI	LOEC:1-5 (30,37-40) IC ₅₀ :0.62-2.3 (30,41)	LOEC:0.03-9.4 (30,37-40,42-44) IC ₅₀ :0.08-0.26 (30,41,45)	LOEC:1-5 (30,37,40)	LOEC:6.3 (30) IC ₅₀ :0.9-281 (30,34,46-51)
Prochloraz	LOEC:1.9-10 (1,13,30,52) 3<IC ₂₅ <10 (32) IC ₅₀ :3.6-13 (30,53-55)	LOEC:1-25 (30,52,56) IC ₅₀ :22-29 (30,56)	LOEC:0.05-1 (33,57) REC ₂₀ :0.71 (58)	LOEC:0.01-5 (17,30,37-40) IC ₅₀ :0.02 (30)	LOEC:0.01-30 (17,30,37-40,59) IC ₅₀ :0.005 (30)	LOEC:0.01-5 (17,30,37,40) EC ₅₀ :0.21 (41)	LOEC:0.3-50 (30,34,38,52,61,62) IC ₅₀ :0.047-15 (30,34,50,51,60-62)
Propiconazole	LOEC:12.5-25 (30,31) 3<IC ₂₅ <10 (32) IC ₅₀ :18 (30)	LOEC:1 (30) IC ₅₀ :55 (30)	LOEC:12.5 (33)	LOEC:1-30 (30,63) IC ₅₀ :0.9 (30)	LOEC:10 (30,63) IC ₅₀ :5.3 (30)	LOEC:0.3 (30)	LOEC:1-50 (30,61,62) IC ₅₀ :0.968-199 (30,34,51,60-62,64)
Tebuconazole	LOEC:0.5-3.8 (1,30,31) 1<IC ₂₅ <3 (32) IC ₅₀ :8.1 (30)	LOEC:1.6 (30) IC ₅₀ :49 (30)	LOEC:6.3 (33)	LOEC:3 (30) IC ₅₀ :1.2 (30)	LOEC:0.1 (30) IC ₅₀ :0.5 (30)	LOEC:10 (30)	LOEC:10 (30) IC ₅₀ :5.8-609 (30,34,51,60)
Scale	x≤0.05	0.05<x≤0.1	0.1<x≤0.5	0.5<x≤1	1<x≤5	x>5	

indicate an inhibition of enzymes involved in the conversion of progesterone to testosterone and might be due, at least partly, to inhibition of CYP17 (Lai et al., 2006), an effect that has been confirmed *in vitro* in H295R cells and in testicular microsomes (Blystone et al., 2007; Ayub and Levell, 1987; Ohlsson et al., 2010). The increased progesterone levels in the dams are suggested to cause virilization of the female offspring (Taxvig et al., 2007), which would explain the increased AGD in females

pups observed after exposure to several of the conazoles (Tab. 4). In addition, the increased progesterone levels in dams could explain the prolonged pregnancy period (Tab. 2) (Vinggaard et al., 2005).

Data on endpoints from *in vivo* developmental toxicity endpoints affected by the conazole fungicides were collected and presented in Table 4. The parameters included common endpoints such as male reproductive organ weight, anogenital



Tab. 4: Overview of data for five conazole compounds causing significant *in vivo* developmental toxicity

Ranges of doses in mg/kg bw/day are shown; ↑↓ indicate an increase or decrease in the investigated parameter, respectively.

Numbers in parentheses are the reference identification numbers as listed in Supplementary materials at

www.altex-edition.org. Italic numbers refer to the tested dose range in mg/kg bw/day in all included studies. AGD = anogenital distance,

NI = parameter not investigated, NS = effect not significant. Lowest-observed-adverse-effect-level (LOAEL) values (in mg/kg bw/day)

were converted into colors using an arbitrary scale selected as shown in the row marked scale. Non-significant effects were assigned

the highest LOAEL value in the scale. Diagonal hatching was assigned to results that indicate an increase in feminization of female offspring or a masculinization of male offspring, respectively.

Conazole fungicide (mg/kg bw/day)	In vivo developmental toxicity endpoints								
	Reproductive organ weight ♂	AGD		No. areolas ♂	Hormone levels in ♂ pups				Other
		♂	♀		Testosterone	Progesterone	17α-hydroxyprogesterone	Androstenedione	
Epoxiconazole ≈ 1-15	↑15 (65)	↑3.75,15 (1,2)	↑3.75-50 (1,2)	NS (2)	NS (7)	NS (7)	NS (2)	NI	Time to vaginal opening ↓150 Time to testes descent ↑150 (66)
Ketoconazole 50	NI	↓50 (7)	↓50 (7)	NI	↓50 (7)	NI	NI	NI	-
Prochloraz 5-500	↓30-250 (12,13,16,17,65)	↓50-150 (13,17)	↑8.75-150 (1,13,17)	↑30-250 (1,12,13,17)	↓30-250 (12,16,17,67)	↓50 ↑7.8-250 (12,16,17,67)	↑7.8-125 (16)	↓31.3,125 (16)	-
Propiconazole ≈ 7.6-414	↑≈36-414 (23)	↑≈145-174 (23)	NS (23)	NI	↑≈36-414 (23)	NI	NI	NI	Female fertility ↓≈36-414 (22,23)
Tebuconazole 12.5-988	↓60-988 (24,29,68)	↑100 (2)	↑12.5-100 (1,2)	↑50,100 (1,2)	↓100 (2)	↑50 (2,7)	↑50,100 (2)	NI	Fertility ↓300 (29)

Scale	LOAEL≤10	10<LOAEL≤30	30<LOAEL≤50	50<LOAEL≤70	70<LOAEL≤100	LOAEL>100
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distance (AGD), number of male areolas, and hormone levels in pups. There is, however, a lack of available data on ketoconazole in that respect. The *in vivo* findings suggest that prochloraz, ketoconazole, and tebuconazole mainly affect male reproductive health endpoints – effects that conceivably are mediated by reduced testosterone during gestation. The findings on epoxiconazole and propiconazole indicate that they mainly affect female reproductive health, e.g., disruption of estrous cycle (Rockett et al., 2006) and time until vaginal opening (de Castro and Maia, 2012) defining the onset of puberty in female rats (Firlit and Schwartz, 1977). These two conazoles also show a weak estrogenic response *in vitro* in the MCF-7 cell proliferation assay (data not included in Tab. 3) (Kjaerstad et al., 2007), a mode of action that might contribute to the effect on female reproduction. Prochloraz, keto-

conazole, and tebuconazole seem to be the most potent male reproductive toxicants affecting either male AGD, nipple retention and/or fetal testosterone. On the other hand, epoxiconazole and propiconazole seem to affect the female pups to a higher degree. Overall, prochloraz ranks the most potent endocrine disruptor based on interference with steroidogenesis *in vitro* (Tab. 3). In agreement with this, *in vivo* data show that prochloraz appears to affect all studied endpoints adversely and propiconazole shows the lowest potency both *in vitro* and *in vivo*. Affinities and potencies for selected CYP enzymes, i.e., CYP17, CYP19, CYP26, and CYP51, appear to be the mechanism eliciting the functional and adverse effects of the conazole fungicides and differential CYP inhibition profiles may explain the observed differences in their embryotoxic and developmental toxic profile.

4 Conclusion

The overall objective of this study was to retrospectively evaluate the predictability of a battery of *in vitro* methods for evaluating embryotoxicity and developmental toxicity for five conazole fungicides. All five conazoles were classified as “weakly embryotoxic” in the EST, and ranking of their potencies based on ID₅₀ values for inhibition of beating occurrence in cardiomyocytes showed fairly good correlation with *in vivo* embryotoxic endpoints. However, in animal studies epoxiconazole appeared to have a stronger embryotoxic potential compared to prochloraz, a difference that was not predicted by EST. The critical mechanism for both imidazoles and triazoles on endpoints of developmental toxicity seems to be inhibition of enzymes involved in sex hormone biosynthesis. Based on data from the H295R steroidogenesis assay prochloraz was found to be the most potent endocrine disruptor *in vitro*, an effect that resulted in a broader variety of adverse *in vivo* developmental effects.

The disparity in influence on adverse *in vivo* developmental and embryotoxic effects observed between the conazole fungicides is likely a result of differences in affinities and potencies for the various CYP enzymes (i.e., CYP17, CYP19, and CYP26) believed to be affected during gestation and/or lactation. In this paper we showed that a battery of *in vitro* test methods, including the H295R steroidogenesis assay and the EST assay can be used as an effective initial approach for ranking of conazoles in relation to adverse health effects such as developmental toxicity and embryotoxicity. Data obtained from the AR, ER, and AhR reporter gene assays and the CYP19 assays contributed with mechanistic information but were not determining factors for the *in vivo* predictability for this class of compounds. Thus, a holistic evaluation of a battery of relevant assays may give important hints on the toxicological outcome in animal studies and hints on the critical mode and mechanisms of action of the chemicals, thereby saving resources and animals in the design of potential future studies.

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Correspondence to

Anne Marie Vinggaard, PhD
National Food Institute
Technical University of Denmark
Mørkhøj Bygade 19
2860 Søborg
Denmark
e-mail: annv@food.dtu.dk