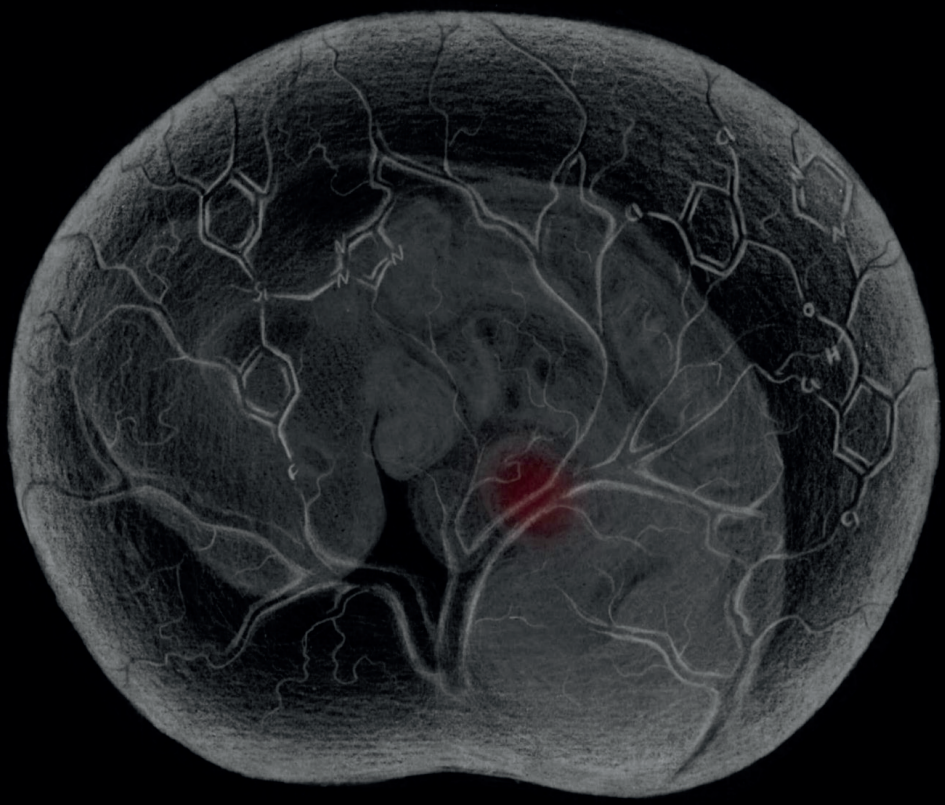
Alternative developmental toxicity models for assessing the *in vivo* embryotoxicity of azoles



Myrto Dimopoulou

Alternative developmental toxicity models for assessing the *in vivo* embryotoxicity of azoles

Myrto Dimopoulou

Thesis committee

Promotors

Prof. Dr B. van Ravenzwaay

Professor of Reproductive and Developmental Toxicology, Wageningen University & Research

Senior Vice President of Experimental Toxicology and Ecology, BASF SE, Ludwigshafen, Germany

Prof. Dr A.H. Piersma

Professor of Reproductive and Developmental Toxicology

Utrecht University & National Institute for Public Health and the Environment (RIVM), Centre for Health Protection, Bilthoven, the Netherlands

Co-promotor

Prof. Dr I.M.C.M. Rietjens

Professor of Toxicology

Wageningen University & Research

Other members

Prof. Dr A.J. Murk, Wageningen University & Research

Prof. Dr J. Kleinjans, Maastricht University, the Netherlands

Dr J. Louisse, KWR Water, Nieuwegein, the Netherlands

Prof. Dr F.G.M. Russel, Radboud University, Nijmegen, the Netherlands

This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Alternative developmental toxicity models for assessing the *in vivo* embryotoxicity of azoles

Myrto Dimopoulou

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 05 March 2018
at 01.30 p.m. in the Aula.

Myrto Dimopoulou

Alternative developmental toxicity models for assessing the *in vivo* embryotoxicity of azoles,

200 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2018)

With references, with summary in English

ISBN: 978-94-6343-731-8

DOI: https://doi.org/10.18174/430573

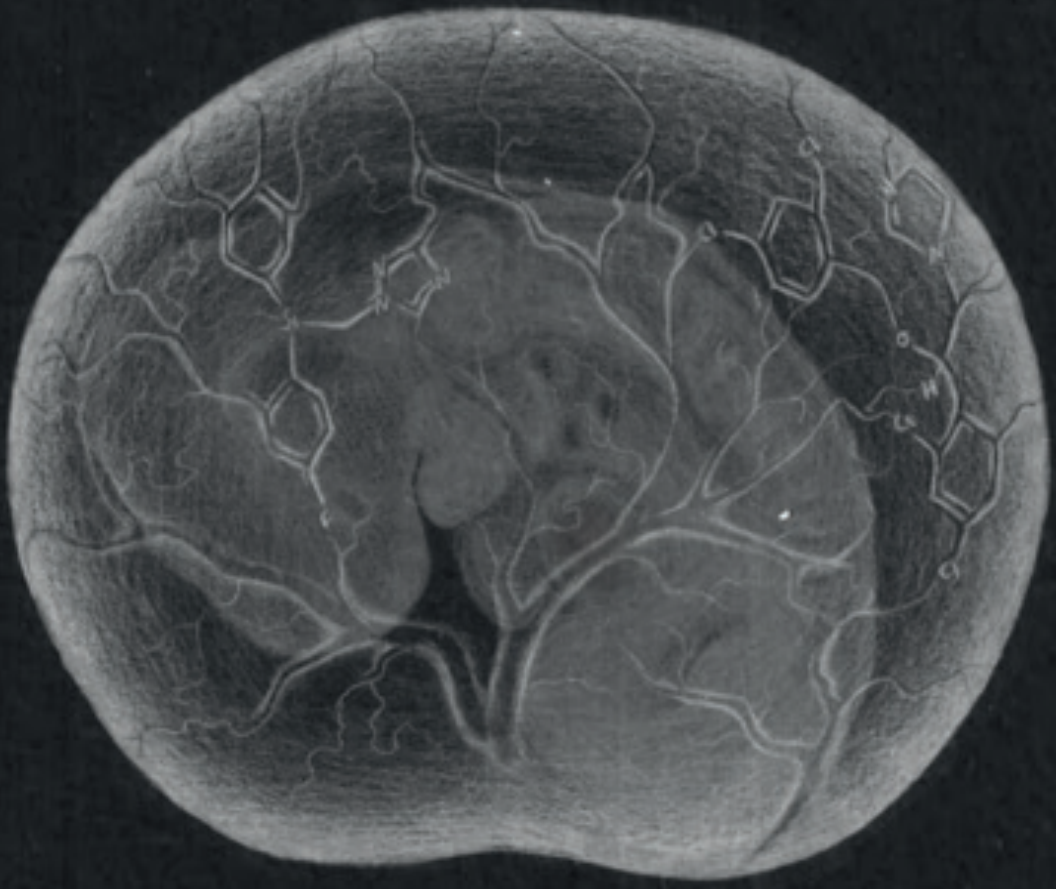
Contents

Abbreviations

ADME	Absorption, distribution, metabolism and excretion
AOP	Adverse outcome pathway
BMD	Benchmark dose
BMR	Benchmark response
CDF	Chip Description File
cDNA	Complementary DNA
CNS	Central nervous system
CTD	Comparative Toxicology Database
DAVID	Database for Annotation, Visualization and Integrated Discovery
DFZ	Difenoconazole
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EB	Embryonic body
ECVAM	European Centre for the Validation of Alternative Methods
ESC	Embryonic stem cells
EST	Embryonic stem cell test
FC	Fold Change
FDR	False discovery rate
FEN	Fenarimol
FLU	Flusilazole
FR	Fold Ratio
GD	Gestational day
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
IC_{xx}	Inhibitory concentration of malformations xx %
ID_{xx}	Inhibitory concentration of differentiation xx %

Abbreviations

KEGG	Kyoto Encyclopedia of Genes and Genomes
KTZ	Ketoconazole
LEL	Lowest effect level
MCZ	Miconazole
MeHg	Methylmercury
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NOAEL	No observed adverse effect level
OECD	Organization for Economic Co-operation and Development
PCA	Principal component analysis
PRO	Propiconazole
PTZ	Prothioconazole
QIVIVE	Quantitative in vitro-in vivo extrapolation
QSAR	Quantitative structure-activity relationship
RA	Retinoic acid
REACH	Registration Authorization and Evaluation of Chemicals
RMA	Robust Multichip Average
RNA	Ribonucleic acid
RT-PCR	Real-time Polymerase Chain Reaction
TDF	Triadimefon
TEB	Tebuconazole
TMS	Total morphological score
WEC	Whole embryo culture
ZET	Zebrafish embryo test



Chapter 1

General introduction

1. The reproductive cycle in mammals

Reproduction in mammals is a complicated process including fertilization, prenatal and postnatal development. It includes a cascade of events such as sexual behaviour, pregnancy, birth, growth and subsequent sexual maturation. The reproductive process is organized and regulated through a variety of mechanisms at the biochemical, molecular, cellular, tissue and organ level, leading to embryonic differentiation and morphogenesis. These processes include the organization of different cells to build three-dimensional structures and, consequently, to form organs and the entire organism [1, 2]. The complexity and sensitivity of the reproductive cycle could be affected by many factors, including malnutrition and stressful environment, or exposure to xenobiotics, biotoxins and radiation [3].

2. Reproductive and developmental toxicology

Reproductive and developmental toxicology is the field of expertise that deals with effects on fertility and with birth defects that could occur during any stage of the reproductive cycle [4]. With the term “birth defects”, structural or functional abnormalities are described, which are identified at birth, can cause physical, intellectual and developmental disabilities and can lead to death of infants during their first year of life. Several birth defects are caused by xenobiotics, called teratogens, coming from the Greek word “teras” that means monster [5]. Teratology belongs to the field of developmental toxicology, and its research objective is to identify the cause of teratogenesis, or otherwise, the cause of morphological adverse outcomes of pregnancy [6]. Historically, I.G. de Saint-Hillaire firstly introduced the term of “teratology” during the 19th century [7]. However, rational biologically based theories of congenital developmental disorders caused by exogenous factors were firstly introduced by J. Warkany in the 1930s and 1940s [8]. Despite the birth defects observed during the first half of the 20th century due to exposure to teratogenic factors such as aminopterin, radiation, estrogens, androgens, cortisone, hypovitaminosis, folic acid, vitamin A and vitamin D supplements, not much attention was given to developmental toxicity studies [7]. The importance of developmental toxicity studies gained the scientific attention in the early 60s when the thalidomide tragedy occurred [9]. Thalidomide, or the commercially so-called Softenon, was prescribed as a sedative to pregnant women against morning sickness, causing severe congenital limb defects (phocomelia) in more than 10,000 children [9, 10]. In 1973, Wilson gave a more comprehensive definition of “teratology”, introducing the terms of mechanism of development, time of exposure, frequency and degree of dosing and different manifestations of deviant development (death, malformation, growth retardation and functional defect), with which he formulated the principles of teratology [6].

Due to the thalidomide incident in the 1960s, since 1980 worldwide guidelines for developmental toxicity testing were incorporated by the Organization for Economic Co-operation and Development (OECD) for improving the assessment of chemical safety and decreasing the risk of other tragedies such as the one caused by thalidomide. The OECD guidelines were designed to assess the developmental toxic effects of pharmaceuticals and pesticides during the entire reproductive cycle, including sexual maturation, fertilization, prenatal and postnatal development by implementing *in vivo* animal testing protocols [11]. The three key phases of reproduction, fertility, pre- and postnatal development, are covered by five OECD test guidelines, which include the prenatal developmental toxicity study (OECD 414), the one-generation (OECD 415) and two-generation studies (OECD 416), as well as the reproductive/developmental toxicity screening test (OECD 421) and the reproductive/developmental toxicity screening study (OECD 422) [12-16]. Later, the OECD 426 study was established for screening developmental neurotoxic effects of chemicals [17]. The application of these guidelines implied the increase of animal testing, experimental costs and time needed for conducting a complete hazard assessment. Additionally, in 2007 the European legislation for Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) was implemented to improve the hazard identification of chemicals and to perform a risk assessment for all chemicals, which are produced or imported in the European Union [18, 19]. The resulting demand to assess the safety of almost 30,000 current and of even more future chemicals implied a dramatic increase of the already high amount of laboratory animals used in chemical safety assessment. Reproductive and developmental toxicity studies required almost 65% of the total number of laboratory animals needed under REACH legislation, which are estimated to be almost 4 - 22 million vertebrates [18, 20-22]. The three Rs concept (reduction, refinement and replacement of animal testing) [23], the increased ethical concerns about animal testing in combination with the drawbacks of *in vivo* testing and issues with interspecies extrapolation, promoted the need for designing alternative *in vitro* methodologies for screening developmental toxicity.

3. *In vitro* developmental toxicity testing

The necessity for improved hazard identification and risk assessment for human health and for reducing the number of laboratory animals leads the scientific community to developing and establishing *in vitro* methodologies for screening the developmental toxicity of chemicals [18, 22, 24, 25]. Due to the complexity of the mammalian reproductive cycle, it can be anticipated that only the combination of many *in vitro* assays, appropriate for studying the various mechanisms of reproductive and developmental toxicity, could mimic the entire reproductive cycle [26]. For developmental toxicity testing, the last decades, more than

twenty *in vitro* assays have been developed, including cell-based methods, organ cultures, organ-on-a-chip concepts, whole embryo cultures and *in silico* simulation models [4, 21, 27]. Among the available alternative systems, three of them have been successfully validated. These are the embryonic stem cell test (EST) [28], the limb bud micromass (MM) [29] and the rat whole embryo culture (WEC) [30], for which standard protocols are available on the website of the European Centre for the Validation of Alternative Methods (ECVAM) [31]. Although these validated methods could importantly decrease the number of laboratory animals, their application is still largely restricted to screening purposes and for further prioritizing *in vivo* testing. Additionally, Hartung *et al*. [32] have proposed that the outcome of mechanistic validation could be a valuable tool for further decision-making based on *in vitro* results. Although the mechanistic approach for screening embryotoxicants is quite appealing, the knowledge in this area is still limited. The current validated studies are based on mathematical prediction models, which simplify the biological responses, while they do not provide mechanistic information. Piersma *et al*. [33] have suggested that for improving the hazard identification, while reducing and replacing animal testing, molecular-based approaches are needed for describing the key events initiated from a chemical stimuli on the molecular to cellular level, organs and, subsequently, to the whole organism, causing any toxic effect. Moreover, in-depth knowledge on the dynamics and kinetics of biological systems may offer a boost in better defining the applicability domain of alternative tests, leading to design of in vitro strategies that are more relevant for a mode-of action based screening of chemical hazards [32, 33].

3.1. The rat whole embryo culture (WEC)

A historical note

In 1960s, the pioneer D. New started the research on developing the rat WEC technique, with which postimplantation embryos were removed from the maternal womb and transferred in their intact visceral yolk sac to an *in vitro* environment, which was ideally designed for supporting normal growth and development during the primary organogenesis phase [34], gestation days (GD) 10-12. This research was inspired by studies of Waddington and Waterman in 1933, who attempted to culture *in vitro* rabbit embryos in plasma clots [35]. Later, the rat WEC was established as a method mimicking organogenesis *in vitro*, including cellular proliferation, interaction, differentiation and finally pattern formation and, consequently, considered ideal for studying the development of mammalian embryos throughout the stage of neurulation and organogenesis [36]. Therefore, the rat WEC was suggested as a useful tool for screening teratogenic outcomes of chemicals [37].

Validation of rat WEC

The first attempt for validating the rat WEC was performed in 1985 by Schmid [38], who suggested that WEC could be a promising screening tool for teratogenicity. The following upgrading of the scoring system, with which many morphological endpoints related to growth and development were additionally considered, improved the predictability of the rat WEC [39, 40]. During the 1980s, the usefulness of this technique as a system for screening teratogenicity of chemicals was widely employed, reaching more than 200 studies [41-47]. In the ECVAM validation study (2001), 20 chemicals were tested in four laboratories in a double blind design. The selected compounds were classified into three categories: non-embryotoxic, weak embryotoxic and strong embryotoxic according to available data describing their *in vivo* embryotoxic profile [31]. The WEC model predicted the classification of the tested compounds with 80% accuracy, while the strong embryotoxicants were all detected (100%) with 83 and 100 % precision, depending on the prediction model used. However, misclassifications of non- or weak embryotoxicants were observed, which suggested the need of further evaluation and introducing more techniques (such as gene expression profiling and toxicokinetics related to placental transfer) for improving the prediction value of the technique [26, 30, 48].

The rat WEC – a validated embryotoxicity test

Today, the rat WEC is an established *in vitro* method with a standardized protocol (Figure 1) for screening embryotoxicants after exposure to chemicals during early organogenesis. Table 1 presents an overview of the total morphological score (TMS) used in the WEC assays performed in the present thesis. Although it is not a fully animal-free method, it is a valuable tool with numerous benefits [49]. The greatest advantage of the rat WEC is that neurulation and early organogenesis could be continuously monitored during the critical window of GD 10 and 12 (Figure 2 and Table 1) [36]. Importantly, the embryonic developmental pace during this time window is in high agreement with the *in vivo* situation for both rat and human embryos [30, 50-52]. However, the restricted experimental duration (48 hours), as well as the lack of maternal interaction and metabolic activity could be limitations for the predictability of the method [53]. Several attempts for adding metabolic activity to the system were not successful [49, 53], while other studies have suggested that individual assessment of metabolites could be a better solution [54, 55]. However, overall, the proposed combination of the WEC with structural- or molecular- based techniques could build a robust system for screening embryotoxicants and prioritizing further *in vivo* testing.

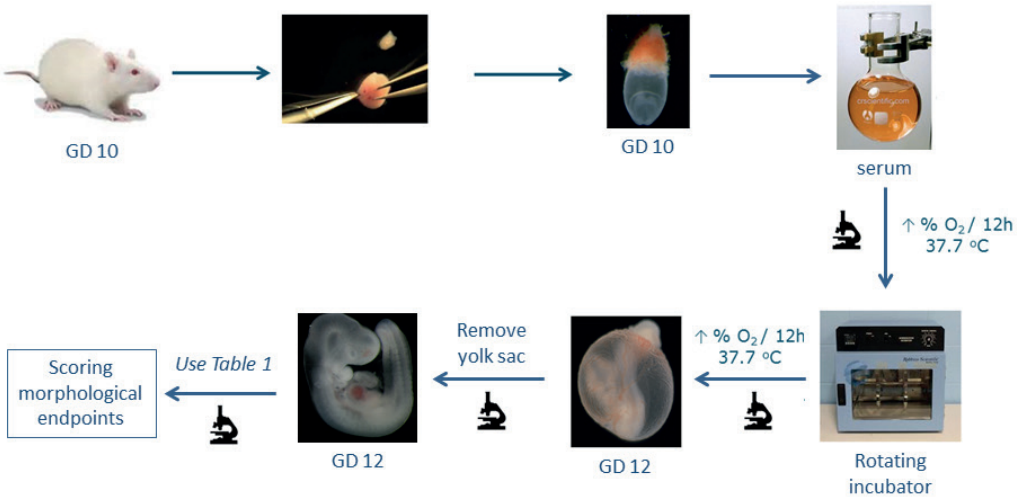


***Figure 1**: Schematic representation of the rat whole embryo culture method. On GD 10, dams are euthanized and their embryos are immediately separated from the uterus. The peripheral trophoblastic cell zone and parietal yolk sac membrane are removed under the microscope leaving both the visceral yolk sac and ectoplacental cone intact. Embryos with 1-5 somites are individually cultured in flasks in 2 mL culture medium. The culture flasks are placed in rotating incubators, which are protected from light and their internal temperature is permanently at 37.7°C. The cultured flasks are oxygenated twice daily for 30 seconds per time, with increasing concentration of oxygen. At the end of culture, the embryos are microscopically evaluated and each morphological item is individually scored. The sum of the individual tissue-scores, the total morphological score (TMS), is a quantitative way for representing the embryonic developmental stage and determining the embryotoxic effect of chemicals on the phenotype, and is summarized in **Table 1** [40].*

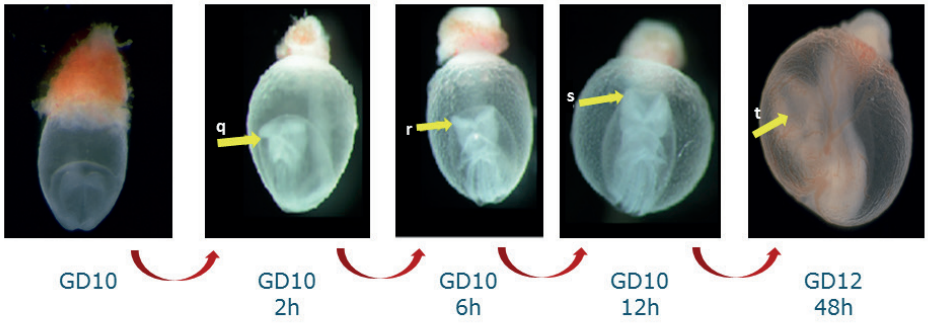


***Figure 2**: The continuous monitoring of the embryonic development during the critical window of the experimental duration (48 hours) is the greatest advantage of the rat WEC.*

The yellow arrows indicate the closure of the neural tube, which is often the first indication of embryonic development, immediately determining any developmental abnormality due to internal (biological) or external (exposure to chemicals) factors. The q, r, s and t letters indicate the process (shape) of the neural tube closure, which is described in more details in ***Table 1***.

***Table 1**: Illustration of the total morphological score (TMS) system, adapted from van Maele-Fabry and Picard [56] and used for examining the rat WEC.*

		Score					
		0	***1***	***2***	***3***	***4***	***5***
Morphological endpoint	***Yolk sac***			 Circulation			
	Allantois						
	Flexion		1 / 4 turning	1 / 2 turning	3 / 4 turning	 C-shaped	 Spiral torsion
	Heart		Neural folds  Heart anlage			3 chambered appearance 2 constrictions	4 chambered appearance
	Caudal neural tube		SECTION			 DORSAL VIEW	
	Hindbrain		TANGENTIAL VIEW		DORSAL VIEW		
	Midbrain		TANGENTIAL VIEW		DORSAL VIEW		
	Forebrain		 TANGENTIAL VIEW		FRONTRAL VIEW		
	Otic system			LATERAL VIEW			
	Optic system				LATERAL VIEW		
	Olfactory system			FRONTAL VIEW			
	Branchial arches			LATERAL VIEW			
	Maxillary process	Head	LATERAL VIEW				
	Mandibular process			VENTRAL VIEW			
	Fore limbs		DORSAL VIEW				
	Hind limbs	LATERAL VIEW	DORSAL VIEW				
	Number of somites	0 - 5	6 - 10	11 - 15	16 - 20	21 - 25	26 - 30

3.2. The embryonic stem cell test

In 1909, A. Maximow firstly introduced the term “stem cells” as a type of cells that participate in the hemapoietic system in the bone marrow during development [57]. The presence of stem cells in murine bone-marrow was explained in the early 60s by McCullough and Till, who discovered also their cellular ability of self-renewing [58, 59]. In the 70s, researchers isolated pluripotent embryocarcinoma cells (ECC) [60, 61], which promoted the idea of isolating embryonic stem cells (ESC) from the inner cell mass of a blastocyst, which was accomplished in the early 80s [62-64]. The use of murine pluripotent ESCs (murine D3 cell line) derived from an 3.5-day old blastocyst was further explored due to their ability to differentiate *in vitro* into endo-, meso-, ecto-derm germ layers; and, therefore, to any type of cells present in the mouse [65]. Therefore, the differentiation of ESCs was further studied as a potential test for screening developmental toxic responses of chemicals. As described by Spielmann [66], following the hanging drop culture technique, in the absence of leukemia inhibitory factor (mLIF) and the presence of fetal bovine serum, the ESCs form aggregates, called embryonic bodies (EBs). The EBs, which mimic the anterior pre-steak embryos, could spontaneously differentiate into foci of beating cardiomyocytes (Figure 3), which is the morphological endpoint for screening developmental toxicity in the 10-day EST assay.

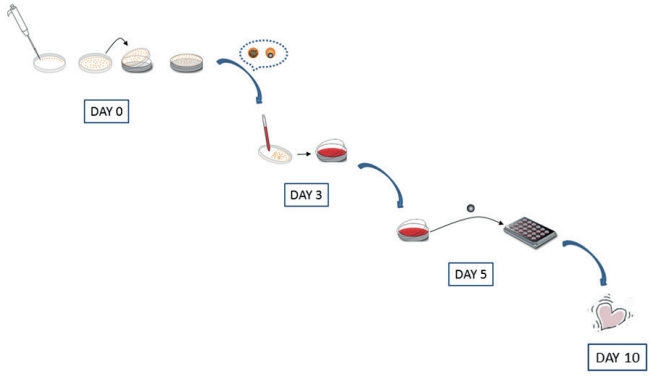


Figure 3*: The validated 10-day differentiation protocol of the EST. On day 0, undifferentiated single cell suspensions are cultured in hanging drops. On day 3, the formed cell aggregates, the EBs, are transferred and further cultured in supplemented cell culture medium. On day 5, single EBs are plated on tissue culture plates, in which they are differentiated into contracting cardiomyocytes, which are microscopically evaluated on day 10.*

In the EVCAM validation study in 2002, 20 chemicals, which were classified as *in vivo* non-, weak or potent embryotoxicants, were assessed with the EST developmental toxicity assay in four laboratories in a blind trial [28, 31, 67]. For concluding on the developmental toxicity,

three endpoints were evaluated: the inhibition of differentiation of D3 cells, as well as the inhibition of growth of D3 cells and 3T3 cells, facilitating the study of differences in sensitivity between embryonic and adult cells. The murine 3T3 cell line derives from mouse fibroblast cells and mimics the maternal side. Based on the improved biostatistical prediction model [31], the EST predicted with 78% overall accuracy the developmental toxicity of the tested compounds [28]. The strong embryotoxicants were 100% predicted with 83% precision. The predictivity of non- and weak embryotoxicants was 73 and 69% [28]. Considering the validation outcome, EST is an applicable assay for classifying a variety of chemicals and, especially, for sufficiently predicting strong embryotoxicants. Given also that it is an animal-free assay, as well as less time consuming and cheaper compared to other *in vitro* assays, the EST is widely accepted and applicable as a test strategy for further prioritizing additional testing of chemicals, especially in the pharmaceutical sector [68]. On the other hand, the EST is relatively simple, as it lacks the interaction of a more complex biological system and metabolic capacity [69]. Additionally, the microscopical evaluation of contracting cardiomyocytes is the single qualitative endpoint that determines the developmental effect of the tested compounds on the differentiation process, which could also lead to a more subjective conclusion [26]. Therefore, the predictivity and applicability domain of EST could be further improved for achieving a read-out useful for regulatory purposes. The addition of quantitative endpoints, the incorporation of toxicokinetic information and the development of *in silico* prediction models could increase the accuracy and predictivity of the model, which could be of use in a battery of alternative tests representing key stages in development [70-72]. Moreover, like with the WEC, the incorporation of metabolic activity could be an additional advantage for building biokinetic systems and, therefore, for improving the applicability of the EST [73]. The extension of the differentiation endpoints to multiple tissues (neural, bone, cartilage and epithelial tissues) and the identification of molecular biomarkers (genes and proteins) of developmental toxicity are some of the further suggestions for improving the classical EST [68, 74].

4. The importance of toxicokinetics in developmental toxicity

4.1. The role of placenta

The placenta is an active endocrine organ with a dynamic profile during pregnancy [75]. Its role is to supply the fetus with oxygen, nutrients and hormones from the mother, as well as to remove metabolic waste from the fetal to maternal side via blood circulation [76, 77]. Mammalian placentas are classified into three groups (haemochorial, endotheliochorial and epitheliochorial) depending on the number of layers that exist between the maternal and fetal side [78]. The human haematochorial placenta includes a layer of tissue, which separates

the maternal from the fetal site, consisting of the endothelium of fetal capillaries and the trophoblast, which contains the cytotrophoblast and syncytiotrophoblast (maternal border) [79, 80]. Similar to the structure of human placenta are those of rat and rabbit, which are the two main species used for studying developmental toxicity [78]. While the placenta was originally considered to function as a barrier for protecting the fetus from harmful substances, after the thalidomide tragedy it has been shown that xenobiotics can cross it and reach the embryos or fetuses, possibly causing developmental adverse effects [77, 80]. The mechanism for placental transfer could vary from passive diffusion, active transport, facilitated diffusion, and filtration to pinocytosis [81]. Passive diffusion is suggested to be the main mechanism of placental transfer of xenobiotics. The amount of transfer of xenobiotics is determined by their physicochemical properties, such as molecular weight, lipophilicity and ionization. The binding affinity of xenobiotics to plasma proteins, such as albumin and a1-acid glycoprotein, is of additional interest as it leads to increased passive placental diffusion with gestation days [82]. Moreover, the placenta has metabolic capacity, which is low compared to the enzymatic metabolic capacity of liver, but notable, considering its capability to transform parent compounds to more potent embryotoxic metabolites [83-85]. Therefore, more emphasis is given to placental toxicokinetic studies, which focus on determining the fate of a chemical when it penetrates the placental barrier and endangers the embryonic development. The combination of *in vitro* or *in silico* models for toxicokinetics with *in vitro* models for studying developmental toxicity are a necessity for improving the predictability of alternatives to animal testing methodologies and extrapolating *in vitro* findings to the *in vivo* situation [86-88].

4.2. Modelling placental transfer – the BeWo approach

In 1968, Pattillo and Gey [89] developed the human BeWo cell line, which originated from a choriocarcinoma (the hormone-synthesizing trophoblast cells *in vitro*). BeWo cells can be grown in transwell models, which are divided into an apical and basolateral compartment, mimicking the maternal and fetal side, respectively [90, 91]. BeWo cells maintain the morphological characteristics of trophoblasts of human placenta, including undifferentiated cytotrophoblasts and syncytiotrophoblast [92], and are useful in placental transfer studies due to their ability to form a polarized and confluent monolayer, mimicking the human placenta [90, 91, 93]. Additionally, similarly to the *in vivo* situation, the BeWo cell line expresses xenobiotic metabolizing enzymes, such as *Cyp1a1*, *Cyp3a4* and *Cyp2c9* and, therefore, it is of use for investigating *in vitro* placental metabolism [81, 94, 95]. Previous studies have shown that the transport rates of xenobiotics measured with the BeWo model satisfactory correlate with the transport rates in the *ex vivo* human placenta perfusion system

[72, 91], which is the *in vitro* system closest to the *in vivo* situation [96]. The advantages of the BeWo model are that it is less time consuming, less expensive and not dependent on the availability of fresh human placental tissue after delivery. However, considering the dynamic profile of the *in vivo* placenta [76], the greatest limitation of both systems is that they cannot accurately predict the function of human placenta during the first and second trimester of pregnancy. On the other hand, previous studies suggested that the evaluation of the *in vitro* developmental toxicity of chemicals in the EST in combination with their placental transfer rates determined with the BeWo model, improved the prediction of the *in vivo* developmental toxicity [97, 98]. Therefore, the addition of the placental transfer models would be valuable for improving the *in vivo* predictability of *in vitro* models and, consequently for promoting the concept of using a battery of multidirectional alternative techniques for improving the classical risk assessment approach.

5. Toxicogenomics

5.1. A molecular-based readout of developmental toxicity

Technological progress has empowered the development and implementation of molecular based approaches, which can investigate thousands of endpoints in one assay and improve the "classical" *in vitro* testing readout [99]. The so-called omics approaches are tools that enable the study of the transcriptome, proteome, epigenome and metabolome of biological systems for providing information for better understanding the biological processes [99-101].

Toxicogenomics is the application of omics technologies for studying responses to chemical substances on the molecular level and identifying or predicting mechanisms of induced toxicity [102]. For identifying such toxic responses, studies on the level of mRNA (transcriptomics), including single-gene studies with RT-PCR, microarrays or next-generation sequencing, gained much attention during the last decades [102].

Compounds from different classes of chemicals might have similar morphological effects, while the underlying mechanism of toxicity could be different. Moreover, alterations on the level of the transcription of the genome may precede morphological toxic responses and, therefore, may present early gene-biomarkers that can have a predictive value in revealing the forthcoming developmental toxicity. Considering also the high conservation on the molecular mechanisms between different species, transcriptomics could facilitate a better interspecies extrapolation based on insight in the mechanisms underlying maldevelopment due to exposure to different classes of compounds [1, 101-103].

The first toxicogenomic studies were performed in the field of carcinogenesis [104], while more studies followed including assessments of developmental toxic responses [105]. The

identification of gene-biomarkers of developmental toxicity is a useful tool for characterizing and classifying chemicals with high sensitivity and predictability [102]. Moreover, they could improve our understanding in the developmental processes and how these are affected after exposure to chemicals. Implementing the available alternative *in vitro* assays (WEC, EST and zebrafish embryo test (ZET)), the developmental toxicity of different classes of chemicals, including endocrine disruptors [106, 107], azole antifungals [108-111], metals [112, 113], retinoic acid [55, 114] and anticonvulsants [115, 116], lead to the elucidation of *in vitro* mechanisms of action, setting also the background for building *in vivo* and *in vitro* correlations and cross-model comparisons.

5.2. *Tools for analysis of transcriptomic data*

The concept of performing transcriptomics in one single experiment using the mRNA array chip technology is based on the rule of hybridization between nucleic acids. All the genes (of the tested species) are represented on the chip by oligonucleotides, amounting to tens of thousands of genes on a single chip, which are immobilized on a solid matrix [117, 118]. Applying fluorescence labels on the biological samples under assessment, the levels of hybridization can be quantified and provide a measure of the expression level of individual genes [119]. The fluorescent signal is read by a scanner and is translated to the effect of the compound on the gene expression [120, 121].

Microarrays studies generate big data sets, in which the information about the specific effects of the tested compounds to gene expression responses is hidden. Once the data set is complete, quality control assessment is required. Normalization of data is also a crucial step for correcting the distribution of intensity values due to technical factors rather than biological variations [122, 123]. The application of statistical stringency criteria, such as *p*-value and a false discovery rate (FDR), for identifying the significance of the regulated genes based on the degree of similarity between the exposed and control groups, is the tool to handle this set of data [124]. The further application of fold change (FD) of gene expression as an additional cutoff value provides a statistical tool to select relevant gene expression changes, taking into account the margin of difference in the expression of genes between exposed and control conditions. The visualization of the relationship of the significantly identified genes is performed with hierarchical clustering and principle component analysis (PCA) methods [125, 126]. The hierarchical clustering better describes the similarities of changes in the gene expression responses, while PCA illustrates the variance due to exposure conditions [127, 128]. Both these methods are classification approaches.

For individual gene annotation and functional analysis, data could be manually obtained from databases such as National Center for Biotechnology Information (NCBI) [129], Gene

Ontology (GO) [129, 130], Comparative Toxicogenome Database (CTD) [131], GeneCards [132] and the Rat Genome Database (RGD) [133].

For practical reasons (size of data) and for interpreting biological relationships between the genes, the whole set of the statistically significant identified genes could be annotated to biological processes, molecular functions (GO) and pathways (KEGG and Wiki Pathways) with the Database for Annotation, Visualization and Integrated Discovery (DAVID) [134-136]. For studying the specific connections of genes within a gene-set, pathway analysis with STITCH, STRING and Wiki Pathways is performed. The visualization of the pathway analysis could be performed with publicly available programs, such as Cytoscape [137] and PathVisio [138] or commercially available tools, such as MetaCore and Ingenuity Pathway Analysis Tool [139].

All the aforementioned tools use a predefined set of genes to identify the molecular functions and pathways that might be involved in the gene-set of question. Tox-profiler and Gene Set Enrichment Analysis (GSEA) programs use the whole expression data set for determining the distribution of the effect of genes, to which their functional role in a pathway is predefined [140, 141]. Following this methodology, even individual genes that do not meet the applied statistical stringency criteria, but with important roles in GO or pathways, could be identified [141-143].

6. The role of retinoic acid in developing embryos

Retinoic acid (RA) is the active metabolite of vitamin A (retinol) [144]. The conversion of vitamin A to RA is a crucial time- and place- dependent event influenced by homeostasis, which is regulated by a cascade of synthesizing and metabolizing enzymes [108, 114, 144, 145]. RA is essential during any developmental and reproductive phase of mammalian species for directing the growth and differentiation of cells, tissues and organs [146]. Deficiency or overload of RA could lead to adverse morphological outcomes in embryos, children or adults [147].

In the 1930s and 1940s, maternal vitamin A deficiency was suspected to cause abnormalities of skeleton, eye and heart in the rat offspring [8, 148]. Later, overdosing of RA was found to be the cause of teratogenicity also in rats including exencephaly, hydrocephaly, cleft palate, facial abnormalities, and spina bifida [148, 149]. In the early 1980s, vitamin A supplements were prescribed to pregnant women, leading to birth defects [150] and, consequently, RA was established as human teratogen causing craniofacial abnormalities [151, 152], heart dysfunction [153] and hypoplasia of thymus [154]. Due to the variety of morphological alterations that either deficiency or overload of RA caused, its importance in

embryogenesis was considered. In the late 1980s, RA was characterized as a morphogen, highlighting its role as regulator of growth and differentiation during embryogenesis [155]. The balanced level of RA drives the patterning of an early developing embryo along the anterior-posterior axis [156]. RA is further located in the paraxial mesoderm of embryos undergoing neurulation and is involved in caudal development as well as brain segmentation and neural differentiation [147, 157]. Additionally, its presence in neural crest cell development and differentiation determines the normal growth of peripheral tissues and organs, as neural crest cells are “donor” cells, directed by RA and contribute to the development of other regions, such as craniofacial, branchial arches, heart and limbs, initiated by this cell migration [158-160]. Concluding, the balance of synthesis and metabolism of RA determines whether it acts as a morphogen or a teratogen in vertebrates. Interestingly, some xenobiotics, for instance azoles [161] and ethanol [162] have been suggested to directly or indirectly disturb the RA balance and lead to RA-related adverse morphological outcomes [146].

7. Azole fungicides – a possible connection with retinoic acid

Azoles form a class of chemicals with antifungal activity and wide applicability in the clinical and agricultural domain [161]. Azoles are sub-divided into imidazoles and triazoles, which contain two and three nitrogen atoms in the five-membered azole ring, respectively. The first report on the antifungal activity of an azole compound appeared in the early 1940s, while more azole compounds were further investigated for this capacity in the 1960s, after the introduction of three novel compounds clotrimazole, miconazole and econazole. Miconazole was the first approved azole in 1978. Imidazoles, the firstly synthesized azoles, were replaced by triazoles after reports concerning their incapability to completely treat persistent fungal infections. Additionally, it was noticed that triazoles have a wider spectrum of applicability, while the induced toxicity was limited compared to imidazoles [163]. On the other hand, since the use of azoles was increased, doubts about their safety arose, with additional focus on their ability to cross the placenta and affect the unborn child [161]. Therefore, more studies on the mechanism of their fungicidal and toxicological mode of action were initiated.

The mechanism of fungicidal action is based on inhibiting the fungal *Cyp51*, the catalyst for converting lanosterol to ergosterol. Elimination of the ergosterol biosynthesis leads to instability and permeability of the cell membrane, resulting in fungal cell death [164-166]. Due to the high conservation of enzymes among the biological systems, mammalian *Cyp51* is also affected during treatment with azoles [167], disturbing the sterol biosynthesis pathway. However, the toxicological mechanism of action of azoles is suggested to be

correlated with the disturbance of *Cyp26a1* function, which is a key regulator of RA homeostasis in mammalian systems by metabolizing excess levels of RA [55, 168, 169]. A variety of similar teratogenic outcomes, including craniofacial malformations, abnormalities in brain segmentation and branchial arches formation, was detected when *in vivo* and *in vitro* embryos were exposed to either azoles or RA, suggesting that they might share the same mode of embryotoxicity [109, 114]. Additional studies on the level of the transcriptome of *in vitro* systems, such as rat WEC [109], EST [110] and ZET [108], supported this theory, showing the upregulation of *Cyp26a1* and other RA related genes upon exposure to azoles.

Since there are limitations on the available *in vivo* data concerning their relative potencies as to developmental toxicity of azoles, more studies are needed. The additional implementation of molecular endpoints to elucidate their mechanism of action may improve the mechanistic understanding of differences in azole embryotoxicity and can be valuable for new risk assessment approaches [108-110].

In the present thesis, twelve azoles (nine known and three new compounds) (Figure 4) were evaluated regarding their developmental toxicity profile in the rat WEC and EST enabling comparison with their *in vivo* potencies (Table 2). Table 2 summarizes the developmental toxicity data of the nine known azoles, including information about the duration of exposure, the doses to which animals were exposed by gavage, as well as the number of fetuses and litters that were examined and the number or percentage of these that showed morphological and/or functional alterations. Since we observed a variety of morphological alterations, Table 2 includes also the morphological endpoint that was considered for each compound for further calculating the dose at which there was a 10% extra incidence of malformations (BMD_{10}). Three of the tested azoles are novel compounds, which BASF SE kindly provided to us together with their *in vivo* prenatal developmental toxicity data. For concluding upon the *in vivo* potency of the tested azoles and given the limited available data of the three novel compounds, a qualitative *in vivo* potency ranking concept was applied in the present thesis. For implementing this approach of *in vivo* analysis, the profiles of the tested compounds were characterized as potent, moderate and weak or non-potent, as illustrated in the last column of Table 2.

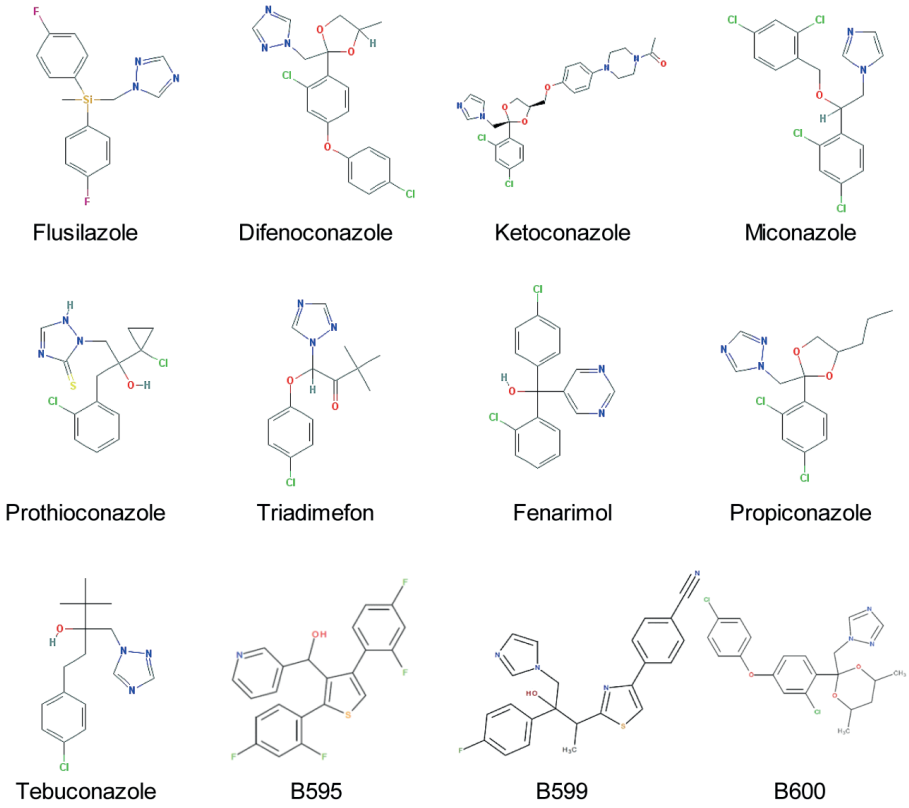


Figure 4: *Chemical structures of the twelve azoles included in this thesis*

Table 2: Developmental toxicity after *in vivo* rat exposure to the twelve azoles under assessment

Compounds	Days of exposure	Dose (mg/kg bw/day)	No. of fetuses/litters	Incidence of morphological alterations in fetuses/litters	Morphological endpoint	Potency group
Flusilazole	GD 6-15	0 0.4 2 10 50 250	-/- -/- -/- -/- -/- -/-	0/0 5/5 2/2 5/5 5/4 4/2	Absence of renal papilla [170]	Potent
Difenoconazole	GD 6-15	0 2 20 100 200	182/25 176/25 172/24 168/23 160/24	0/0 1/1 0/0 2/2 4/3	Ossification, deformation thoracic [171]	Weak
Ketoconazole	GD 6-17	0 25 75	-/22 4/22 -/3	-/0 -/3 -/3	Cleft palate [172]	Potent
Miconazole	GD 7-17	0 10 30 100	128/34 122/32 158/26 144/21	0/1 0/0 2/0 0/0	Skeletal malformations [173]	Intermediate
Prothioconazole	GD 6-19	0 20 80 750	-/- -/- -/- -/-	23.5%/95.2% 18.2%/77.8% 27.6%/88.9% 33.6%/95.7%	Rudimentary ribs [174]	Intermediate
Triadimefon	GD 6-15	0 10 30 100	48/20 52/17 54/19 84/22	1/1 16/10 13/10 5719	Supernumerary ribs [175]	Intermediate
Fenarimol	GD 6-15	0 5 13 35	-/- -/- -/- -/-	9%/25% 0%/0% 0%/0% 30%/62%	Hydronephrosis [176]	Intermediate
Propiconazole	GD 6-15	0 30 90 300	129/22 136/21 146/22 137/22	0/0 1/1 4/4 53/16	Rudimentary ribs [177]	Weak
Tebuconazole	GD 6-15	0 30 60 120	144/24 137/24 127/22 116/24	29/14 40/17 38/17 48/20	Supernumery ribs, ossification [178]	Intermediate
B595	GD 6-19	0 200 600	95/10 74/8 †	1/1 0/0 †	No morphological alteration (BASF)	Weak
B599	GD 6-19	0 100 250	95/10 99/10 †	1/1 12/2 †	Cleft palate (BASF)	Potent
B600	GD 6-19	0 200 600	95/10 99/10 104/10	1/1 0/0 5/2	Cleft palate (BASF)	Intermediate

-: data not available; †: lethality of fetuses and litters

8. Objective and outline of this thesis

8.1. *Objective of the thesis*

The objective of the research described in this thesis is to improve the detection of developmental toxicity of azole compounds with *in vitro* models (WEC and EST) and enhance the knowledge on the molecular level providing a mechanistic insight into the embryotoxicity of these compounds. Both the WEC and EST are validated *in vitro* methods for studying the developmental toxic capacity of many chemical substances. The WEC is based on exposing embryos to chemicals starting on gestational day 10 for 48 hours, while the major part of organogenesis occurs, similar to the *in vivo* situation. Continuous monitoring and final morphological evaluation of the embryonic development (including endpoints of both development and differentiation) are considered for concluding about the embryotoxicity of chemicals. Similarly, following a 10-day protocol, in the EST, stem cells are exposed to chemicals and their differentiation to contracting cardiomyocytes is the endpoint for concluding upon the induced embryotoxicity. The addition of the BeWo model, which mimics the placental barrier, may enhance the predictive value of both developmental toxicity tests by combining relative placental transfer rates with the classical endpoints of WEC and EST assays. Previous studies already demonstrated that this combination with BeWo assay data increased the accuracy of EST based predictions [97, 98]. The evaluation of global or specific gene expression changes with microarrays (transcriptomics) and Real Time-PCR could also increase the mechanistic knowledge, as well as the sensitivity and predictability of both WEC and EST assays [52, 102, 110]. The consequent identification of a set of biomarkers for the toxicological and pharmacological mode of action may improve the characterization of the embryotoxicity of compounds, and the optimization of development new chemicals, while reducing animal experimentation. The choice of azoles as the chemical group under assessment is based on their potential to induce teratogenicity in a dose dependent manner, while they are widely used in medicine and agriculture as fungicides. Moreover, previous studies suggested that azoles and RA share the same morphological teratogenic outcome, revealing also similar gene expression changes [114]. Overall, the implementation of alternative *in vitro* assays, together with toxicokinetic data and combined with molecular-based approaches may be valuable for improving the mechanistic elucidation of the *in vivo* developmental toxicity of azoles, providing a testing approach that notably eliminates or reduces the use of animals. A schematic overview of the experiments included in this thesis is illustrated in Figure 5.

8.2. Outline of the thesis

Considering that embryotoxic responses are critically dependent on the timing of exposure during embryo development, in **chapter 2** we examined the time- dependent developmental effects in the rat WEC exposed to flusilazole, and their link to RA mediated pathways. To this end, we assessed the effects of 4-hour exposure of WEC rat embryos to 300μM FLU during four developmental time windows (0-4, 4-8, 24-28 and 44-48 h), evaluating morphological parameters, expression and localization of five gene-biomarkers of the RA, differentiation and sterol biosynthesis pathways.

Subsequently, in **chapter 3** we compared the morphological changes in the WEC induced by exposure to six azoles with their early (0-4 h) gene expression profiling (transcriptomics) at equipotent concentrations for any of the tested compounds. Additionally, we studied the differences in gene signatures after exposing the same system for a longer period (0-24 h). Considering that toxicological responses are much dependent on the applied dose or concentration, we performed also a dose-response assessment of the gene regulation after shortly (0-4 h) exposing rat WEC to flusilazole.

In **chapter 4**, we evaluated the gene specific responses by implementing the transcriptomics approach in the WEC upon exposure to six additional azoles for 4 hours (0-4 h) and we combined them with previously (obtained from chapter 3) collected transcriptomics data of the WEC. Applying the identified gene-sets, indicators of toxicological and fungicidal mechanism of action, we compared the morphological adverse outcomes of the tested twelve azoles in both *in vivo* and WEC *in vitro* systems, with the responses of the selected molecular pathways.

Chapter 5 provides a combined analysis of the twelve azoles tested in the WEC and EST system. The addition of placental transfer rates (toxicokinetics) and re-calculation of effective concentrations of the twelve azoles in both *in vitro* systems are taken into account for correlations with the *in vivo* collected data. Supplementary targeted gene expression was evaluated for revealing the usefulness of pre-selected biomarkers of developmental toxicity and fungicidal action.

Chapter 6 summarizes the results of the previous chapters of this thesis and provides a discussion on alternative *in vitro* approaches for testing the developmental toxicity of chemical substances with greater focus on azoles. Emphasis is also given on the implementation of transcriptomics as a tool for increasing the predictability of *in vitro* techniques. Future perspectives for improving the current testing approaches in developmental toxicology are also presented.

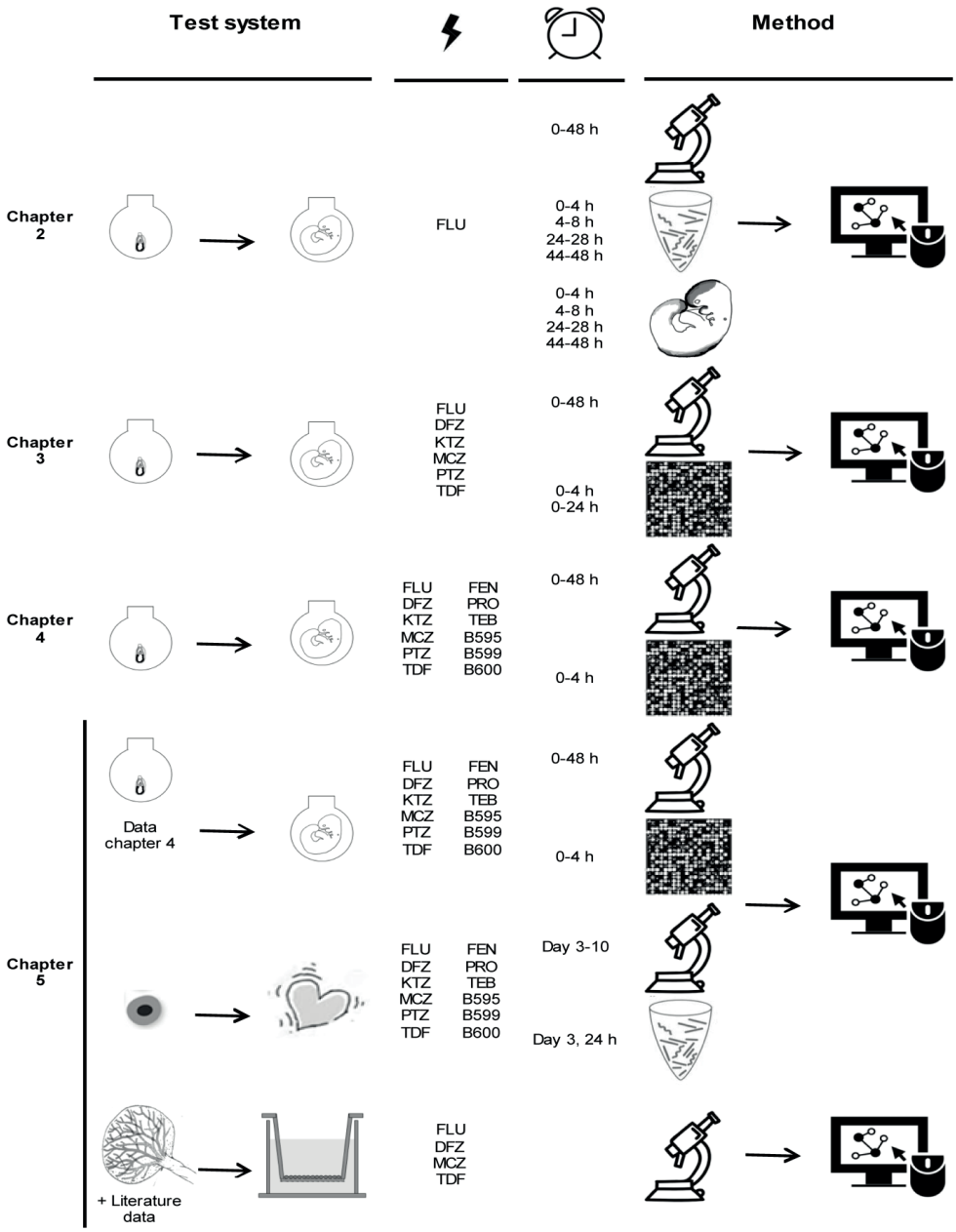


***Figure 5**: Schematic overview of the experimental designs as described in each chapter of this thesis, including information for the test system or the combination of them, the exposure conditions (compounds and duration of treatment) and the methods of analysis.*

References

1. Adler, S., et al., *Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010.* Arch Toxicol, 2011. **85**(5): p. 367-485.
2. Spielmann, H., *Reproduction and development.* Environ Health Perspect, 1998. **106 Suppl 2**: p. 571-6.
3. Spielmann, H., *Predicting the risk of developmental toxicity from in vitro assays.* Toxicol Appl Pharmacol, 2005. **207**(2 Suppl): p. 375-80.
4. Spielmann, H., *The way forward in reproductive/developmental toxicity testing.* Altern Lab Anim, 2009. **37**(6): p. 641-56.
5. Friedman, J.M., *The principles of teratology: are they still true?* Birth Defects Res A Clin Mol Teratol, 2010. **88**(10): p. 766-8.
6. Wilson, J.G., *Environment and birth defects [by] James G. Wilson*. Environmental sciences research report. 1973, New York: Academic Press.
7. Ujhazy, E., et al., *Teratology - past, present and future.* Interdiscip Toxicol, 2012. **5**(4): p. 163-8.
8. WARKANY, J. and R.C. NELSON, *APPEARANCE OF SKELETAL ABNORMALITIES IN THE OFFSPRING OF RATS REARED ON A DEFICIENT DIET.* Science, 1940. **92**(2391): p. 383-384.
9. Lenz, W., *A short history of thalidomide embryopathy.* Teratology, 1988. **38**(3): p. 203-215.
10. Greek, R., N. Shanks, and M.J. Rice, *The History and Implications of Testing Thalidomide on Animals.* Journal of Philosophy Science and Law, 2011. **11**.
11. OECD, *Draft guidance document on mammalian reproductive toxicity testing and assessement.* OECD Environment, Health and Safety Publications. , 2007. **Series on Testing and Assessment No. 43**.
12. OECD, *Test No. 414: Prenatal Development Toxicity Study*. OECD Publishing.
13. OECD, *Test No. 415: One-Generation Reproduction Toxicity Study*. OECD Publishing.
14. OECD, *Test No. 416: Two-Generation Reproduction Toxicity*. OECD Publishing.
15. OECD, *Test No. 421: Reproduction/Developmental Toxicity Screening Test*. OECD Publishing.
16. OECD, *Test No. 422: Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test*. OECD Publishing.
17. OECD, *Test No. 426: Developmental Neurotoxicity Study*. OECD Publishing.
18. van der Jagt K., M.S., Tørsløv J. & de Bruijn J. , *Alternative approaches can reduce the use of test animals under REACH* EUROPEAN COMMISSION DIRECTORATE GENERAL JRC JOINT RESEARCH CENTRE Institute for Health and Consumer Protection, http://home.kpn.nl/reach/downloads/reducingtheuseoftestanimalsunderreachihcprepor.pdf, 2004.
19. Lilienblum, W., et al., *Alternative methods to safety studies in experimental animals: role in the risk assessment of chemicals under the new European Chemicals Legislation (REACH).* Arch Toxicol, 2008. **82**(4): p. 211-36.
20. Hofer, T., et al., *Animal testing and alternative approaches for the human health risk assessment under the proposed new European chemicals regulation.* Arch Toxicol, 2004. **78**(10): p. 549-64.
21. Piersma, A.H., *Alternative methods for developmental toxicity testing.* Basic Clin Pharmacol Toxicol, 2006. **98**(5): p. 427-31.
22. Scialli, A.R., *The challenge of reproductive and developmental toxicology under REACH.* Regul Toxicol Pharmacol, 2008. **51**(2): p. 244-50.
23. Russell, W.M.S. and R.L. Burch, *The principles of humane experimental technique.* 1959.
24. Augustine-Rauch, K., C.X. Zhang, and J.M. Panzica-Kelly, *In vitro developmental toxicology assays: A*

review of the state of the science of rodent and zebrafish whole embryo culture and embryonic stem cell assays. Birth Defects Res C Embryo Today, 2010. **90**(2): p. 87-98.

25. Scialli, A.R. and A.J. Guikema, *REACH and reproductive and developmental toxicology: still questions.* Syst Biol Reprod Med, 2012. **58**(1): p. 63-9.
26. Sogorb, M.A., et al., *An integrated approach for detecting embryotoxicity and developmental toxicity of environmental contaminants using in vitro alternative methods.* Toxicol Lett, 2014. **230**(2): p. 356-67.
27. Lee, H.Y., et al., *Alternative models in developmental toxicology.* Syst Biol Reprod Med, 2012. **58**(1): p. 10-22.
28. Genschow, E., et al., *Validation of the embryonic stem cell test in the international ECVAM validation study on three in vitro embryotoxicity tests.* Altern Lab Anim, 2004. **32**(3): p. 209-44.
29. Spielmann, H., et al., *Validation of the rat limb bud micromass test in the international ECVAM validation study on three in vitro embryotoxicity tests.* Altern Lab Anim, 2004. **32**(3): p. 245-74.
30. Piersma, A.H., et al., *Validation of the postimplantation rat whole-embryo culture test in the international ECVAM validation study on three in vitro embryotoxicity tests.* Altern Lab Anim, 2004. **32**(3): p. 275-307.
31. Genschow, E., et al., *The ECVAM international validation study on in vitro embryotoxicity tests: results of the definitive phase and evaluation of prediction models. European Centre for the Validation of Alternative Methods.* Altern Lab Anim, 2002. **30**(2): p. 151-76.
32. Hartung, T., S. Hoffmann, and M. Stephens, *Mechanistic validation.* Altex, 2013. **30**(2): p. 119-30.
33. Piersma, A.H., et al., *A critical appraisal of the process of regulatory implementation of novel in vivo and in vitro methods for chemical hazard and risk assessment.* Crit Rev Toxicol, 2014. **44**(10): p. 876-94.
34. New, D.A.T. and K.F. Stein, *Cultivation of post-implantation mouse and rat embryos on plasma clots.* Journal of Embryology and Experimental Morphology, 1964. **12**(1): p. 101-111.
35. Waddington, C.H. and A.J. Waterman, *The Development in vitro of Young Rabbit Embryos.* J Anat, 1933. **67**(Pt 3): p. 355-70.
36. New, D.A.T., P.T. Coppola, and D.L. Cockroft, *Comparison of growth in vitro and in vivo of post-implantation rat embryos.* Journal of Embryology and Experimental Morphology, 1976. **36**(1): p. 133-144.
37. New, D.A.T., *WHOLE-EMBRYO CULTURE AND THE STUDY OF MAMMALIAN EMBRYOS DURING ORGANOGENESIS.* Biological Reviews, 1978. **53**(1): p. 81-122.
38. Schmid, B.P., *Xenobiotic influences on embryonic differentiation, growth and morphology in vitro.* Xenobiotica, 1985. **15**(8-9): p. 719-26.
39. Brown, N.A., *Teratogenicity testing in vitro: status of validation studies.* Arch Toxicol Suppl, 1987. **11**: p. 105-14.
40. Brown, N.A. and S. Fabro, *Quantitation of rat embryonic development in vitro: a morphological scoring system.* Teratology, 1981. **24**(1): p. 65-78.
41. Bechter, R. and H. Schon, *Use of the whole-embryo culture system in drug safety assessment?* Toxicol In Vitro, 1988. **2**(3): p. 195-203.
42. Cicurel, L. and B.P. Schmid, *Postimplantation embryo culture for the assessment of the teratogenic potential and potency of compounds.* Experientia, 1988. **44**(10): p. 833-40.
43. Flynn, T.J., *Teratological research using in vitro systems. I. Mammalian whole embryo culture.* Environmental Health Perspectives, 1987. **72**: p. 203-210.
44. Smith, M.K., et al., *A selection of candidate compounds for in vitro teratogenesis test validation.* Teratog Carcinog Mutagen, 1983. **3**(6): p. 461-80.
45. Tesh, J.M., *The application of whole-embryo culture to new product development.* Toxicology in Vitro, 1988. **2**(3): p. 189-194.
46. Van Maele-Fabry, G. and J.J. Picard, *Evaluation of the embryotoxic potential*

of ten chemicals in the whole mouse embryo culture. Teratology, 1987. **36**(1): p. 95-106.

47. Bechter, R. and B.P. Schmid, *Teratogenicity in vitro-A comparative study of four antimycotic drugs using the whole-embryo culture system.* Toxicol In Vitro, 1987. **1**(1): p. 11-5.
48. Ellis-Hutchings, R.G. and E.W. Carney, *Whole embryo culture: a "New" technique that enabled decades of mechanistic discoveries.* Birth Defects Research Part B: Developmental and Reproductive Toxicology, 2010. **89**(4): p. 304-312.
49. Piersma, A.H., *Whole embryo culture and toxicity testing.* Toxicol In Vitro, 1993. **7**(6): p. 763-8.
50. Fang, H., et al., *Transcriptome analysis of early organogenesis in human embryos.* Dev Cell, 2010. **19**(1): p. 174-84.
51. Irie, N. and S. Kuratani, *Comparative transcriptome analysis reveals vertebrate phylotypic period during organogenesis.* Nature Communications, 2011. **2**: p. 248.
52. Robinson, J.F., A. Verhoef, and A.H. Piersma, *Transcriptomic analysis of neurulation and early organogenesis in rat embryos: an in vivo and ex vivo comparison.* Toxicol Sci, 2012. **126**(1): p. 255-66.
53. Webster, W.S., P.D. Brown-Woodman, and H.E. Ritchie, *A review of the contribution of whole embryo culture to the determination of hazard and risk in teratogenicity testing.* Int J Dev Biol, 1997. **41**(2): p. 329-35.
54. Flick, B., et al., *Embryotoxic potential of N-methyl-pyrrolidone (NMP) and three of its metabolites using the rat whole embryo culture system.* Toxicol Appl Pharmacol, 2009. **237**(2): p. 154-67.
55. Luijten, M., et al., *Application of a metabolizing system as an adjunct to the rat whole embryo culture.* Toxicol In Vitro, 2008. **22**(5): p. 1332-6.
56. Van Maele-Fabry, G., F. Delhaise, and J.J. Picard, *Morphogenesis and quantification of the development of post-implantation mouse embryos.* Toxicol In Vitro, 1990. **4**(2): p. 149-56.
57. A., M., *Der Lymphozyt als gemeinsame Stammzelle der verschiedenen Blutelemente in der embryonalen Entwicklung und im postfetalen Leben der Säugetiere.* Folia Haematologica 1909. **8**: p. 125-134.
58. Becker, A.J., C.E. Mc, and J.E. Till, *Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells.* Nature, 1963. **197**: p. 452-4.
59. Till, J.E. and C.E. Mc, *A direct measurement of the radiation sensitivity of normal mouse bone marrow cells.* Radiat Res, 1961. **14**: p. 213-22.
60. Kahan, B.W. and B. Ephrussi, *Developmental Potentialities of Clonal In Vitro Cultures of Mouse Testicular Teratoma2.* JNCI: Journal of the National Cancer Institute, 1970. **44**(5): p. 1015-1036.
61. Martin, G.R. and M.J. Evans, *The morphology and growth of a pluripotent teratocarcinoma cell line and its derivatives in tissue culture.* Cell, 1974. **2**(3): p. 163-72.
62. Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos.* Nature, 1981. **292**(5819): p. 154-6.
63. Martin, G.R., *Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells.* Proc Natl Acad Sci U S A, 1981. **78**(12): p. 7634-8.
64. Thomson, J.A., et al., *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-7.
65. Keller, G., *Embryonic stem cell differentiation: emergence of a new era in biology and medicine.* Genes Dev, 2005. **19**(10): p. 1129-55.
66. Spielmann, H., et al., *The embryonic stem cell test (EST), an in vitro embryotoxicity test using two permanent mouse cell lines: 3T3 fibroblasts and embryonic stem cells.* In Vitro Toxicology, 1997. **10**: p. 119-127.
67. Hartung, T., et al., *A modular approach to the ECVAM principles on test validity.* Altern Lab Anim, 2004. **32**(5): p. 467-72.

68. Marx-Stoelting, P., et al., *A review of the implementation of the embryonic stem cell test (EST). The report and recommendations of an ECVAM/ReProTect Workshop.* Altern Lab Anim, 2009. **37**(3): p. 313-28.

69. Spielmann, H., et al., *The practical application of three validated in vitro embryotoxicity tests. The report and recommendations of an ECVAM/ZEBET workshop (ECVAM workshop 57).* Altern Lab Anim, 2006. **34**(5): p. 527-38.

70. Inselman , A.L., et al., *Reevaluation of the embryonic stem cell test.* International Journal of Regulatory Science, 2013. **1**(1): p. 32-49.

71. Piersma, A.H., et al., *Evaluation of an alternative in vitro test battery for detecting reproductive toxicants.* Reprod Toxicol, 2013. **38**: p. 53-64.

72. Li, H., et al., *Assessment of an in vitro transport model using BeWo b30 cells to predict placental transfer of compounds.* Arch Toxicol, 2013. **87**(9): p. 1661-9.

73. Suter-Dick, L., et al., *Stem cell-derived systems in toxicology assessment.* Stem Cells Dev, 2015. **24**(11): p. 1284-96.

74. Buesen, R., et al., *Trends in improving the embryonic stem cell test (EST): an overview.* Altex, 2004. **21**(1): p. 15-22.

75. Fox, H., *A contemporary view of the human placenta.* Midwifery, 1991. **7**(1): p. 31-9.

76. Benirschke , K., P. Kaufmann, and R. Baergen, *Pathology of the Human Placenta.* Springer New York, 2006.

77. Prouillac, C. and S. Lecoeur, *The role of the placenta in fetal exposure to xenobiotics: importance of membrane transporters and human models for transfer studies.* Drug Metab Dispos, 2010. **38**(10): p. 1623-35.

78. van der Aa, E.M., et al., *Mechanisms of drug transfer across the human placenta.* Pharm World Sci, 1998. **20**(4): p. 139-48.

79. Griffiths, S.K. and J.P. Campbell, *Placental structure, function and drug transfer.* Continuing Education in Anaesthesia Critical Care & Pain, 2015. **15**(2): p. 84-89.

80. Syme, M.R., J.W. Paxton, and J.A. Keelan, *Drug transfer and metabolism by the human placenta.* Clin Pharmacokinet, 2004. **43**(8): p. 487-514.

81. Myllynen, P. and K. Vähäkangas, *Placental transfer and metabolism: An overview of the experimental models utilizing human placental tissue.* Toxicology in Vitro, 2013. **27**(1): p. 507-512.

82. Herman, N.L., et al., *Transfer of methohexital across the perfused human placenta.* J Clin Anesth, 2000. **12**(1): p. 25-30.

83. Hakkola, J., et al., *Xenobiotic-metabolizing cytochrome P450 enzymes in the human feto-placental unit: role in intrauterine toxicity.* Crit Rev Toxicol, 1998. **28**(1): p. 35-72.

84. Miller, R.K., et al., *The role of the human placenta in embryonic nutrition. Impact of environmental and social factors.* Ann N Y Acad Sci, 1993. **678**: p. 92-107.

85. Fabian, E., et al., *Activities of xenobiotic metabolizing enzymes in rat placenta and liver in vitro.* Toxicol In Vitro, 2016. **33**: p. 174-9.

86. Blaauboer, B.J., *Biokinetic modeling and in vitro-in vivo extrapolations.* J Toxicol Environ Health B Crit Rev, 2010. **13**(2-4): p. 242-52.

87. Coecke, S., et al., *Toxicokinetics as a key to the integrated toxicity risk assessment based primarily on non-animal approaches.* Toxicol In Vitro, 2013. **27**(5): p. 1570-7.

88. Strikwold, M., et al., *Combining in vitro embryotoxicity data with physiologically based kinetic (PBK) modelling to define in vivo dose-response curves for developmental toxicity of phenol in rat and human.* Arch Toxicol, 2013. **87**(9): p. 1709-23.

89. Pattillo, R.A. and G.O. Gey, *The establishment of a cell line of human hormone-synthesizing trophoblastic cells in vitro.* Cancer Res, 1968. **28**(7): p. 1231-6.

90. Bode, C.J., et al., *In Vitro Models for Studying Trophoblast Transcellular Transport.* Methods in molecular medicine, 2006. **122**: p. 225-239.

91. Poulsen, M.S., et al., *Modeling placental transport: Correlation of in vitro BeWo cell permeability and ex vivo human placental perfusion.*

Toxicology in Vitro, 2009. **23**(7): p. 1380-1386.

92. Wolfe, M.W., *Culture and transfection of human choriocarcinoma cells.* Methods Mol Med, 2006. **121**: p. 229-39.

93. Mitra, P. and K.L. Audus, *In Vitro Models and Multidrug Resistance Mechanisms of the Placental Barrier*, in *Drug Absorption Studies: In Situ, In Vitro and In Silico Models*, C. Ehrhardt and K.-J. Kim, Editors. 2008, Springer US: Boston, MA. p. 368-396.

94. Avery, M.L., C.E. Meek, and K.L. Audus, *The presence of inducible cytochrome P450 types 1A1 and 1A2 in the BeWo cell line.* Placenta, 2003. **24**(1): p. 45-52.

95. Pavek, P., et al., *Examination of Glucocorticoid receptor alpha-mediated transcriptional regulation of P-glycoprotein, CYP3A4, and CYP2C9 genes in placental trophoblast cell lines.* Placenta, 2007. **28**(10): p. 1004-11.

96. Hutson, J.R., et al., *The Human Placental Perfusion Model: A Systematic Review and Development of a Model to Predict In Vivo Transfer of Therapeutic Drugs.* Clinical Pharmacology & Therapeutics, 2011. **90**(1): p. 67-76.

97. Li, H., et al., *Extended evaluation on the ES-D3 cell differentiation assay combined with the BeWo transport model, to predict relative developmental toxicity of triazole compounds.* Arch Toxicol, 2016. **90**(5): p. 1225-37.

98. Li, H., et al., *Use of the ES-D3 cell differentiation assay, combined with the BeWo transport model, to predict relative in vivo developmental toxicity of antifungal compounds.* Toxicol In Vitro, 2015. **29**(2): p. 320-8.

99. Daston, G.P., *Genomics and developmental risk assessment.* Birth Defects Res A Clin Mol Teratol, 2007. **79**(1): p. 1-7.

100. Daston, G.P. and J.M. Naciff, *Predicting developmental toxicity through toxicogenomics.* Birth Defects Res C Embryo Today, 2010. **90**(2): p. 110-7.

101. Waters, M.D. and J.M. Fostel, *Toxicogenomics and systems toxicology: aims and prospects.* Nat Rev Genet, 2004. **5**(12): p. 936-48.

102. Robinson, J.F. and A.H. Piersma, *Toxicogenomic approaches in developmental toxicology testing.* Methods Mol Biol, 2013. **947**: p. 451-73.

103. Hartung, T., *Toxicology for the twenty-first century.* Nature, 2009. **460**(7252): p. 208-12.

104. Kramer, J.A., et al., *Acute molecular markers of rodent hepatic carcinogenesis identified by transcription profiling.* Chem Res Toxicol, 2004. **17**(4): p. 463-70.

105. van Dartel, D.A., et al., *Early gene expression changes during embryonic stem cell differentiation into cardiomyocytes and their modulation by monobutyl phthalate.* Reprod Toxicol, 2009. **27**(2): p. 93-102.

106. Miller, H.D., et al., *Anchoring Ethinylestradiol Induced Gene Expression Changes with Testicular Morphology and Reproductive Function in the Medaka.* PLOS ONE, 2012. **7**(12): p. e52479.

107. Naciff, J.M., et al., *Gene Expression Changes Induced in the Testis by Transplacental Exposure to High and Low Doses of 17α-Ethynyl Estradiol, Genistein, or Bisphenol A.* Toxicological Sciences, 2005. **86**(2): p. 396-416.

108. Hermsen, S.A., et al., *Concentration-response analysis of differential gene expression in the zebrafish embryotoxicity test following flusilazole exposure.* Toxicol Sci, 2012. **127**(1): p. 303-12.

109. Robinson, J.F., et al., *Triazole induced concentration-related gene signatures in rat whole embryo culture.* Reproductive Toxicology, 2012. **34**(2): p. 275-283.

110. van Dartel, D.A., et al., *Concentration-dependent gene expression responses to flusilazole in embryonic stem cell differentiation cultures.* Toxicol Appl Pharmacol, 2011. **251**(2): p. 110-8.

111. Goetz, A.K. and D.J. Dix, *Mode of action for reproductive and hepatic toxicity inferred from a genomic study of triazole antifungals.* Toxicol Sci, 2009. **110**(2): p. 449-62.

112. Robinson, J.F., et al., *Cadmium-Induced Differential Toxicogenomic Response in Resistant and Sensitive Mouse Strains Undergoing Neurulation.* Toxicological Sciences, 2009. **107**(1): p. 206-219.

113. Robinson, J.F., et al., *Arsenic- and Cadmium-Induced Toxicogenomic Response in Mouse Embryos Undergoing Neurulation.* Toxicology and applied pharmacology, 2011. **250**(2): p. 117-129.

114. Robinson, J.F., et al., *A comparison of gene expression responses in rat whole embryo culture and in vivo: time-dependent retinoic acid-induced teratogenic response.* Toxicol Sci, 2012. **126**(1): p. 242-54.

115. Kultima, K., et al., *Valproic Acid Teratogenicity: A Toxicogenomics Approach.* Environmental Health Perspectives, 2004. **112**(12): p. 1225-1235.

116. Hill, E.J., et al., *Effects of Lithium and Valproic Acid on Gene Expression and Phenotypic Markers in an NT2 Neurosphere Model of Neural Development.* PLOS ONE, 2013. **8**(3): p. e58822.

117. Sawada, H., K. Taniguchi, and K. Takami, *Improved toxicogenomic screening for drug-induced phospholipidosis using a multiplexed quantitative gene expression ArrayPlate assay.* Toxicology in Vitro, 2006. **20**(8): p. 1506-1513.

118. Duggan, D.J., et al., *Expression profiling using cDNA microarrays.* Nature Genetics 1999. **21**: p. 10 - 14.

119. Wang, E., *RNA amplification for successful gene profiling analysis.* Journal of Translational Medicine, 2005. **3**: p. 28-28.

120. Yang, Y.H., M.J. Buckley, and T.P. Speed, *Analysis of cDNA microarray images.* Briefings in Bioinformatics, 2001. **2**(4): p. 341-349.

121. Arteaga-Salas, J.M., et al., *An overview of image-processing methods for Affymetrix GeneChips.* Brief Bioinform, 2008. **9**(1): p. 25-33.

122. Smyth, G.K. and T. Speed, *Normalization of cDNA microarray data.* Methods, 2003. **31**(4): p. 265-73.

123. Bolstad, B.M., et al., *A comparison of normalization methods for high density oligonucleotide array data based on variance and bias.* Bioinformatics, 2003. **19**(2): p. 185-93.

124. Benjamini, Y. and Y. Hochberg, *Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing.* Journal of the Royal Statistical Society. Series B (Methodological), 1995. **57**(1): p. 289-300.

125. Pearson, K., *LIII. On lines and planes of closest fit to systems of points in space.* Philosophical Magazine, 1901. **2**(11): p. 559-572.

126. Eisen, M.B., et al., *Cluster analysis and display of genome-wide expression patterns.* Proc Natl Acad Sci U S A, 1998. **95**(25): p. 14863-8.

127. Shlens, J., *A Tutorial on Principal Component Analysis.* 2014.

128. Hayes, K.R. and C.A. Bradfield, *Advances in toxicogenomics.* Chem Res Toxicol, 2005. **18**(3): p. 403-14.

129. NCBI, *Database resources of the National Center for Biotechnology Information.* Nucleic Acids Res, 2013. **41**(Database issue): p. D8-d20.

130. The Gene Ontology, C., et al., *Gene Ontology: tool for the unification of biology.* Nature genetics, 2000. **25**(1): p. 25-29.

131. Davis, A.P., et al., *The Comparative Toxicogenomics Database: update 2017.* Nucleic Acids Res, 2017. **45**(D1): p. D972-d978.

132. Ben-Ari Fuchs, S., et al., *GeneAnalytics: An Integrative Gene Set Analysis Tool for Next Generation Sequencing, RNAseq and Microarray Data.* Omics, 2016. **20**(3): p. 139-51.

133. Shimoyama, M., et al., *The Rat Genome Database 2015: genomic, phenotypic and environmental variations and disease.* Nucleic Acids Res, 2015. **43**(Database issue): p. D743-50.

134. Kanehisa, M., et al., *KEGG: new perspectives on genomes, pathways, diseases and drugs.* Nucleic Acids Research, 2017. **45**(D1): p. D353-D361.

135. Kutmon, M., et al., *WikiPathways: capturing the full diversity of pathway knowledge.* Nucleic Acids Research, 2016. **44**(D1): p. D488-D494.

136. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.* Nat Protoc, 2009. **4**(1): p. 44-57.

137. Shannon, P., et al., *Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks.* Genome Research, 2003. **13**(11): p. 2498-2504.

138. Kutmon, M., et al., *PathVisio 3: An Extendable Pathway Analysis Toolbox.* PLOS Computational Biology, 2015. **11**(2): p. e1004085.

139. Krämer, A., et al., *Causal analysis approaches in Ingenuity Pathway Analysis.* Bioinformatics, 2014. **30**(4): p. 523-530.

140. Boorsma, A., et al., *T-profiler: scoring the activity of predefined groups of genes using gene expression data.* Nucleic Acids Research, 2005. **33**(Web Server issue): p. W592-W595.

141. Subramanian, A., et al., *Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles.* Proc Natl Acad Sci U S A, 2005. **102**(43): p. 15545-50.

142. Tarca, A.L., G. Bhatti, and R. Romero, *A Comparison of Gene Set Analysis Methods in Terms of Sensitivity, Prioritization and Specificity.* PLOS ONE, 2013. **8**(11): p. e79217.

143. Song, S. and M.A. Black, *Microarray-based gene set analysis: a comparison of current methods.* BMC Bioinformatics, 2008. **9**: p. 502.

144. Metzler, M.A. and L.L. Sandell, *Enzymatic Metabolism of Vitamin A in Developing Vertebrate Embryos.* Nutrients, 2016. **8**(12): p. 812.

145. Tonk, E.C., J.L. Pennings, and A.H. Piersma, *An adverse outcome pathway framework for neural tube and axial defects mediated by modulation of retinoic acid homeostasis.* Reprod Toxicol, 2015. **55**: p. 104-13.

146. Piersma, A.H., E.V. Hessel, and Y.C. Staal, *Retinoic acid in developmental toxicology: Teratogen, morphogen and biomarker.* Reprod Toxicol, 2017.

147. Rhinn, M. and P. Dolle, *Retinoic acid signalling during development.* Development, 2012. **139**(5): p. 843-58.

148. Warkany, J. and E. Schraffenberger, *Congenital malformations induced in rats by maternal vitamin a deficiency: I. defects of the eye.* Archives of Ophthalmology, 1946. **35**(2): p. 150-169.

149. Cohlan, S.Q., *Congenital anomalies in the rat produced by excessive intake of vitamin A during pregnancy.* Pediatrics, 1954. **13**(6): p. 556-67.

150. Rosa, F.W., *Megadoses of vitamin A.* Teratology, 1987. **36**(2): p. 272.

151. Morriss-Kay, G., *Retinoic acid and craniofacial development: molecules and morphogenesis.* Bioessays, 1993. **15**(1): p. 9-15.

152. Webster, W.S., et al., *Isotretinoin embryopathy and the cranial neural crest: an in vivo and in vitro study.* J Craniofac Genet Dev Biol, 1986. **6**(3): p. 211-22.

153. Pan, J. and K.M. Baker, *Retinoic acid and the heart.* Vitam Horm, 2007. **75**: p. 257-83.

154. Cohen, M., et al., *Thymic hypoplasia associated with isotretinoin embryopathy.* Am J Dis Child, 1987. **141**(3): p. 263-6.

155. Slack, J.M.W., *We have a morphogen!* Nature, 1987. **327**(6123): p. 553-554.

156. Shimozono, S., et al., *Visualization of an endogenous retinoic acid gradient across embryonic development.* Nature, 2013. **496**(7445): p. 363-6.

157. Maden, M., *Retinoids and spinal cord development.* J Neurobiol, 2006. **66**(7): p. 726-38.

158. Keyte, A. and M.R. Hutson, *The Neural Crest in Cardiac Congenital Anomalies.* Differentiation; Research in Biological Diversity, 2012. **84**(1): p. 25-40.

159. Martinez-Morales, P.L., et al., *FGF and retinoic acid activity gradients control the timing of neural crest cell emigration in the trunk.* J Cell Biol, 2011. **194**(3): p. 489-503.

160. Minoux, M. and F.M. Rijli, *Molecular mechanisms of cranial neural crest cell migration and patterning in craniofacial development.* Development, 2010. **137**(16): p. 2605-21.

161. Marotta, F. and G.M. Tiboni, *Molecular aspects of azoles-induced*

teratogenesis. Expert Opin Drug Metab Toxicol, 2010. **6**(4): p. 461-82.

162. Kane, M.A., et al., *Ethanol elevates physiological all-trans-retinoic acid levels in select loci through altering retinoid metabolism in multiple loci: a potential mechanism of ethanol toxicity.* Faseb j, 2010. **24**(3): p. 823-32.

163. Maertens, J.A., *History of the development of azole derivatives.* Clin Microbiol Infect, 2004. **10 Suppl 1**: p. 1-10.

164. Prasad, R., A.H. Shah, and M.K. Rawal, *Antifungals: Mechanism of Action and Drug Resistance.* Adv Exp Med Biol, 2016. **892**: p. 327-349.

165. Georgopapadakou, N.H., *Antifungals: mechanism of action and resistance, established and novel drugs.* Curr Opin Microbiol, 1998. **1**(5): p. 547-57.

166. White, T.C., K.A. Marr, and R.A. Bowden, *Clinical, cellular, and molecular factors that contribute to antifungal drug resistance.* Clin Microbiol Rev, 1998. **11**(2): p. 382-402.

167. Yoshida, Y., et al., *Sterol 14-Demethylase P450 (CYP51) Provides a Breakthrough for the Discussion on the Evolution of Cytochrome P450 Gene Superfamily.* Biochemical and Biophysical Research Communications, 2000. **273**(3): p. 799-804.

168. Giavini, E. and E. Menegola, *Are azole fungicides a teratogenic risk for human conceptus?* Toxicol Lett, 2010. **198**(2): p. 106-11.

169. Di Renzo, F., et al., *Early genetic control of craniofacial development is affected by the in vitro exposure of rat embryos to the fungicide triadimefon.* Birth Defects Research Part B: Developmental and Reproductive Toxicology, 2011. **92**(1): p. 77-81.

170. Lamontia CL, S.R., Alvarez L., *Embryo-fetal toxicity and teratogenicity study of INH-6573-39 by gavage in the rat.* Unpublished reports No. HLR 444-83 and HLR 142-84 from Haskell Laboratory for Toxicology and Industrial Medicine, Wilmington, Delaware, USA. Submitted to WHO by E.I. du Pont de Nemours & Co., Inc., Wilmington, Delaware, USA. Summarized in JMPR Monograph—Flusilazole, Pesticide residues in food: 1995 evaluations Part II Toxicological & EnvironmentEnvironment. http://www.inchem.org/documents/jmpr/jmpmono/v95pr08.htm, 1984.

171. Lochry, E.A., *Developmental toxicity study of CGA-169374 technical (FL-851406) administreted orally via gavage to Crl:COBS CD (SD) BR presumed pregnant rats.* Unpublished report No. 203-005 from Novartis Crop Protection AG., Basel, Switzerland and Argus Research Lab. Inc., Horsham, USA, Submitted to WHO by SYgenta Crop Protection AG, Basel, Switzerland. http://apps.who.int/iris/bitstream/10665/44064/1/9789241665230_eng.pdf?ua=1, 1987.

172. Tachibana, M.N., Y. and Monro, A.M., *Toxicology of fluconazole in experimental animals.* In Fromtling, R.A. Recent trends in the discovery, development, and evaluation of antifungal agents, JR Prous, Barcelona, 1987: p. 93-102.

173. Ito, C., Y. Shibutanil, K. Inoue, K. Nakano and H. Ohnishi, *Toxicologocal Studies of Miconazole (II) Teratological Studies of Miconazole in Rats* IYAKUHIN KENKYU, 1976. **7**(3): p. 367-376.

174. Stahl, B., *JAU 6476 - Developmental toxicity study in rats after oral administration. Unpublished report No. M-012279-01-1 from Bayer AG, Wuppertal, Germany. Submitted to WHO by Bayer CropScience AG, Germany.* *https://www.google.nl/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&ved=0ahUKEwjcjNCx5-HPAhWDrxoKHebSDzYQFgghMAE&url=http%3A%2F%2Fapps.who.int%2Fpesticide-residues-jmpr-database%2FDocument%2F110&usg=AFQjCNGpjQOE2xfb0QbWPWwA5-avbrC8kQ&sig2=ZFM7G3fgK4EdIA0e_zX0hQ&cad=rja.* 1997.

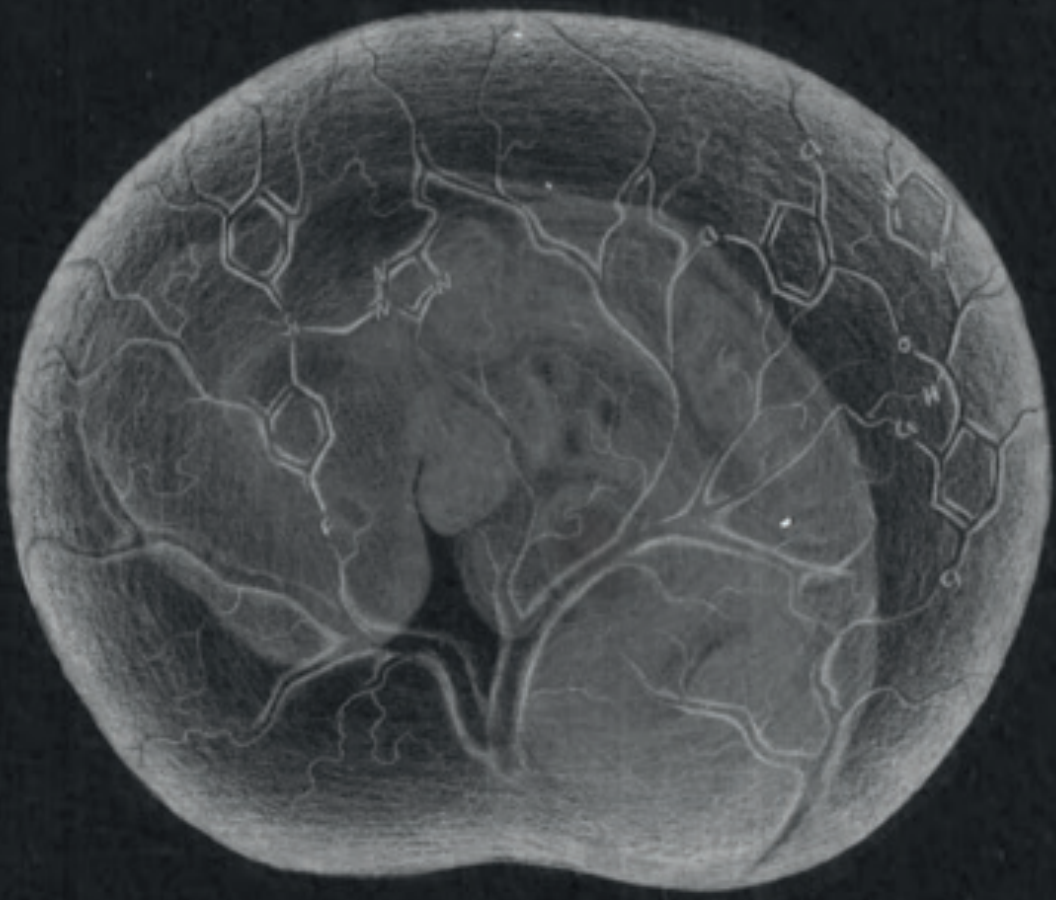
175. Unger, T.M., Van Goethem, D. & Shellenberger, *A teratological evaluation of Bayleton in mated female rats.* Midwest Research Institute, Report No. 324 to Mobay Chemical Corporation Agricultural Chemical Division submitted to WHO by Bayer AG. (Unpublished),

http://www.inchem.org/documents/jmpr/jmpmono/v83pr39.htm, 1982.

176. Hoffman, D.G., Owen, N.V. & Adams, E.R., *A teratology study with EL-222 in the rat.* Unpublished report No. R06279 form Lilly research Laboratories, USA. Submitted to WHO by DowElanco Europe, Wantage, Oxon, United Kingdom, 1980.

177. Giknis, M.L.A., *A teratology (segment II) study in rats.* Unpublished report no.: 86004 from Ciba-Geigy Pharmaceutical division, New Jersey. Submitted to WHO by Ciba-Geigy Ltd., Basle, Switzerland 1987.

178. Becker, H., Vogel, W. & Terrier, C., *Embryotoxicity study (including teratogenicity) with HWG 1608 technical in rat*, in *Unpublished report Ref. No. R 4451, prepared by Research & Consulting Co. AG (RCC), Itingen, Switzerland. Submitted to WHO by Bayern AG, Leverkusen, Germany*. 1988.



Chapter 2

Flusilazole induces spatio-temporal expression patterns of retinoic acid-, differentiation- and sterol biosynthesis- related genes in the rat Whole Embryo Culture

Myrto Dimopoulou, Aart Verhoef, Bennard van Ravenzwaay, Ivonne M. C. M. Rietjens and Aldert H. Piersma

Reproductive Toxicology (2016) 64; 77-85

Abstract

Embryotoxic responses are critically dependent on the timing of exposure during embryo development. Here, we examined the time- dependent developmental effects in rat embryos exposed to flusilazole (FLU), and their link to retinoic acid (RA) mediated pathways. To this end, we assessed the effects of 4-hour exposure of rat embryos *in vitro* to 300μM FLU during four developmental time windows (0-4, 4-8, 24-28 and 44-48 h), evaluating morphological parameters, expression and localization of five genes directly or indirectly linked with the RA pathway. These were RA- (*Cyp26a1* and *Dhrs3*), differentiation- (*Gbx2* and *Cdx1*) and sterol biosynthesis- (*Cyp51*) related genes. Extended exposure for 48 hours to 300μM FLU resulted in morphological changes, typical for triazoles and RA, while the 4h exposure times did not. Time dependent significant upregulation of the five selected genes was observed. These results corroborate that the embryotoxic responses to FLU are correlated with the regulation of the RA pathway. Thus, these gene expression markers can be considered early biomarkers of FLU-induced potential developmental toxicity later in the development.

Key words: Whole embryo culture; Flusilazole; Retinoic acid; time-dependent; developmental toxicity; gene expression; *in situ* hybridization

Introduction

On 1 July 2007, the Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) was implemented by the European Union (EU) to decrease chemical risks for both humans and the environment. Consequently, there is a demand to assess the safety of all current and future chemicals, which will utilize millions of laboratory animals. The assessment of possible developmental toxicants through the application of alternative techniques may reduce animal use and enhance mechanistically driven risk assessment [1-4].

According to ECVAM (European Centre for the Validation of Alternative Methods), the rat post-implantation whole embryo culture (WEC) is a standardized alternative *in vitro* method for identifying developmental toxicants after exposure during early organogenesis [2, 5-7]. The advantage of this method is that early organogenesis and neurulation can be continuously monitored during this 48-hour critical period [2, 6], while there is a high level of concordance between human and rodent *in vivo* development during this embryonic stage [6, 8, 9]. On the other hand, possible limitations of this model could be the lack of maternal metabolism and the restricted 48- hour experimental window. However, validation studies have proved that by using the WEC assay, the developmental toxicity of chemical substances can be studied taking into consideration a variety of both morphological and molecular endpoints [5, 6, 10-12].

Triazoles are a category of fungicides widely used in both medicine and agriculture [13, 14]. The triazoles' mechanism of pharmacological action is based on inhibiting the fungal *Cyp51*, the catalyst for converting lanosterol to ergosterol. This inhibition causes increased fungal cell wall (exterior membrane) permeability and cell death [15, 16]. In mammalian systems some triazoles may induce developmental toxicity [17, 18]. One of the triazoles, Flusilazole (FLU), has been extensively used as a model compound in the study of induced developmental toxicity [18-20].

In vivo and *in vitro* studies have suggested that the mechanism of developmental toxicity of FLU is associated with the expression changes of genes, such as *Cyp26a1* and *Dhrs3*, which participate in the mechanisms of growth and differentiation [21, 22]. In detail, data from *in vitro* techniques have suggested that the embryotoxicity of triazoles is promoted through the retinoic acid (RA) pathway, on account of similarities in both morphological embryotoxic outcome and read-outs of selected biomarkers after exposure to either triazoles or RA [2, 23-25]. Furthermore, recent animal studies demonstrate commonly regulated morphological characteristics,

which support the aforementioned hypothesis concerning the involvement of the RA pathway [22]. The most common malformations introduced by FLU and RA are craniofacial and posterior axis defects, disturbance of neural crest cell (NCC) migration, altered branchial arch development and abnormal hindbrain segmentation [26, 27].

Given that developmental toxicity may be highly dependent on the embryonic stage when exposure occurs, it is of interest to compare different exposure time windows, and compare the differences in the magnitude of gene expression responses that may underlie developmental toxicity upon exposure during these different time windows. This allows determination of time windows which would be most sensitive to detect gene expression changes as biomarkers of developmental toxicity induced by specific groups of compounds, such as the triazole group. The two selected genes for this study, *Cyp26a1* and *Dhrs3*, are actively involved in balancing embryonic RA concentration and, therefore, respond to RA- like exposures [2, 28, 29]. Furthermore, the next two RA-related genes, *Gbx2* and *Cdx1*, are involved in craniofacial development, posterior axis, early embryonic patterning and cardiovascular formation [2, 30-34], while the last selected gene, *Cyp51*, is linked to steroid biosynthesis [21]. Changes in expression of these genes have been shown to be associated with a malformation when there was an over-accumulation of RA in the embryonic body [35]. The role of these genes in the toxicological mechanism of FLU's action could be the key for explaining FLU-induced teratogenicity in a stage- dependent manner during early embryonic development. In this study, we assess the time- dependent induced teratogenicity after exposure of rat WEC to FLU during different time windows by monitoring phenotypic and genotypic alterations, the latter with RT-PCR and *in situ* hybridization.

Materials and Methods

Animal care

Animal studies were approved and performed in concordance with institutional and federal regulations at the National Institute of Public Health and the Environment (RIVM). Wistar rats (HsdCpd:WU) (Harlan, The Netherlands) were housed at the RIVM Animal Care facility in a climate controlled room with a 12h light cycle (04:00-16:00 dark). After acclimatizing for 14 days, virgin female rats were mated with male rats for 3 hours (9:00-12:00). The presence of copulatory plug in the female rats was considered evidence of pregnancy, and more precisely, as gestational day 0 (GD0). The dams were housed in different cages where their clinical condition was monitored daily.

Rat whole embryo culture

The whole embryo culture (WEC) technique was performed in accordance with the validated method of Piersma [6]. Previous experiments were conducted in the same laboratory following the same protocol [4, 13, 36]. On the 10^{th} gestational day, between 9:00 and 12:00 AM, dams were euthanatized by intracardiac injection of $T61^{R}$ (Intervet, The Netherlands). Rat embryos were immediately explanted from the mother's uterus. The peripheral trophoblastic cell zone and the parietal yolk sac membrane were removed under the microscope leaving both the visceral yolk sac and ectoplacental cone intact. Embryos with 1-5 somites were cultured for morphological assessment while embryos with 2-4 somites were cultured for gene expression studies. Each embryo was placed individually in a flask with 2 mL culture medium. The medium was a mix of 90% pregnant bovine serum and 10% rat serum (Biochrom, Berlin, Germany), diluted with 14% Hank's solution (Gibco) and supplemented with 1.57 mg/mL D-glucose and L-methionine (Sigma-Aldrich, Zwijndrecht, The Netherlands). The culture flasks with embryos in culture medium were placed in rotating incubators where they were completely protected from light and were stabilized at 37.7^{o}C. A gas mixture was also provided 5 times for 30 seconds during the culture period, with an increasing content of oxygen: on the first day (GD10) at 9:00 and 16:00 (5% O_2 : 5% CO_2 : 90% N_2), on the second day (GD11) at 9:00 and 16:00 (20% O_2 : 5% CO_2 : 75% N_2) and on the third day at 9:00 (40% O_2 : 5% CO_2 : 55% N_2).

Flusilazole exposure

Flusilazole (FLU, CAS number 85509-19-9, Sigma-Aldrich, Zwijndrecht, The Netherlands) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Zwijndrecht) and added to the culture medium at a final concentration of 300μM FLU and 0.1% DMSO. In agreement with previous studies, DMSO control embryos were also cultured, to enable assurance that the morphological or gene expression alterations were due to FLU exposure. The exposure dose was indicated by a pilot experiment on cultured embryos at 0, 30, 100 and 300μM of FLU for 48 h. The embryos were further exposed to 300μM FLU during one of four 4-hour timeframes during the whole culture period of 48 hours: 0-4h, 4-8h, 24-28h, and 44-48h. Rat samples, which were cultured for study the morphological outcome after exposing to 300μM FLU, were collected in the end of the 48 h WEC culture period. Morphological alterations were also assessed for embryos exposed to 300μM of FLU during the whole culture period (48 h in total). Gene expression signature was studied for embryos that were exposed according to the aforementioned methodology (0-4h, 4-8h, 24-28h, and 44-48h). These embryos were collected for further studying the expression of the selecting genes immediately after the end of their exposure (4h, 8h, 28h and 48h). In addition to these samples, we exposed rat embryos to the same concentration of FLU (300μM) during 0-4h and we continued their culture into refreshed medium without FLU until the end of the culture period of 48h. For studying the localization of the genes with *in situ* hybridization technique, additional embryos were exposed to 300μM FLU following the same methodology as in the gene expression experiments. The embryos were immediately collected after the end of their exposure (4h, 8h, 28h and 48h).

Morphological Scoring

Embryos, that were cultured for 48 h (whole culture period), were scored according to the Total Morphological Score (TMS) system taking into account a variety of morphological endpoints [37]. The morphological endpoints include growth parameters (crown-rump length, head diameter, number of somites and yolk sac diameter) and developmental parameters, such as yolk sac blood circulation, heart, embryo turning, caudal neural tube, optic and otic system, fore- and hind- limb, branchial arches, mandibular and maxillary process and the shape and size of somites.

Whole embryo RNA isolation

For the gene expression analysis, cultured embryos were quickly scored on the basis of their somite number, their position in the yolk sac, the neural tube developmental stage, the crown-rump length and the head diameter. They were further isolated from the yolk sac and ectoplacental cone, placed in 200μM RNAlater (Ambion, Austin, Texas), stored for one week at 4^{o}C, and then at -80^{o}C for further processing with the lowest possible amount of RNAlater. After the embryos were thawed on ice, they were homogenized by passing them 10 times though a 1mL syringe with a 26G needle for small (4h-, 8h- and 28h-) embryos or with a 22G needle for large (48h-) embryos. The RNA of the homogenized lysate was further isolated by using the RNeasy Micro Plus RNA isolation kit (CAS number 74034, Qiagen, the Netherlands). Eluting with 14μM of RNA-free H_2O, final volumes of 12μL RNA were obtained and tested on both Nanodrop (Nanodrop Technologies Inc., Wilmington, Delaware) and 2100 BioAnalyzer (Agilent Technologies, Palo Alto, California) to establish the RNA quality and quantity. The RNA samples with an absorbance value between 1.9 and 2.2 (ratio 260mm/280mm) and RNA integrity number (RIN) higher than 7, were further used for gene expression analysis with Real Time-PCR. The RNA samples were stored at -80^{o}C.

cDNA synthesis

For performing the RT-PCR analysis, cDNA was synthesized by using the QuantiTect® Reverse Transcription Kit (CAS number 205210, Qiagen, The Netherlands), in accordance with the manufacturer's instructions. The produced embryonic cDNA template was used for the later standardization of the Real-Time PCR by mixing the cDNA templates of all the samples from the same category (exposed during 0-4h, 4-8h, 24-28h, and 44-48h and cultured till 4th, 8th, 28th and 48th hour, respectively, or exposed during 0-4h and cultured till 48th hour). In addition to the synthesis of cDNA template (300ng/μL) of each sample, both No Reverse Transcription (No RT) and No Template Control (NTC) were prepared for excluding the possibility of contamination with genomic DNA (gDNA) and assessing the efficiency of the method and selected experimental conditions.

Gene expression – Quantitative RT-PCR

We quantified the expression levels of the five aforementioned selected genes in the rat embryos by performing Real-Time PCR with the Rotor-Gene Q (Qiagen, the

Netherlands). Moreover, we used three housekeeping genes to ensure the consistency of the expression levels of the genes under assessment. The following QuantiTect SYBR Primer Assays were purchased by Qiagen (The Netherlands): primers for genes under assessment; *Cyp26a1* (Rn_Cyp26a1_1_SG QuantiTect Primer Assay, QT00191191), *Gbx2* (Rn_RGD:621866_1_SG QuantiTect Primer Assay, QT00416738), *Cdx1* (Rn_Cdx1_1_SG QuantiTect Primer Assay, QT00482013), *Cyp51* (Rn_Cyp51_1_SG QuantiTect Primer Assay, QT00195552), *Dhrs3* (Rn_Dhrs3_1_SG QuantiTect Primer Assay, QT01615348), and primers for housekeeping genes *Hprt1* (Rn_Hprt1_2_SG QuantiTect Primer Assay, QT00365722), *Polr2a* (Rn_Polr2a_1_SG QuantiTect Primer Assay, QT00379477) and *Actb* (Rn_Actb_1_SG QuantiTect Primer Assay, QT00193473). For the quantification of the gene expression of each gene in the WEC samples, eight different standard curves were constructed for the five genes under assessment and the three housekeeping genes for every developmental time window, because of the complicated and unstable nature of the WEC. For preparing the standard curve, a pool mixture, consisting of 2 μL of each sample from the same time window-group, was used for the quantification of every gene. According to the manufacturer's instructions, 12.5μL of QuantiFast SYBR Green Master mix was mixed with 2.5μL of QuantiTect Primer Assay of every primer. 10μL of template cDNA diluted with RNase-free H_2O was added and a three-step RT-PCR with melting curve was performed. Before the cDNA amplification, a hold step of 5 min at 95^{o}C was preceded for activation of the HotStarTAq *Plus* DNA Polymerase. The cDNA was amplified in a 3-step cycling of 40 cycles. The pre-denaturation step of the sample was the "hold-step" of 10 min at 95^{o}C. The denaturation of the sample was performed for 10 s at 95^{o}C, followed by combined annealing for 15 s at 60^{o}C and, then, for 20 s at 72^{o}C. The No Template Control (NTC) and No Reverse Transcriptase (no RT) control were included in every RT-PCR run for assessing the reliability of the produced results. The melting curve of every PCR product was also studied after every run in order to assure the efficiency and sensitivity of both primers and assay. The standard, cycling and melting curves, as well as the threshold, were automatically calculated by the software Rotor-Gene 2.3.1.49. We normalized the expression of the genes under assessment with the housekeeping gene *Hprt1*, which was constantly expressed among all the different developmental time windows under assessment compared to *Polr2a* and *Actb*.

RNA probes

Before starting the whole mount *in situ* hybridization of rat embryos, anti-sense RNA digoxigenin (DIG) -labelled probes were synthesized for the five genes under assessment. Anti-sense RNA probes were designed with the Primer-BLAST tool on the NCBI website by following the complete RefSeq sequences of the five genes of interest (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were purchased from Invitrogen (Life Technologies, Breda, the Netherlands). The summary of their sequences are presented in table 1. Primers were used for one-step RT-PCR with Titan One Tube RT-PCR System (Roche Applied Science). After the purification of the acquired PCR product with the QIAquick PCR purification kit (Qiagen, Venlo, the Netherlands), a nested PCR was performed in order to amplify the specific PCR product. Another purification step of the obtained DNA template followed. For synthesizing the RNA DIG-labelled probes, an incubation step of the PCR product with T3 and T7 polymerases followed. Before using the DIG-labelled probes, a step with post-reaction clean-up columns (Sigma spin^{TM} Sequencing Reaction Clean-up) purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands) was conducted for cleaning the obtained product.

Table 1: Designed primer sequences used for RNA probe synthesis

Primer	Strand	Type of PCR	Sequence
Cyp26a1	Forward	One step RT-PCR	GAAGTGAGCGGTTGTCTGGA
Cyp26a1	Reverse	One step RT-PCR	TAGTGAAGCTGTCTGCCACG
Cyp26a1	Forward	Nested PCR	ATTAACCCTCACTAAAGGGAGAAGTGAGCGGTTGTCTGGA
Cyp26a1	Reverse	Nested PCR	TAATACGACTCACTATAGGGAGAGGAGCTCTGTGGACGAT
Dhrs3	Forward	One step RT-PCR	TTTTGTCCACCGCCTCCTAC
Dhrs3	Reverse	One step RT-PCR	CCCTGAAACATCTCGGTGCT
Dhrs3	Forward	Nested PCR	ATTAACCCTCACTAAAGGGAGAGGAGATTCGGCAGATGGG
Dhrs3	Reverse	Nested PCR	TAATACGACTCACTATAGGGCCCTGAAACATCTCGGTGCT
Gbx2	Forward	One step RT-PCR	CACCGGCTACCCCATGTTTA
Gbx2	Reverse	One step RT-PCR	CCGTCTTGGAATTGGCGTTG
Gbx2	Forward	Nested PCR	ATTAACCCTCACTAAAGGGAGGCAAGGGAAAGACGAGTCA
Gbx2	Reverse	Nested PCR	TAATACGACTCACTATAGGGCCGTCTTGGAATTGGCGTTG
Cdx1	Forward	One step RT-PCR	GACAAGGACTCCCCCGTGTA
Cdx1	Reverse	One step RT-PCR	GCATTGGTGGGGCATAGACT
Cdx1	Forward	Nested PCR	ATTAACCCTCACTAAAGGGAAACTTGGGTCTCACAGAGCG
Cdx1	Reverse	Nested PCR	TAATACGACTCACTATAGGGGAGTGGGCAAGCTACTTGGT
Cyp51	Forward	One step RT-PCR	GCCTGGATGGGCTTCTTTCT
Cyp51	Reverse	One step RT-PCR	TCCAAGCCGGAATCTCCCTA
Cyp51	Forward	Nested PCR	ATTAACCCTCACTAAAGGGAGGGAGGATCTGCCTCCCTTA
Cyp51	Reverse	Nested PCR	TAATACGACTCACTATAGGGCACACTGGCTCCTTGTTCCT

In Situ Hybridization

Embryos exposed to 300 μM FLU during the time frames 0-4, 4-8, 24-28 or 44-48h, respectively, were morphologically scored and one of each group was used for performing *in situ* hybridization. The whole mount *in situ* hybridization was strictly performed only on embryos that appeared morphologically intact. The *in situ* hybridization technique was performed by following the protocol previously described by Wilkinson [38]. The application of this method required modifications for analysing the expression patterns of the five selected genes (*Cyp26a1*, *Dhrs3*, *Gbx2*, *Cdx1* and *Cyp51*) depending on the developmental stage of the rat embryos. In brief, both control and FLU exposed embryos were washed in 1x phosphate-buffered saline (PBS, Invitrogen), fixed overnight at 4^{o}C in 4% Paraformaldehyde (PFA, Sigma-Aldrich) dissolved in 1x PBS and progressively dehydrated in 1x PBS-Methanol (MeOH, Merck) dilutions with gradually increased concentration of MeOH. The dehydrated embryos were stored in 100% MeOH at -20^{o}C for several months. Before starting the *in situ* hybridization, the embryos were gradually rehydrated in 1x PBS-MeOH dilutions with increased concentration of 1x PBS followed by four washing steps with PTW (1x PBS with 0.1% Tween (Merck)). For increasing the permeability of the outer embryonic membrane, embryos were treated with 10μg/mL Proteinase K (Roche) in PTW at room temperature. For embryos in GD10, 10μg/mL Proteinase K were applied for 7 min, while for embryos in their GD11 and GD12 the treatment lasted for 10 and 12 min, respectively. After this, embryos were washed three times with PTW and fixed in 4% PFA. Afterwards, the embryos were prehybridized for 3 hours at 70^{o}C in hybridization buffer containing 50 % deionized formamide (Ambion), 5x SSC (Invitrogen), 500 μg/mL tRNA (Sigma-Aldrich), 50 μg/mL heparine (Sigma-Aldrich), 0.1% Tween20 (Merck), adjusted at pH 6.0 with 1 M citric acid. The hybridization step was followed by replacing the hybridization buffer with a mixture of fresh hybridization buffer with RNA DIG-labelled probe for the gene of interest in a final concentration of 1 μg/mL, pre-heated for 10 min. The incubation at 70^{o}C was continued overnight for completing the hybridization step. During the second day of this procedure, five washing steps with dilutions of SSC [38] were carried out in order to remove the probe that was not annealed in order to target the embryonic RNA of the gene of interest. Pre-block of embryos for 3h at room temperature in a mixture of PTW with 2% sheep serum and 0.2% Bovine Serum Albumin (BSA) (Sigma-Aldrich) was introduced for avoiding increased background noise. Overnight incubation of embryos was performed at 4^{o}C with anti-DIG antibody solution in PBT at a final concentration of 0.5 μg/mL on a shaking platform. During the third day of the *in situ*

hybridization protocol, the embryos were washed six times with PBT and staining buffer, which contained 100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween20, 5 mM levamisole (Sigma-Aldrich). Then, embryos were stained with a staining mixture, containing 20 µL NBT/BCIP (Roche) per 1mL staining buffer. The staining step was terminated when the obtained signal was optimal by microscopic observation, which was additionally dependent on both the developmental stage of every embryo under assessment and the gene of interest. The staining procedure was completed by washing the embryos three times in PTW followed by overnight fixation in 4% PFA at 4^{o}C. For making photomicrographs of the stained embryos, an overnight incubation step in 100 % glycerol (Sigma-Aldrich) at room temperature in the dark on a shaking plate was additionally required.

Statistical Analysis for Morphological endpoints

For determining the significance of the differences in morphological endpoints between the FLU treated groups of WEC and their controls, we performed the parametric Student's *t*-test (unpaired) and the non-parametric Mann Whitney test, for both two-sided, with 95% confidence intervals. Each group included 8 rat embryos cultured individually in separate culture flasks. These two statistical approaches revealed similarities in the significance of effects detected for each morphological endpoint. Therefore, the statistically significant difference of the experimental values that has been calculated by the Student's *t*-test is presented in the figures and tables in this research paper.

Gene expression analysis - data processing

The parametric Student's *t*-test two-sided mathematical approach was used to assess statistical significance on the rat WEC gene expression analysis. Every exposure window group included data from 8 treated embryos tested versus 8 control embryos.. An exception was the group, in which the embryos were exposed for the first 4 hours and further cultured for 44 hours in refreshed culture medium, that included only 2 treated embryos versus 2 control embryos. In any case the confidence interval was set to 95%.

Results

Morphological analysis of embryos

Table 2 summarizes the TMS results of embryo development after exposure to 300 µM FLU during the exposure time windows: 0-4, 4-8, 24-28, 44-48 and 0-48 h. We observed a statistically significantly lower TMS in the 4-8h, 24-48h and 0-48 h groups compared to controls. The 0-48-hour exposure caused the largest effect on TMS, including significant embryonic malformations. As illustrated in both Figure 1 and Table 2, we observed a statistically significant malformed profile of the rat embryos that were continuously exposed to 300µM FLU for 48 hours. We found that the most common morphological alterations after exposure for 48 hours were the failure in closure of the caudal neural tube, and malformations of the branchial arches and mandibular and maxillary process. Additionally, we detected general growth retardation in these embryos (reduced crown-rump length and head diameter), including retarded brain segmentation and reduced limb growth.

Specific morphological defects were limited and scattered among the different groups of 4-hour treatment with FLU. More precisely, these defects included abnormal development of the otic system after exposure to 300µM FLU during the 4-8th hour of culture. After exposure between the 24-28th hour of culture there was an effect on the quality of the formed somite shape.

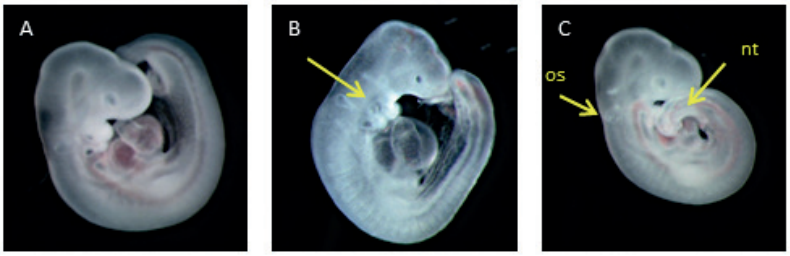


Figure 1*: Rat WEC cultured in 0.1% DMSO (A), 300µM FLU for 48h (B and C). Compared to the control embryo (A), observed malformations are indicated with the yellow arrows: abnormal mandibular formation (B), open caudal neural tube (C, nt) and failure of the formation of the otocyst (C, os)*

Table 2: Overview of morphological effects of 300 µM FLU in the rat WEC assay. Each number represents a mean ± SD (N=8, Student's *t*-test: * p < 0.05, ** p < 0.005, *** p < 0.0005, **** p < 0.0001).

Compound	Concentration (µM)	Time of exposure (h)	TMS	CRL (mm)	S_{48h} - S_{0h}	FORE	MID	HIND	CAUD	OTIC	OPTIC	BRAN	MAND-MAX	LIMB	SOM	HEART
FLU	0	0-48 h	67.88 ± 1.41	3.95 ± 0.22	23.62 ± 0.74	5 ± 0	5 ± 0	5 ± 0	5 ± 0	4.6 ± 0.44	4.1 ± 0.23	5 ± 0	4.9 ± 0.35	4.4 ± 0.35	5 ± 0	5 ± 0
FLU	300	0-4 h	67.44 ± 1.15	3.93 ± 0.10	23 ± 0.46	5 ± 0	5 ± 0	5 ± 0	4.9 ± 0.35	4.5 ± 0.38	4.2 ± 0.26	5 ± 0	4.75 ± 0.46	4.25 ± 0.27	4.8 ± 0.35	5 ± 0
FLU	300	4-8 h	66.6 ± 1.06*	3.8 ± 0.12	23.9 ± 0.83	4.8 ± 0.37	5 ± 0	5 ± 0	5 ± 0	4.1 ± 0.23*	4 ± 0	5 ± 0	4.6 ± 0.5	4.1 ± 0.58	5 ± 0	5 ± 0
FLU	300	24-28 h	65.8 ± 1.4**	4.02 ± 0.11	26.7 ± 0.5	5 ± 0	5 ± 0	5 ± 0	5 ± 0	4.8 ± 0.36	4 ± 0	5 ± 0	4.8 ± 0.4	4.1 ± 0.42	4.5 ± 0.53 *	5 ± 0
FLU	300	44-48 h	66.5 ± 2.09	4.01± 0.06	23.4 ± 0.74	5 ± 0	5 ± 0	5 ± 0	5 ± 0	4.6 ± 0.23	4.06 ± 0.18	5 ± 0	4.75 ± 0.46	4.5 ± 0	5 ± 0	5 ± 0
FLU	300	0-48 h	44 ± 2 ****	3.35 ± 0.13 ***	19.75 ± 0.96 ****	3.5 ± 0.58 ****	4 ± 0	2.75 ± 0.96 ****	1 ± 0 ****	3 ± 0 ****	3.25 ± 0.96 *	1.25 ± 0.5 ****	2.75 ± 1.5 *	3 ± 0 ****	5 ± 0	5 ± 0

CRL: crown-rump length; S_{48h}- S_{0h}: number of somites that formed during the culture period of rat WEC; FORE: forebrain; MID: midbrain; HIND: hindbrain; CAUD: caudal neural tube; OTIC: otic system; OPTIC: optic system; BRAN: branchial arches; MAND-MAX: mandibular and maxillary process; LIMB: fore- and hind- limb formation; SOM: quality of somites and HEART: heart.

Gene expression analysis

Figure 2 presents the somitogenesis of the embryos of which gene expression was further analysed with qRT-PCR. These embryos were firstly assessed in terms of any morphological alteration. The results presented in Figure 2 reveal that exposed embryos had a normal somite number. These embryos were used for gene expression analysis. In this way, we excluded rat embryos of which the phenotype was affected either due to their exposure to FLU or biological variability, because we focused on investigating possible early alterations on the transcriptome level provoked by exposure to FLU.

As shown in Figure 3, when rat embryos were exposed during various 4-hour time windows, a general increased trend in the expression of each of the five selected genes was detected. The highest average fold induction gene expression was observed in embryos that were exposed during the 44-48 h time window, however, the variation in response was also highest in this group.

In more detail, *Cyp26a1* expression shows a gradual increase of approximately 2.5-, 3.5-, 3.5- and 6- fold induction among the developmental windows of 0-4, 4-8, 24-28 and 44-48 hours of exposure. Relatively similar results were observed for the gene *Dhrs3*.

Gbx2 and *Cdx1* showed statistically significant upregulation after exposure during 0-4 and 4-8 hours only. For both of these genes, an upward trend of their expression was revealed by taking into consideration the first two time windows of exposure. However, when the embryos were exposed at their 24th hour of development for 4h (till their 28th hour of culture), the expression of both *Gbx2* and *Cdx1* was lower than that observed for the control levels. On the other hand, *Gbx2* and *Cdx1* expression levels after exposure to 300μM FLU during 44-48 h of culture were raised approximately to the same level observed for the 4-8 h time window.

Cyp51 did not show any significant regulation in these experiments, with expression levels ranging from 1.5 to 2.4-fold higher than control, albeit not statistically significant.

Finally, when embryos were left until 48 hours culture after exposure between 0 and 4 hours, the expression of these genes seems to rebound to lower levels than those observed for unexposed 48-hr embryos with approximately 0.6 to 0.8 fold induction, depending on the gene under assessment.

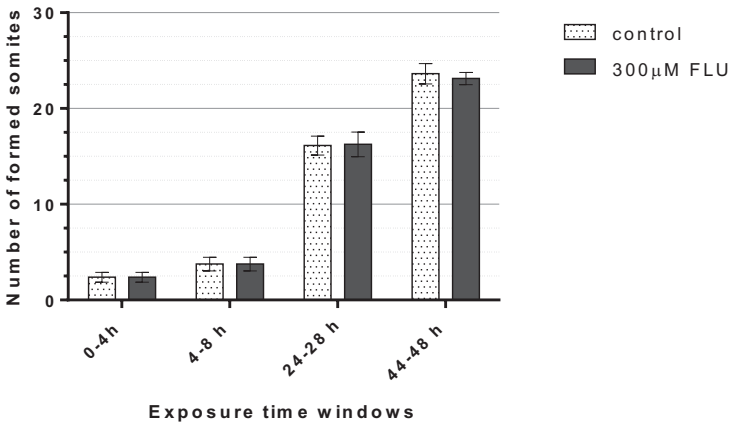


***Figure 2**: Somitogenesis in rat embryos exposed to 300 µM FLU during four different developmental time windows. A comparison on the number of somites between control and treated embryos at the end of the exposure period. No statistically significant differences were observed. Each bar represents a mean ± SD (N=8 for 0-4, 4-8, 24-28, 44-48 h, t-test, p > 0.05).*

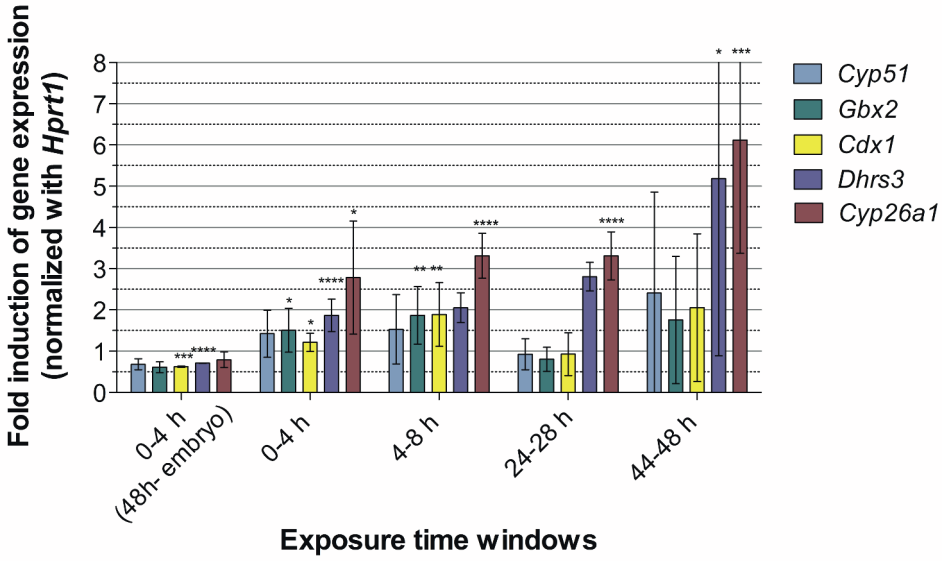


***Figure 3**: Effect of exposure to 300µM FLU for 4 hours in the rat WEC during different developmental time windows. The relative fold induction of gene expression of compound-treated versus vehicle-treated embryos within exposure window groups was calculated after normalizing with the reference housekeeping gene Hprt1 and expressing the control (0.1%*

DMSO) for each time point as 1. Each bar represents a mean ± SD (N=8 vs. 8 for 0-4, 4-8, 24-28, 44-48 h and N=2 vs. 2 for 0-4h exposure and till 48th hour culture). Embryos were analysed after 48 hours of culture (five bars on the left) or directly after exposure (all other bars).

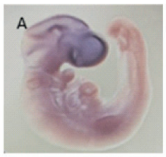
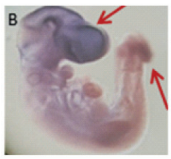
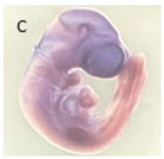
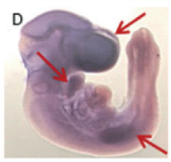
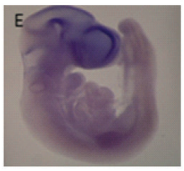
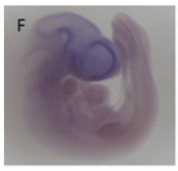
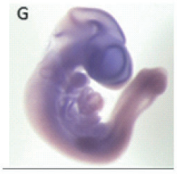
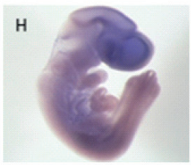
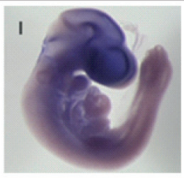
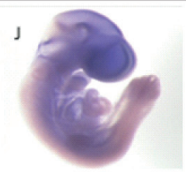
In situ hybridization

Table 3 summarizes the results after hybridization of representative GD12 embryos exposed to FLU for the last 4 hours of culture, as this experimental group showed the clearest changes in gene expression as observed under the microscope. There is a clear FLU-related effect on the expression of *Cyp26a1* and *Dhrs3*. *Cyp26a1* is localized in the head region (fore- and mid-brain cavity), on the 1st branchial arch and the forelimbs (Table 3 pictures A and B). However, there is a higher expression in the tail and head regions of the embryo exposed to FLU as compared to the untreated control embryo. Embryos exposed to FLU showed a higher expression of *Dhrs3* in the fore-, mid- and hind-brain regions than control embryos. Similar to the case of *Cyp26a1*, *Dhrs3* was notably localized on the 1st branchial arch. Particular localization of *Dhrs3* was also observed in the forelimbs.

Gbx2, *Cdx1* and *Cyp51* transcripts did not show any specific expression pattern change after exposure to FLU. As previously mentioned, *Gbx2* and *Cdx1* have a function in rhombomere segmentation and axial patterning, respectively. These two genes were indeed expressed in the lower rhombomeres, where NCC migration is occurring later in the development for establishing the branchial arches and the otic system.

Cyp51, a gene involved in sterol biosynthesis, is expressed in all tissues, nucleated cells and at acrosomes on the outer membrane of the Golgi apparatus [39]. This gene is abundantly expressed in 48h- embryos without exhibiting a particular pattern, in both control and FLU-exposed rat embryos (Table 3, pictures I and J).

Table 3: Whole mount in situ hybridization of five genes in control and 300 µM FLU-treated embryos on GD12 after exposure during the 44-48 hour time window. Arrows indicate areas of expression changes after FLU exposure (see text for explanation).

	DMSO control	300 µM FLU
Cyp26a1		
Dhrs3		
Gbx2		
Cdx1		
Cyp51		

Discussion

Exposure of rat embryos in culture for 48h to 300μM FLU caused typical morphological changes including abnormal craniofacial and neural development, and ear and branchial arches defects. This pattern of malformations is similar to that observed after exposure to RA [2, 26, 35]. This commonly abnormal phenotype of embryos exposed to either FLU or RA supports the hypothesis that the phenotype induced by FLU is caused by disturbance of RA-mediated pathways. In addition, we observed specific responses of rat embryos on both the morphological and molecular level after exposure to FLU during different developmental time windows, which is reminiscent of earlier observations [24, 40].

A transcriptome analysis of embryos exposed to FLU and other triazoles have revealed the disturbance of RA-related gene families [23, 35]. Therefore, we hypothesized that representatives from this gene family could be used as biomarkers for detecting toxic effects induced by FLU during the selected time-windows.

Effects of exposure to FLU on the genes studied could be established even in the very early developmental time window (0-4 h). This is even earlier than the 4-6 h time window, which has been shown before as a sensitive period for studying effects of xenobiotic exposures on the level of the transcriptome [2, 41, 42].

Robinson *et al*. [35], using a transcriptomic analysis of rat embryos exposed to FLU during the 0-4 hour time window, has clearly revealed the activation of pathways related to RA metabolism, hindbrain development and neurogenesis. More concrete gene expression analysis has pointed out that the most pronounced gene targets of these bioprocesses were two of the gene-targets of our study, *Cyp26a1* and *Dhrs3* [35]. As already mentioned, both these genes are involved in the metabolism of RA and in maintaining optimal RA concentrations along the anterior-posterior axis during growth and differentiation. When RA levels increase in the embryonic body, both *Cyp26a1* and *Dhrs3* are upregulated, resulting in an increased breakdown of RA, as well as in inhibition of its production. High RA exposures have been shown to induce developmental toxicity in a time- and dose- dependent manner [8, 23, 43, 44]. An increased expression of these directly related RA genes, *Cyp26a1* and *Dhrs3*, is clearly illustrated in our study. In agreement with the quantification of the gene expression by RT-PCR, *Cyp26a1* and *Dhrs3* are both highly expressed in 48h- embryos at the sites of the observed malformations, in agreement with published data [28, 45-47]. The effects on the head and otic system are connected with the disturbance of these two genes after prolonged exposure to FLU or other xenobiotics with a similar mode of action. Moreover, deficient caudal neural tube closure due to extended exposure is associated with overexpression of *Cyp26a1* in the tail end of the embryo, which aims to restore the balance

of an increased concentration of RA. Conclusively, both FLU and RA result in gene expression changes indicating disturbed RA balance, leading to embryonic malformations.

Gbx2 has an important role in hindbrain development, in combination with the growth factor *FgF8* and *Hox* genes. All these genes are known as specific "pattern forming" transcriptional factors [23, 26, 42], which are also controlled by RA- related pathways. When rat embryos were exposed to either RA or triazoles, the downregulation of growth and transcriptional factors led to the abnormal upregulation of *Gbx2*, probably aiming to restoring the disturbed balance [18]. Figure 3 illustrates an upward trend on the *Gbx2* expression after FLU exposure in the different developmental windows addressed. This could be explained by its crucial role on initiating the NCC migration and rhombomeres segmentation in the early embryonic stages following by the later hindbrain development. The expression of *Gbx2* was more pronounced after FLU exposure and the possible over-accumulation of RA in the rat embryos [26]. However, *in situ* localization of *Gbx2* did not reveal any specific localization during the developmental stages assessed.

The upregulation of *Cdx1* could also be involved in the morphological effects of long-term exposure of rat embryos to FLU. *Cdx1* expression is initiated by an increased embryonic RA level and activation of *Wnt* signalling in a tissue- and stage- specific manner. This affects early axis patterning, as well as later limb formation and intestinal development through regulation of e.g. *Fgf, Wnta3* and *Hox* genes [48-52]. Prinos *et al*. [49] have proposed a model for *Cdx1* expression in mouse studies, in which they have shown an increased expression during GD 7.5, followed by its stabilization during GD 8.5 due to the activation of a *Cdx1* autoregulatory loop [53]. In mammalian systems, *Cdx1* has an altered role after GD 9 and 11 in mouse and rat, respectively [50], actively participating in the organization of the gastrointestinal tract development in collaboration with *Cdx2* [53]. The dysmorphology of rat embryos exposed to FLU for 48 h is likely related to the abnormal expression of *Cdx1*.

In agreement with this, our study shows the same upward expression pattern for both *Gbx2* and *Cdx1* in early embryonic developmental stages, which drops in the 24-28h (11^{th} gestational day) developmental time window under assessment. The decreased expression of these two genes may be related to the embryonic stage specificity of their function, regulated by local RA-concentrations. Concurrent changes in *Cyp26a1* and *Dhrs3* indeed indicate changes in levels of RA.

The pharmacological mechanism of action of FLU is based on the inhibition of the enzyme *Cyp51*, which is important in the fungal sterol biosynthesis pathway [21]. Therefore, *Cyp51* was selected as a potential biomarker in the present study. Interestingly, previous studies on the Embryonic Stem Cell test (EST) and Zebrafish Embryo Test (ZET) have shown upregulation of this gene after exposure to FLU [23, 54]. Additionally, a study from de Jong

et al. [13], has illustrated the relatively high correlation of developmental effects between EST, ZET and WEC. However, *Cyp51* expression was not significantly affected in our study. This suggests that sterol/cholesterol biosynthesis may be less sensitive compared to the RA metabolism or embryonic development bioprocesses during early development [18]. Therefore, the applied 4-h exposure in our study appeared inadequate for observing an effect of FLU on *Cyp51* in RT-PCR. Our *in situ* staining shows that *Cyp51* is abundantly expressed throughout the rat embryos, but is not visibly affected by exposure to FLU during any of the four developmental stages assessed. The abundant expression is in agreement with the general role of this gene during embryo development. Studies with ZET have suggested that intestine, liver and central nervous system (CNS) development are greatly dependent on *Cyp51* because of its crucial role in lipid and glucose metabolism [55-57]. An additionally indirect effect of *Cyp51* embryonic expression could be induced via its participation to the fibroblast growth factor receptor (*Fgfr2*) mediated pathway. *Fgfr2*, in combination with the *Wnt* signalling pathway, has a crucial role in the heart development by modulating the myocyte proliferation [58]. Increased levels of RA trigger and downregulate both *Fgfr2* and *Wnt* pathways [59]. Huang *et al.* [60] have suggested that the mutations on *Fgfr2* could be associated with abnormal differentiation, as well as skeletal and cardiac abnormalities. Moreover, all these abnormalities have been clinically reported from Antley-Bixler syndrome patients, who were exposed to Fluconazole, another antifungal azole related to RA [60-62]. Therefore, there would be an indirect link of disturbed *Cyp51* expression to RA related malformations

In summary, there is a stage- specific gene expression response of cultured rat embryos exposed to FLU, which could be detected with early molecular biomarkers during crucial time- windows of early development, in the absence of concurrent morphological effects. The directly RA-related genes, *Cyp26a1* and *Dhrs3*, are the most significantly upregulated after embryonic exposure to FLU during all four selected time windows. Moreover, they revealed a pronounced pattern of induction at the sites of the most common FLU-induced abnormalities. Furthermore, *Gbx2* and *Cdx1* expression changes after FLU-exposure were observed to be dependent on the applied exposure windows. In conclusion, the response of selected gene biomarkers is dependent on the time window and precedes the development of morphologically observable malformations. Such biomarkers can therefore be employed as useful tools for early detection of possible teratogenic properties of xenobiotic compounds which belong in the group of triazoles.

Acknowledgements

This work was supported by BASF SE.

Notes

The authors declare that they have no conflict of interest.

References

1. Dreisig, K., et al., *Predictive value of cell assays for developmental toxicity and embryotoxicity of conazole fungicides.* Altex, 2013. **30**(3): p. 319-30.
2. Luijten, M., et al., *Transcriptomics analysis of retinoic acid embryotoxicity in rat postimplantation whole embryo culture.* Reprod Toxicol, 2010. **30**(2): p. 333-40.
3. Piersma, A.H., et al., *Evaluation of an alternative in vitro test battery for detecting reproductive toxicants.* Reprod Toxicol, 2013. **38**: p. 53-64.
4. Robinson, J.F., et al., *Embryotoxicant-Specific Transcriptomic Responses in Rat Postimplantation Whole-Embryo Culture.* Toxicological Sciences, 2010. **118**(2): p. 675-685.
5. Ellis-Hutchings, R.G. and E.W. Carney, *Whole embryo culture: a "New" technique that enabled decades of mechanistic discoveries.* Birth Defects Research Part B: Developmental and Reproductive Toxicology, 2010. **89**(4): p. 304-312.
6. Piersma, A.H., *Validation of alternative methods for developmental toxicity testing.* Toxicol Lett, 2004. **149**(1-3): p. 147-53.
7. Sogorb, M.A., et al., *An integrated approach for detecting embryotoxicity and developmental toxicity of environmental contaminants using in vitro alternative methods.* Toxicol Lett, 2014. **230**(2): p. 356-67.
8. Fang, H., et al., *Transcriptome analysis of early organogenesis in human embryos.* Dev Cell, 2010. **19**(1): p. 174-84.
9. Irie, N. and S. Kuratani, *Comparative transcriptome analysis reveals vertebrate phylotypic period during organogenesis.* 2011. **2**: p. 248.
10. Chapin, R., et al., *State of the art in developmental toxicity screening methods and a way forward: a meeting report addressing embryonic stem cells, whole embryo culture, and zebrafish.* Birth Defects Res B Dev Reprod Toxicol, 2008. **83**(4): p. 446-56.
11. Giavini, E. and E. Menegola, *Biomarkers of teratogenesis: suggestions from animal studies.* Reprod Toxicol, 2012. **34**(2): p. 180-5.
12. Harris, C., *Rodent whole embryo culture.* Methods Mol Biol, 2012. **889**: p. 215-37.
13. de Jong, E., et al., *Comparison of the mouse Embryonic Stem cell Test, the rat Whole Embryo Culture and the Zebrafish Embryotoxicity Test as alternative methods for developmental toxicity testing of six 1,2,4-triazoles.* Toxicol Appl Pharmacol, 2011. **253**(2): p. 103-11.
14. Martin, M.T., et al., *Toxicogenomic study of triazole fungicides and perfluoroalkyl acids in rat livers predicts toxicity and categorizes chemicals based on mechanisms of toxicity.* Toxicol Sci, 2007. **97**(2): p. 595-613.
15. Kjaerstad, M.B., et al., *Endocrine disrupting effects in vitro of conazole antifungals used as pesticides and pharmaceuticals.* Reprod Toxicol, 2010. **30**(4): p. 573-82.
16. Trosken, E.R., et al., *Comparison of lanosterol-14 alpha-demethylase (CYP51) of human and Candida albicans for inhibition by different antifungal azoles.* Toxicology, 2006. **228**(1): p. 24-32.
17. Giavini, E. and E. Menegola, *Are azole fungicides a teratogenic risk for human conceptus?* Toxicol Lett, 2010. **198**(2): p. 106-11.
18. Robinson, J.F., et al., *Triazole induced concentration-related gene signatures in rat whole embryo culture.* Reprod Toxicol, 2012. **34**(2): p. 275-83.
19. Menegola, E., et al., *Study on the common teratogenic pathway elicited by the fungicides triazole-derivatives.* Toxicol In Vitro, 2005. **19**(6): p. 737-48.
20. Menegola, E., et al., *Effects of mixtures of azole fungicides in postimplantation rat whole-embryo cultures.* Arch Toxicol, 2013. **87**(11): p. 1989-97.
21. Goetz, A.K., et al., *Disruption of testosterone homeostasis as a mode of action for the reproductive toxicity of triazole fungicides in the male rat.* Toxicol Sci, 2007. **95**(1): p. 227-39.

22. Mineshima, H., et al., *Malformation spectrum induced by ketoconazole after single administration to pregnant rats during the critical period - comparison with vitamin A-induced malformation spectrum.* J Appl Toxicol, 2012. **32**(2): p. 98-107.

23. Hermsen, S.A., et al., *Triazole-induced gene expression changes in the zebrafish embryo.* Reprod Toxicol, 2012. **34**(2): p. 216-24.

24. Marotta, F. and G.M. Tiboni, *Molecular aspects of azoles-induced teratogenesis.* Expert Opin Drug Metab Toxicol, 2010. **6**(4): p. 461-82.

25. Pennimpede, T., et al., *Analysis of Cyp26b1/Rarg compound-null mice reveals two genetically separable effects of retinoic acid on limb outgrowth.* Dev Biol, 2010. **339**(1): p. 179-86.

26. Menegola, E., et al., *Relationship between hindbrain segmentation, neural crest cell migration and branchial arch abnormalities in rat embryos exposed to fluconazole and retinoic acid in vitro.* Reprod Toxicol, 2004. **18**(1): p. 121-30.

27. Menegola, E., et al., *Craniofacial and axial skeletal defects induced by the fungicide triadimefon in the mouse.* Birth Defects Res B Dev Reprod Toxicol, 2005. **74**(2): p. 185-95.

28. Billings, S.E., et al., *The retinaldehyde reductase DHRS3 is essential for preventing the formation of excess retinoic acid during embryonic development.* Faseb j, 2013. **27**(12): p. 4877-89.

29. Kam, R.K., et al., *Dhrs3 protein attenuates retinoic acid signaling and is required for early embryonic patterning.* J Biol Chem, 2013. **288**(44): p. 31477-87.

30. Li, J.Y. and A.L. Joyner, *Otx2 and Gbx2 are required for refinement and not induction of mid-hindbrain gene expression.* Development, 2001. **128**(24): p. 4979-91.

31. Li, K., J. Zhang, and J.Y. Li, *Gbx2 plays an essential but transient role in the formation of thalamic nuclei.* PLoS One, 2012. **7**(10): p. e47111.

32. Lickert, H., et al., *Wnt/(beta)-catenin signaling regulates the expression of the homeobox gene Cdx1 in embryonic intestine.* Development, 2000. **127**(17): p. 3805-13.

33. Meyer, B.I. and P. Gruss, *Mouse Cdx-1 expression during gastrulation.* Development, 1993. **117**(1): p. 191-203.

34. Millet, S., et al., *A role for Gbx2 in repression of Otx2 and positioning the mid/hindbrain organizer.* Nature, 1999. **401**(6749): p. 161-4.

35. Robinson, J.F., et al., *A comparison of gene expression responses in rat whole embryo culture and in vivo: time-dependent retinoic acid-induced teratogenic response.* Toxicol Sci, 2012. **126**(1): p. 242-54.

36. Tonk, E.C., et al., *Valproic acid-induced gene expression responses in rat whole embryo culture and comparison across in vitro developmental and non-developmental models.* Reprod Toxicol, 2013. **41**: p. 57-66.

37. Brown, N.A. and S. Fabro, *Quantitation of rat embryonic development in vitro: a morphological scoring system.* Teratology, 1981. **24**(1): p. 65-78.

38. Wilkinson, D.G. and M.A. Nieto, *Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts.* Methods Enzymol, 1993. **225**: p. 361-73.

39. Debeljak, N., M. Fink, and D. Rozman, *Many facets of mammalian lanosterol 14alpha-demethylase from the evolutionarily conserved cytochrome P450 family CYP51.* Arch Biochem Biophys, 2003. **409**(1): p. 159-71.

40. Lee, Y.M., et al., *Retinoic acid stage-dependently alters the migration pattern and identity of hindbrain neural crest cells.* Development, 1995. **121**(3): p. 825-37.

41. Balmer, N.V., et al., *From transient transcriptome responses to disturbed neurodevelopment: role of histone acetylation and methylation as epigenetic switch between reversible and irreversible drug effects.* Arch Toxicol, 2014. **88**(7): p. 1451-68.

42. Robinson, J.F., A. Verhoef, and A.H. Piersma, *Transcriptomic analysis of neurulation and early organogenesis in rat embryos: an in vivo and ex vivo comparison.* Toxicol Sci, 2012. **126**(1): p. 255-66.

43. del Corral, R.D., et al., *Opposing FGF and Retinoid Pathways Control Ventral Neural Pattern, Neuronal Differentiation, and Segmentation during Body Axis Extension.* Neuron, 2003. **40**(1): p. 65-79.

44. Rhinn, M. and P. Dolle, *Retinoic acid signalling during development.* Development, 2012. **139**(5): p. 843-58.

45. Abu-Abed, S., et al., *The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures.* Genes Dev, 2001. **15**(2): p. 226-40.

46. MacLean, G., et al., *Cloning of a novel retinoic-acid metabolizing cytochrome P450, Cyp26B1, and comparative expression analysis with Cyp26A1 during early murine development.* Mech Dev, 2001. **107**(1-2): p. 195-201.

47. Tahayato, A., P. Dolle, and M. Petkovich, *Cyp26C1 encodes a novel retinoic acid-metabolizing enzyme expressed in the hindbrain, inner ear, first branchial arch and tooth buds during murine development.* Gene Expr Patterns, 2003. **3**(4): p. 449-54.

48. Beland, M., et al., *Cdx1 autoregulation is governed by a novel Cdx1-LEF1 transcription complex.* Mol Cell Biol, 2004. **24**(11): p. 5028-38.

49. Prinos, P., et al., *Multiple pathways governing Cdx1 expression during murine development.* Dev Biol, 2001. **239**(2): p. 257-69.

50. Rankin, E.B., et al., *Putative intestine-specific enhancers located in 5' sequence of the CDX1 gene regulate CDX1 expression in the intestine.* Am J Physiol Gastrointest Liver Physiol, 2004. **286**(5): p. G872-80.

51. van den Akker, E., et al., *Cdx1 and Cdx2 have overlapping functions in anteroposterior patterning and posterior axis elongation.* Development, 2002. **129**(9): p. 2181-93.

52. Young, T. and J. Deschamps, *Hox, Cdx, and anteroposterior patterning in the mouse embryo.* Curr Top Dev Biol, 2009. **88**: p. 235-55.

53. Grainger, S., A. Hryniuk, and D. Lohnes, *Cdx1 and Cdx2 exhibit transcriptional specificity in the intestine.* PLoS One, 2013. **8**(1): p. e54757.

54. van Dartel, D.A., et al., *Concentration-dependent gene expression responses to flusilazole in embryonic stem cell differentiation cultures.* Toxicol Appl Pharmacol, 2011. **251**(2): p. 110-8.

55. Archer, A., et al., *Transcriptional activity and developmental expression of liver X receptor (lxr) in zebrafish.* Dev Dyn, 2008. **237**(4): p. 1090-8.

56. Archer, A., et al., *Fasting-induced FGF21 is repressed by LXR activation via recruitment of an HDAC3 corepressor complex in mice.* Mol Endocrinol, 2012. **26**(12): p. 1980-90.

57. Pinto, C.L., et al., *Lxr regulates lipid metabolic and visual perception pathways during zebrafish development.* Mol Cell Endocrinol, 2016. **419**: p. 29-43.

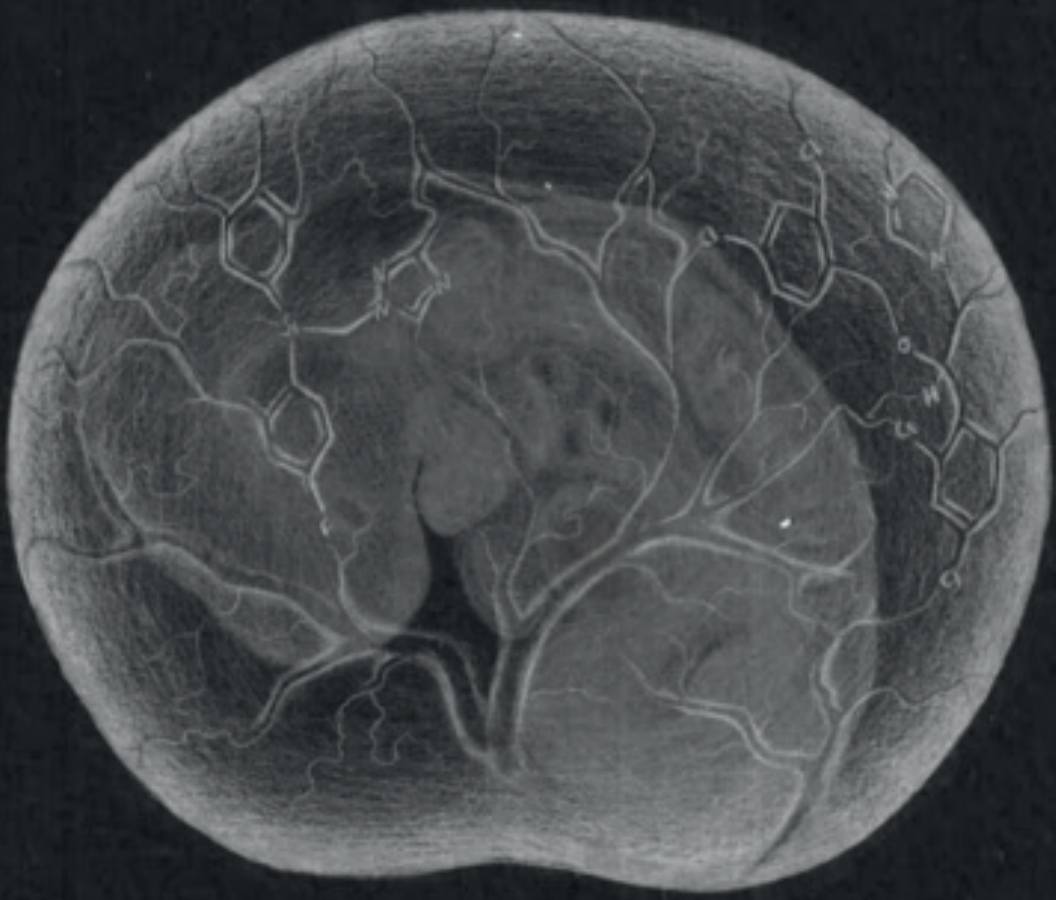
58. Merki, E., et al., *Epicardial retinoid X receptor alpha is required for myocardial growth and coronary artery formation.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18455-60.

59. Jagtap, S., et al., *All-trans retinoic acid and basic fibroblast growth factor synergistically direct pluripotent human embryonic stem cells to extraembryonic lineages.* Stem Cell Res, 2013. **10**(2): p. 228-40.

60. Huang, N., et al., *Diversity and function of mutations in p450 oxidoreductase in patients with Antley-Bixler syndrome and disordered steroidogenesis.* Am J Hum Genet, 2005. **76**(5): p. 729-49.

61. Keber, R., et al., *Mouse knockout of the cholesterogenic cytochrome P450 lanosterol 14alpha-demethylase (Cyp51) resembles Antley-Bixler syndrome.* J Biol Chem, 2011. **286**(33): p. 29086-97.

62. Schmidt, K., et al., *Cholesterol metabolism: the main pathway acting downstream of cytochrome P450 oxidoreductase in skeletal development of the limb.* Mol Cell Biol, 2009. **29**(10): p. 2716-29.



Chapter 3

Embryotoxic and pharmacologic potency ranking of six azoles in the rat Whole Embryo Culture by morphological and transcriptomic analysis

Myrto Dimopoulou, Aart Verhoef, Jeroen L.A. Pennings, Bennard van Ravenzwaay, Ivonne M.C.M. Rietjens and Aldert H. Piersma

Toxicology and Applied Pharmacology (2017) 322; 15-26

Abstract

Differential gene expression analysis in the rat Whole Embryo Culture (WEC) assay provides mechanistic insight into the embryotoxicity of test compounds. In our study, we hypothesized that comparative analysis of the transcriptomes of rat embryos exposed to six azoles (flusilazole, triadimefon, ketoconazole, miconazole, difenoconazole and prothioconazole) could lead to a better mechanism-based understanding of their embryotoxicity and pharmacological action. For evaluating embryotoxicity, we applied the total morphological scoring system (TMS) in embryos exposed for 48 hours. The compounds tested showed embryotoxicity in a dose-response fashion. Functional analysis of differential gene expression after 4 hours exposure at the ID_{10} (effective dose for 10% decreased TMS), revealed the sterol biosynthesis pathway and embryonic development genes, dominated by genes in the retinoic acid (RA) pathway, albeit in a differential way. Flusilazole, ketoconazole and triadimefon were the most potent compounds affecting the RA pathway, while in terms of regulation of sterol function, difenoconazole and ketoconazole showed the most pronounced effects. Dose-dependent analysis of the effects of flusilazole revealed that the RA pathway related genes were already differentially expressed at low dose levels while the sterol pathway showed strong regulation at higher embryotoxic doses, suggesting that this pathway is less predictive for the observed embryotoxicity. A similar analysis at the 24-hour time point indicated an additional time-dependent difference in the aforementioned pathways regulated by flusilazole. In summary, the rat WEC assay in combination with transcriptomics could add a mechanistic insight into the embryotoxic potency ranking and pharmacological mode of action of the tested compounds.

Keywords: Whole Embryo Culture, toxicogenomics, azoles, retinoic acid, embryonic development, sterol biosynthesis

Introduction

Supranational regulatory guidelines, such as the European legislation of Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), demand the use of large numbers of experimental animals for the risk assessment of chemicals [1]. Reproductive and developmental toxicity studies require almost 65% of experimental animals needed overall [1, 2]. The necessity of reduction, refinement and replacement of animal testing has stimulated the design and application of alternative assays for the hazard identification of developmental toxicants [3-5]. Alternative techniques vary from cell-based methods to organ culture, organ-on-a-chip, whole embryo cultures to *in silico* simulation models [4, 6, 7].

The rat Whole Embryo Culture (WEC) technique is an alternative method for assessing possible developmental toxicants, and it is used for screening studies due to its numerous benefits [8, 9]. This technique allows the continuous monitoring of embryonic development during gestational days (GD) 10 to 12, when a major part of organogenesis occurs [10]. Additionally, the complexity of the entire embryo is included, and development mimics the *in vivo* situation in terms of both morphology and gene expression signatures [11]. The limitations of this method include the restricted experimental duration and the absence of metabolic activity, as well as the lack of maternal interaction. Various metabolic systems added to WEC have shown activity [12, 13]. However, even with the addition of metabolic activity, some classes of proembryotoxicants would not be classified properly in *in vitro* testing systems, such as the WEC [14]. Furthermore, it has been suggested that direct testing of the parent compounds and metabolites individually might provide a solution if metabolic pathways are known [13-15]. Overall, the rat WEC is considered a valuable tool for screening xenobiotics and prioritizing further steps in risk assessment of possible embryotoxicants [16-18]. Previous studies have shown that apart from screening morphological endpoints for evaluating possible developmental toxicants in the WEC model, the implementation of technologically advanced molecular-based assays could improve its value as a predictive assay [4, 19, 20]. Previous studies have shown that gene expression changes, related to toxic responses of biological systems, are not only associated with morphological outcomes, but also precede them [21]. Additionally, literature data indicate that different compounds might cause a similar adverse outcome, while the underlying mechanisms of toxicity might be different. Transcriptomics approaches could enhance mechanistic knowledge of embryotoxicants [20, 22]. An additional advantage of transcriptomics is that the evaluation of molecular signatures could contribute to the identification of biomarkers for detecting embryotoxicity among different classes of compounds [20, 23]. Furthermore, due to the high conservation of many molecular pathways, these biomarkers could improve the extrapolation of results obtained from *in vitro*

studies to the *in vivo* situation and contribute to understanding interspecies differences [22, 24].

Azoles form a class of compounds, known for their potential to induce teratogenic effects in mammals in a dose dependent manner [25, 26]. They are fungicides with either agricultural or clinical use. Their mode of pharmacological action in the fungal cell is based on the inhibition of the conversion of lanosterol to ergosterol via disturbing the enzyme involved being *Cyp51*, which is the mediator for securing the robustness of fungal membranes [27, 28]. The toxicological mechanism of azoles is partly unknown. However, when *in vivo* and *in vitro* mammalian systems are exposed to either azoles or retinoic acid (RA), a variety of similar teratogenic responses have been observed, including abnormalities in craniofacial development, brain segmentation and branchial arches formation [29-32]. Therefore, azoles might have a shared mechanism of toxicological action with RA-induced embryotoxicity, which is supported by gene expression studies showing that the enzyme *Cyp26a1*, a key regulator of embryonic RA concentration, was upregulated in a common manner [32-34]. Thus, *Cyp26a1* and *Cyp51* appear as candidate biomarkers for embryotoxicity and pharmacologic activity of azoles, respectively. However, this does not exclude that other mechanisms may be involved as well.

In order to assess the usefulness of transcriptomics readouts in assessing relative embryotoxic potencies and the underlying modes of action, we have studied the morphologic and transcriptomic responses of six azoles in WEC and compared their potencies with existing *in vivo* data. We included flusilazole (FLU) [35, 36], triadimefon (TDF) [37, 38] and ketoconazole (KTZ) [39, 40] as relatively strong embryotoxicants *in vivo*; miconazole (MCZ) [41] as weak embryotoxicant; difenoconazole (DFZ) [42] and prothioconazole (PTZ) [43] as non- embryotoxicants.

Materials and Methods

Animal care

Animal studies were approved and performed in concordance with institutional and federal regulations at the National Institute of Public Health and the Environment (RIVM). Wistar rats (HsdCpd:WU) (Harlan, The Netherlands) were housed at the RIVM Animal Care facility in a climate-controlled room with a 12h light cycle (04:00-16:00 dark). Water and food were provided ad libitum. After acclimating for 2 weeks, virgin female rats were housed with male rats for a 3-hour mating period (9:00-12:00) (GD 0). Mated dams were individually housed. The clinical condition of all the animals was monitored daily.

Rat Whole Embryo Culture

The WEC technique was performed in accordance with the validated method of Piersma [9]. On GD 10, between 9:00 and 12:00 a.m., dams were euthanized by intracardiac injection of $T61^R$ (Intervet, The Netherlands). Rat embryos were immediately separated from the uterus. The peripheral trophoblastic cell zone and parietal yolk sac membrane were removed under the microscope leaving both the visceral yolk sac and ectoplacental cone intact. Embryos with 1-5 somites were cultured for morphological assessment while embryos with 2-4 somites were cultured for gene expression studies, which increases precision of the embryonic stage sufficient for gene expression analysis [32]. Embryos were separately cultured in flasks with 2 mL culture medium, which was a mixture of 90% pregnant bovine serum and 10% rat serum (Biochrom, Berlin, Germany), diluted with 14% Hank's solution (Gibco) and supplemented with 1.6 mg/mL D-glucose and 75 μg/mL L-methionine (Sigma-Aldrich, Zwijndrecht, The Netherlands). Afterwards, the culture flasks were placed in rotating incubators, which were completely protected from light and their internal temperature was permanently at 37.7°C. The cultured flasks were oxygenated twice daily for 30 seconds per time, with increasing concentration of oxygen: on the first day (GD10) at 9:00 and 16:00 (5% O_2, 5% CO_2, 90% N_2), on the second day (GD11) at 9:00 and 16:00 (20% O_2, 5% CO_2, 75% N_2) and on the third day (GD12) at 9:00 (40% O_2, 5% CO_2, 55% N_2).

Morphological assessment and statistical analysis of individual endpoints

Embryos (exposed to test compounds and controls) were cultured for 48 h (whole culture period; 0-48h) and were scored according to the TMS system taking into account 20 morphological endpoints [44]. These morphological endpoints were sub-divided into groups, which included growth parameters (yolk sac diameter, crown-rump length, head diameter and number of somites) and developmental/functional parameters, such as yolk sac and

allantoic blood circulation, heart formation and heart beating, embryo- turning, caudal neural tube, optic and otic system, fore- and hind- limb, branchial arches, mandibular and maxillary process and the shape and size of somites. Average scores of each of the morphological endpoints were calculated for identifying any possible specific and selective embryotoxic effects of the tested compounds in rat embryos.

Within each exposure group, including also the vehicle control (DMSO), 8 rat embryos were evaluated. For normalizing the obtained data and eliminating daily variation, the embryos within the same exposure group were derived from different dams and they were cultured over different culture days. Statistical analysis was performed using the parametric Student's *t*-test (unpaired), two-sided, and with 95% confidence intervals. Images of the examined embryos (exposed for 48 hours to either DMSO or tested compounds) were obtained using an Olympus SZX9 camera and Olympus DP software. The pictures were taken at x20 magnification. For specific observations, x32 magnification was used.

Test compounds and exposure concentrations

The following six azoles were tested in rat WEC for 48 hours (0-48h) in a range of concentrations with the lowest concentration inducing no morphological effect to the highest being the maximal achievable concentration in culture:

difenoconazole (DFZ; CAS# 119446-68-3, Sigma-Aldrich, Zwijndrecht, The Netherlands) at 20, 60, 200 and 600 μM; flusilazole (FLU; CAS# 85509-19-9, Sigma-Aldrich, Zwijndrecht, The Netherlands) at 2, 6, 20, 60, 200 and 600 μM; ketoconazole (KTZ; CAS# 65277-42-1, Sigma-Aldrich, Zwijndrecht) at 2, 6, 20, 60 and 200 μM; miconazole (MCZ; CAS# 22916-47-8, Sigma-Aldrich, Zwijndrecht, The Netherlands) at 2, 6, 20, 60, 200 and 600 μM; prothioconazole (PTZ; CAS# 178928-70-6, Sigma-Aldrich, Zwijndrecht, The Netherlands) at 60, 200 and 600 μM and triadimefon (TDF; CAS# 43121-43-3, Sigma-Aldrich, Zwijndrecht, The Netherlands) at 20, 60, 200 and 600 μM. All the compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Zwijndrecht, The Netherlands), and all embryos were exposed to a final DMSO concentration of 0.1%. As it has been previously described, this concentration of DMSO did not significantly alter the morphology (4, 24 and 48h) and has limited effects on gene expression after either 4h or 24h of exposure at the same concentration (0.1%) [11, 24, 45]. For finding the appropriate concentration at which the microarrays were conducted, we calculated the concentration which results to 10% reduction of the control TMS (ID_{10}) with both PROAST [46] and Graphpad software (www.graphpad.com). Therefore, for microarray analysis, rat WEC were exposed for 4 hours (0-4h) to FLU at testing concentrations of 0.002, 0.02, 0.2, 2, 25, 200 μM (tenfold

concentration intervals) for 4 hours (0-4h), while they were also exposed to the established ID_{10} of 25μM for 24 hours (0-24h). The remaining compounds were tested in the rat WEC for 4 hours (0-4h) at their ID_{10} values: DFZ at 110μM, KTZ at 40μM, MCZ at 25μM and TDF at 150μM, as derived from the concentration response curves on TMS. Rat embryos which were treated with PTZ were exposed to the calculated ID_{10} (250 μM) and at 60 μM. All the treated embryos were immediately collected after the end of their exposure and stored for performing the microarray analysis.

Whole embryo RNA isolation

For transcriptomics, cultured embryos were quickly scored on the basis of their somite number, their position in the yolk sac, neural tube developmental stage, crown-rump length and head diameter. They were then isolated from the yolk sac and ectoplacental cone, placed in 200μM RNAlater (Ambion, Austin, Texas), stored for one week at 4^{o}C, and then stored for further processing at -80^{o}C with the lowest possible amount of RNAlater. After the embryos were thawed on ice, they were separately homogenized by passing them 10 times though a 1mL syringe with a 26G needle. The RNA of the homogenized lysate was further isolated by using the RNeasy Micro Plus RNA isolation kit (CAS number 74034, Qiagen, the Netherlands). Eluting with 14μM of RNase-free H_2O, final volumes of 12μL RNA were obtained and tested on both Nanodrop (Nanodrop Technologies Inc., Wilmington, Delaware) and 2100 BioAnalyzer (Agilent Technologies, Palo Alto, California) to establish the RNA quality and quantity. The RNA samples with an absorbance value between 1.9 and 2.2 (ratio 260mm/280mm) and RNA integrity number (RIN) higher than 7, were further used for performing the microarray analysis. RNA samples were stored at -80^{o}C.

Microarray hybridization

RNA hybridization and microarray experimentation were performed by the Dutch Service and Support Provider (MAD) of the University of Amsterdam, the Netherlands. In brief, for every sample, RNA was amplified, biotin-labelled and hybridized to Affymetrix GeneChip HT RG-230 PM Array Plates according to the provided protocols by Affymetrix (Santa Clara, CA). After staining, the HT Array plate was read by the Affymetrix GeneChip® HT Scanner and analyzed by the Affymetrix GeneChip® Operating Software. For performing the aforementioned steps, the GeneTitan® Hybridization, Wash, and Stain Kit for 3' IVT Arrays (cat no. 901530) was used. In total, 112 arrays were analyzed. For the exposure to compounds tested at singe concentrations, 8 embryos per compound were prepared, while for the exposure to FLU for which more than one concentration was tested, 7 embryos for each concentration were included.

Microarray analysis and data processing

The quality control (QC) and the normalization of the microarray data were performed using the ArrayAnalysis.org webpage (www.arrayanalysis.org) [47], designed by the Department of Bioinformatics in Maastricht University. Raw microarray values were inspected for their quality by assessing the 3´/5´ ratios for β-actin and GAPDH, RNA degradation, background intensity, signal quality and the probe-set homogeneity with NUSE (Normalized Unscaled Standard Error) and RLE (Relative Log Expression). After the QC, two samples were excluded from the analysis because they did not fulfil the aforementioned quality criteria. The Affymetrix CEL files were further normalized by using the Robust Multichip Average (RMA) algorithm [48] and the Brainarray custom CDF version 19 probe set annotation (http://brainarray.mbni.med.umich.edu/Brainarray/default.asp) [49]. In total, 13,877 probe sets, each corresponding to an Entrez Gene ID, were further evaluated by performing a statistical analysis in R (www.R-project.org) and Microsoft Excel.

Identification of differentially expressed genes

Normalized data was transformed to log scale. For each exposure group (i.e. a compound at a concentration and time point), gene expression data were compared to the appropriate control (unexposed embryos at the same time point), for calculating absolute average fold changes of individual gene expression. Differentially expressed genes were identified by using ANOVA, using a p-value < 0.001 and a False Discovery Rate (FDR) of 10%, as stringency criteria. Genes differentially expressed in at least one of the 8 or 7 rat WEC samples from the corresponding exposure groups were combined for further analysis.

Gene expression responses were visualized using a heatmap combined with hierarchical clustering (Euclidean distance, Ward linkage) as well as Principal Component Analysis (PCA). Each bar in the heatmap represents the average of the gene expression in the experimental group compared to the appropriate control group.

Functional interpretation analysis of differentially expressed genes

Functional annotation and overrepresentation analysis were performed using DAVID (https://david.ncifcrf.gov/) [50]. Additionally, functional annotations were added from the literature [51, 52]. For genes involved in RA pathway, general development and sterol biosynthesis pathway, gene expression data per pathway were combined to absolute average fold changes per pathway. Next, the absolute average fold changes per exposure group were plotted against the compound concentration used. In the case of FLU multiple concentrations were tested, allowing data for other compounds to be compared against the

flusilazole dose-response curve to assess the relative potency in each pathway. Finally, gene expression and functionality were visualized as a network using Cytoscape version 2.8.3 (www.cytoscape.org) [53].

In vivo data analysis

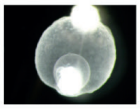
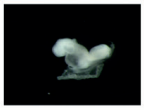
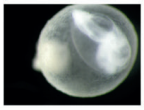
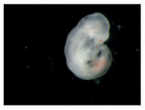
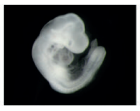
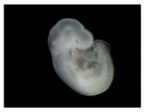
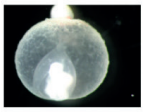
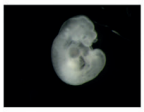
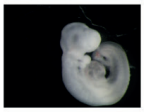
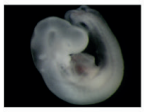
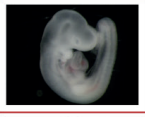
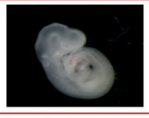
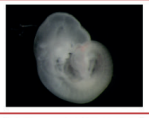
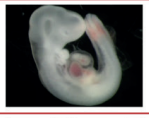
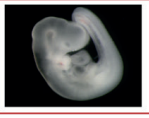
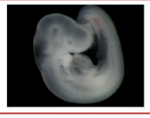
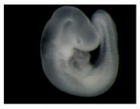
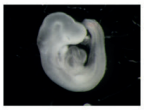
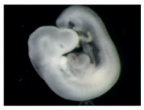
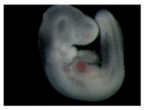
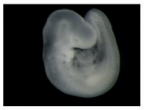
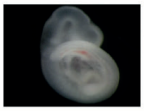
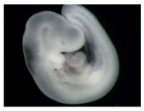
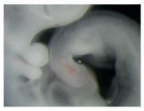
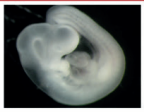
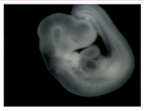
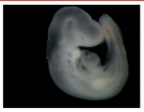
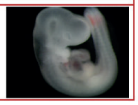
A literature survey was performed to determine the *in vivo* developmental toxic profile of the six tested azoles. We selected studies performed in rats orally exposed to the tested compounds during either GD6-15 or GD7-16 at multiple dose regimes. Studies with at least one control group and two dose groups were selected to allow analysis using the Benchmark Dose (BMD) approach. The BMD values were calculated on the basis of an increase in the incidence of skeletal malformations or cleft palate, selected as sensitive endpoints of *in vivo* developmental toxicity. A concentration-response curve was fitted to the data to determine the BMD for the selected benchmark response (BMR) for each tested azole. The BMD was defined as 10% additional incidence of skeletal malformations or cleft palate (BMD_{10}). The BMD_{10} of each compound was calculated with the PROAST software [46] using dichotomous concentration-response models (quantal data). Several models were fitted, included gamma, logistic, loglogistic, probit, logprobit, multistage and Weibull. The selection of the best model was determined based on the goodness of fit (p-value>0.05), given also the lowest BMD value.The developmental lowest effect levels (dLEL), obtained from the EPA ToxREF database [54], were also considered for comparison.

Results

Morphological assessment and definition of ID_{10} values

All azoles induced developmental toxicity in a dose dependent manner in the WEC assay (Tables 1&2). Highest concentrations without statistically significant effects on TMS were 20 µM for FLU, KTZ and MCZ, and 60 µM for DFZ, PTZ and TDF, respectively. At embryotoxic concentrations, FLU, KTZ and MCZ mostly affected the closure of the neural tube, the formation of branchial arches and early organogenesis of the optic cup. PTZ and DFZ were less potent but showed comparable abnormalities such as somitogenesis and disturbance of closure of the neural tube, as well as retarded development of both otic and optic cups at high doses. On the other hand, TDF affected the development of the branchial arches and the neural tube, while not affecting any of the other morphological endpoints. After evaluating the TMS concentration-response curves in the rat WEC, the ranking of decreasing potency of the six tested azoles was KTZ, MCZ, FLU, DFZ, TDF and PTZ (Figure 1). The ID_{10} concentrations of all tested compounds were calculated from Figure 1 to apply them as exposure concentrations in subsequent WEC experiments for performing transcriptomic analysis.

Table 1: Representative pictures of rat WEC exposed to the tested azoles at a range of concentrations for 48 hours. The morphology of embryos exposed at the lowest concentration of each compound was identical with those in the control group (DMSO).

	DFZ	FLU	KTZ	MCZ	PTZ	TDF	DMSO
600 μM							
200 μM							
60 μM							
20 μM							
6 μM							
2 μM							
0 μM							

The pictures were taken at x20 magnification. For specific observations, x32 magnification was used

Table 2: Overview of morphological effects of the tested azoles in the rat WEC assay.

Compound	Concentration (µM)	TMS	CRL (mm)	$S_{48h} - S_{0h}$	FORE	MID	HIND	CAUD	OTIC	OPTIC	BRAN	MAND-MAX	SOM	HEART
DMSO	0	67.4±0.8	4.2±0.2	24±1.1	-	-	-	-	-	-	-	-	-	-
DFZ	20	66.1±2.1	4.1±0.2	24±0.5	-	-	-	-	-	-	-	-	-	-
	60	65.4±1.4	4.1±0.2	23±1.4	-	-	-	*	-	-	-	-	-	-
	200	50.2±2.2**	3.1±0.2****	18±1.6*	*	**	**	**	**	*	*	**	*	*
	600	14.8±0.9****	#	#	***	**	***	***	****	***	***	****	***	***
FLU	2	66.6±0.5	4.2±0.2	24±1.2	-	-	-	-	-	-	-	-	-	-
	6	66.0±1	4.2±0.2	24±0.3	-	-	-	-	-	-	-	-	-	-
	20	60.8±4.7	4.0±0.3	22±1.6	-	-	-	-	-	-	-	-	-	-
	60	53.9±2.6*	3.9±0.2	17±2.0	-	-	-	*	-	-	-	-	-	-
	200	37.4±9.1***	3.2±0.2****	15±3.0*	*	**	**	**	***	*	*	**	-	*
	600	18.6±2.5****	1.5±0.5****	9±0.7**	***	**	***	****	****	**	**	****	**	***
KTZ	2	66.9±1.2	4.2±0.2	23±0.7	-	-	-	-	-	-	-	-	-	-
	6	66.8±1.2	4.2±0.2	24±0.5	-	-	-	-	-	-	-	-	-	-
	20	63.3±2.5	4.1±0.3	23±1.7	-	-	-	-	-	-	-	-	-	-
	60	54.4±3.7*	3.9±0.3	16±1.8	-	-	-	*	-	-	-	-	-	-
	200	15.6±0.5***	#	#	*	**	**	**	***	*	*	*	**	*
MCZ	2	66.7±0.8	4.1±0.2	24±0.5	-	-	-	-	-	-	-	-	-	-
	6	63.0±7.6	4.1±0.3	23±2.5	-	-	-	-	-	-	-	-	-	-
	20	61.0±3.4	4.0±0.2	22±1.5	-	-	-	-	-	-	-	-	-	-
	60	58.3±6.2*	4.1±0.3	23±1.2	-	-	-	*	-	-	-	-	-	-
	200	30.2±6.1***	2.9±0.7****	13±2.7*	*	**	**	**	***	*	*	**	-	*
	600	20.6±3.6****	#	12±2.1**	***	***	***	****	****	**	**	****	**	***
PTZ	60	65.8±4.2	4.1±0.3	24±1.1	-	-	-	-	-	-	-	-	-	-
	200	62.4±2.6**	3.9±0.2	23±1.1	*	*	*	*	**	*	*	*	**	-
	600	33.1±9.8****	2.7±0.4****	10±2.7**	***	***	***	***	***	***	**	***	***	***
TDF	20	66.4±1.1	4.1±0.2	24±0.8	-	-	-	-	-	-	-	-	-	-
	60	65.4±1.1	4.1±0.2	24±0.9	-	-	-	*	-	-	-	-	-	-
	200	55.8±5.1**	3.8±0.2**	20±2.2*	*	**	**	**	**	*	*	**	*	*
	600	18.8±2.4****	2.4±0.2****	10±3.4***	***	***	***	***	****	***	***	****	***	***

Each number represents a mean ± SD (N=8, Student's *t*-test: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$). CRL: crown-rump length; S_{48h}- S_{0h}: number of somites that formed during the culture period of rat WEC; FORE: forebrain; MID: midbrain; HIND: hindbrain; CAUD: caudal neural tube; OTIC: otic system; OPTIC: optic system; BRAN: branchial arches; MAND-MAX: mandibular and maxillary process; SOM: quality of somites and HEART: heart; "#": could not be measured.

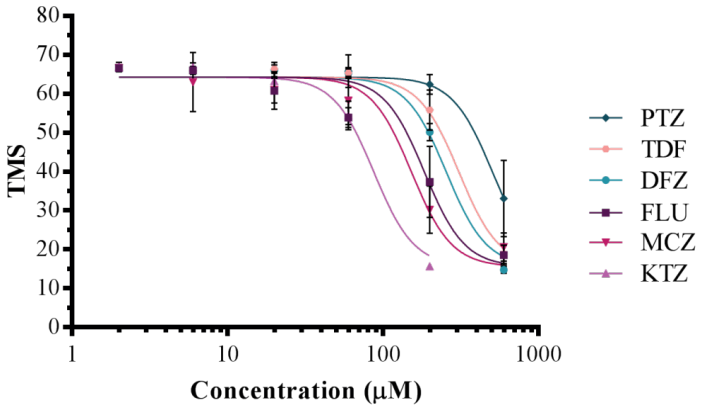


***Figure 1**: Total Morphological Score (TMS) concentration responses of 6 azoles in rat WEC after 48 hours of culture. Each point represents a mean ±SD (N=8).*

Microarrays and identification of significant responses

Rat embryos were exposed to ID_{10} concentrations of azoles detected in the WEC assay based on the TMS score to study effects on the level of their transcriptome. Embryos exposed to ID_{10} concentrations for either 4 or 24 hours had not yet developed any morphologically observable alterations. For example, somitogenesis, a general hallmark of the progress of embryo development, was unaffected (Figure 2, A and B).

Gene expression data were analysed by comparing each compound with the control group using ANOVA with significance threshold $p \leq 0.001$ (*t*-test) and an FDR value of 10%. Figure 3 shows an overview of gene expression changes of the 87 genes showing statistically significant responses. Using DAVID for functional annotation of significantly regulated genes, three main enriched gene groups were identified: RA pathway, general development and sterol biosynthesis pathway. The numbers of genes that were included in these pathways was 12, 29 and 17, respectively (Table 3). The genes in the RA pathway were a subset of the list referred to as the general development related genes.

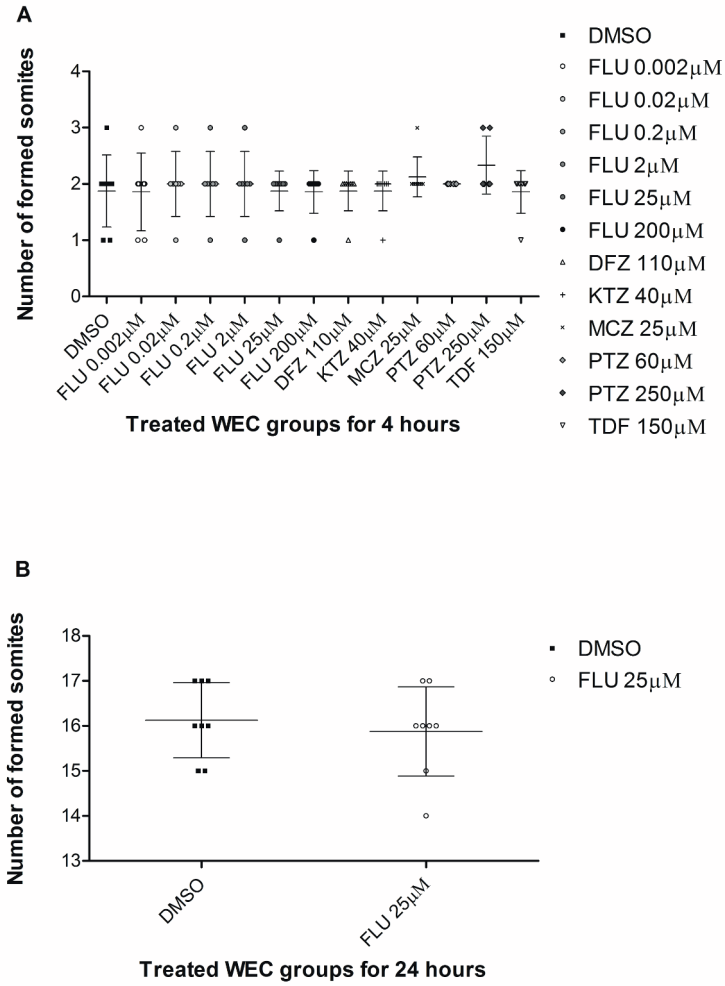


Figure 2: *Somitogenesis in rat embryos exposed to the six tested azoles for 4 hours (A) or 24 hours (B) and further used for microarray analysis. Each point represents the mean ±SD of embryos which belong in the same group of exposure. The number of embryos per group is N=8 for single doses per treatment (For 4-hour treatments: DMSO, FLU 25μM, DFZ 110μM, KTZ 40μM, MCZ 25μM, TDF 150μM, and for 24-hour treatments: DMSO, FLU 25μM). The number of embryos exposed for 4 hours to FLU is N=7 for each dose (0.002, 0.02, 0.2, 2, 200μM).*

Table 3: Gene-set compositions of the three main enriched gene groups: RA pathway, general development and sterol biosynthesis pathway. The numbers of genes included in these pathways is 12, 29 and 17, respectively.

RA pathway (12)	General development (29)	Sterol biosynthesis pathway (17)
Hoxa1	*Hoxa1*	*Pcsk9*
Gbx2	*Gbx2*	*Srebf2*
Fgf4	*Fgf4*	*Sc5d*
Cyp26a1	*Cyp26a1*	*Msmo1*
Hoxc4	*Hoxc4*	*Scd1*
Rarb	*Rarb*	*Cyp51*
Fgfr4	*Fgfr4*	*Hmgcr*
Cyp26b1	*Cyp26b1*	*Sqle*
Dhrs3	*Dhrs3*	*Hsd17b7*
Hoxc10	*Hoxc10*	*Fdft1*
Mafb	*Mafb*	*Hmgcs1*
Wnt5a	*Wnt5a*	*Nsdhl*
	Jund	*Pmvk*
	Ngfr	*Abca1*
	Alx1	*Dhcr7*
	Dlx5	*Mvd*
	Lhx1	*Idi1*
	Sostdc1	
	Mecom	
	Ifrd1	
	Btg2	
	Pcsk9	
	Srebf2	
	Glis2	
	Tbx4	
	Nrip1	
	Cbln1	
	Fgfbp3	
	Tgm2	

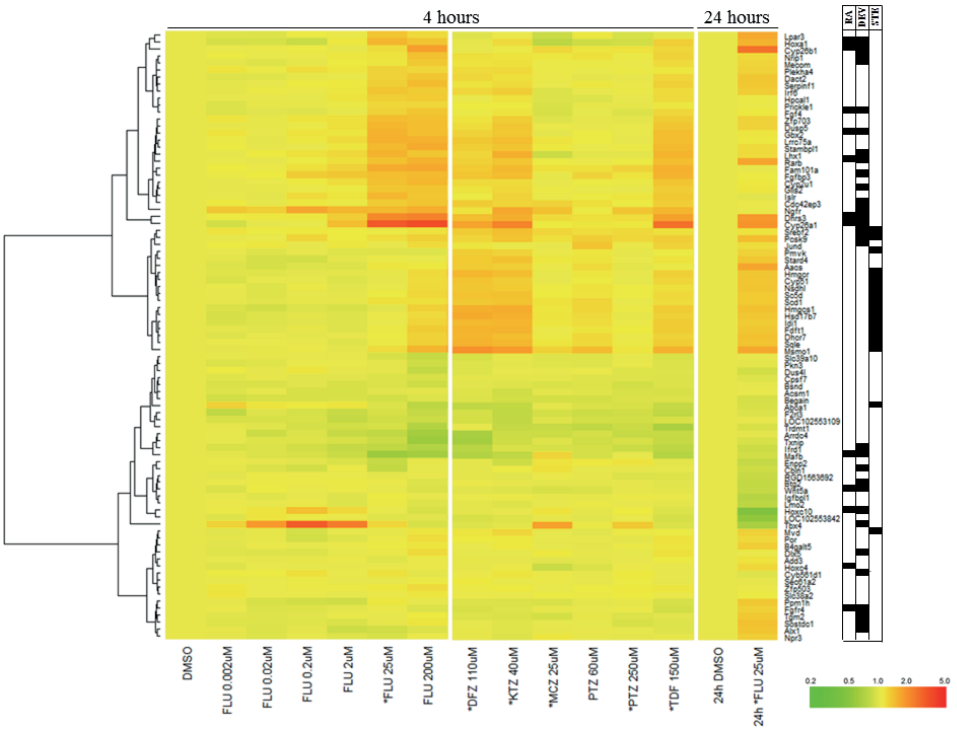


Figure 3: *Hierarchical clustering of gene expression in rat embryos exposed to six azoles. In total 87 genes were significantly regulated after at least one of the treatments. Each bar in the heatmap represents the average of the gene expression in the experimental group compared to the appropriate control group. The 87 genes were further categorized into three groups of biological processes RA pathway (RA), general embryonic development (DEV) and sterol biosynthesis pathway (STE), illustrated as black bars on the right part of this figure.*

Network analysis: Relating exposures to pathways and responsive genes

Figure 4 illustrates the relationships between exposures, genes and pathways as observed in the present study (Figure 3) using Cytoscape network visualization. Generally, this network analysis demonstrates that embryonic 4-hour exposure to FLU and TDF at or, for FLU, above the ID_{10} provoked an up- or down-regulation of genes involved in general embryonic development and in the RA pathway. In contrast, embryonic exposure to KTZ or DFZ at their respective ID_{10}, for 4 hours was primarily linked to the dysregulation of genes related to the sterol biosynthesis pathway. Prolonged exposure of rat embryos at 25µM of

FLU caused a notable upregulation of genes related to the sterol biosynthesis pathway, while the response of the genes participating in the general embryonic development and the RA pathway was varied from up- to down- regulation. PTZ and MCZ were the two compounds with the least effects on the embryonic pathways and the involved genes under investigation.

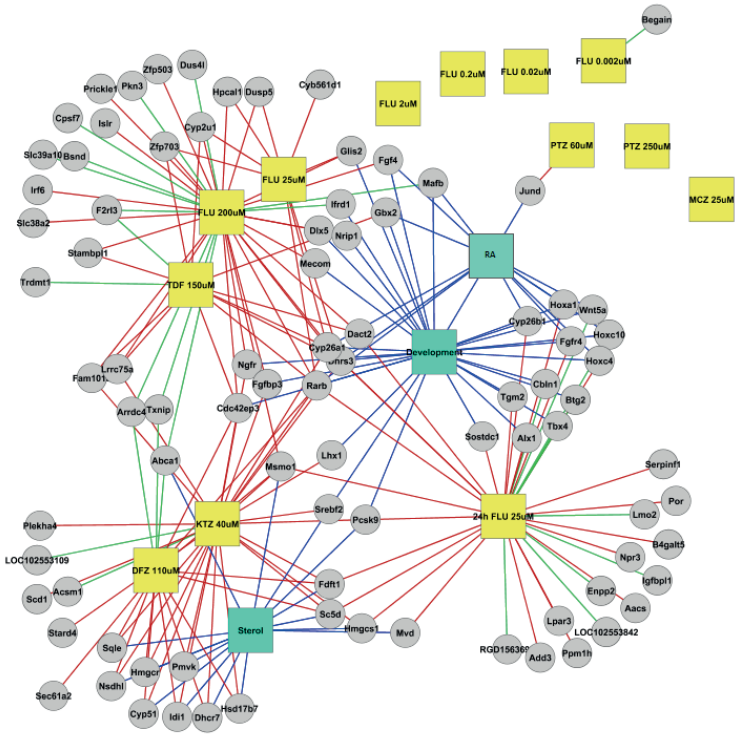


Figure 4: *Cytoscape network visualization of 87 significantly regulated genes due to rat WEC exposure to azoles. Grey cycled nodes represent the 87 genes, yellow and green squared nodes illustrate the compounds and the associated biological processes, respectively. Connections between exposure and regulated pathways demonstrate upregulation (red line), or downregulation (green line) and links of genes to pathways (blue line).*

Concentration-dependence of gene expression changes

Figure 5 illustrates the absolute average fold change of the expression of genes participating in the three aforementioned biological pathways upon exposure to increasing concentrations of FLU. Clearly, the lowest FLU concentration tested already showed regulation of each of

the three pathways considered. All pathways responded concentration dependently on exposure to FLU. The RA pathway showed the highest magnitude of regulation. The sterol biosynthesis pathway regulation was substantially affected at the highest concentration tested (200μM), whereas the general development pathway already showed a response at lower concentrations. At the ID_{10} of FLU (25μM), the RA pathway and general embryonic development showed a more pronounced effect compared to the sterol biosynthesis pathway, at given fold change of 1.41, 1.27 and 1.07, respectively.

Within pathways, individual genes showed differences in responses. In the general development gene set, *Tbx4* was upregulated in a concentration-dependent manner, while *Hoxa1* was downregulated with the same trend (Figure 3). However, the expression of both genes was almost not altered when rat embryos were exposed at 25μM (ID_{10}). As to the RA pathway and general development, in 4h- exposed embryos at 25μM of FLU there was a pronounced up regulation of *Cyp26a1*, *Dhrs3*, *Gbx2*, *Lhx1*, *Hoxa1* and *Wnt5a*. Their expression was quantified to be respectively 3.0, 2.0, 1.5, 1.5, 1.5 and 1.2 fold higher compared to the control. *Tbx4* and *Mafb* were expressed in a more pronounced way at concentrations lower than the ID_{10}. Sterol-related genes were expressed with slight fluctuations and revealed a remarkable 1.2 fold upward trend when embryos were exposed to 200μM. Some of the genes in this pathway that did not follow this general trend were *Abca1*, which showed an upward trend at the lowest dose of 0.002μM of FLU, while *Pcsk9* and *Msmo1*had an upward trend of expression at 25 and 200μM. Some other representative genes which contributed to the enrichment of the sterol biosynthesis pathway, such as *Cyp51*, *Sqle*, and *Hmgcr*, did show a regular rise of their absolute fold change in response, up to 1.3 fold at the highest concentration of FLU tested.

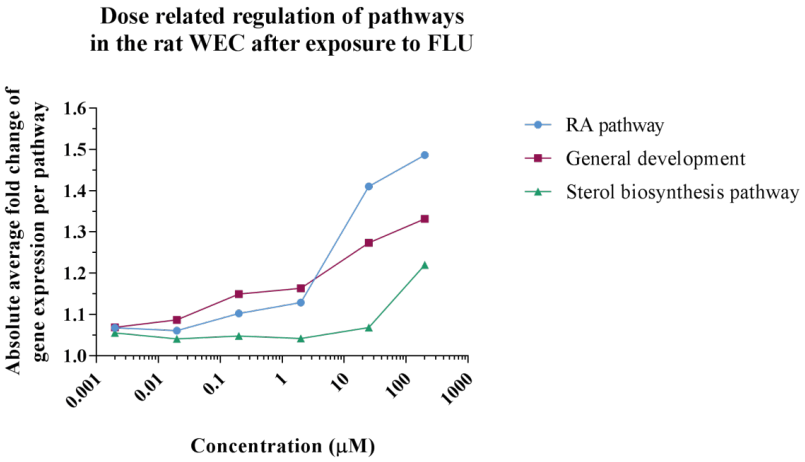


Figure 5: *Concentration-dependent effects on enriched biological processes of FLU in rat WEC, relative to the vehicle control (=1.0), expressed as an average absolute fold change of all genes in each pathway.*

Comparing gene set responses at the morphological ID_{10} of six azoles

The potency of the six tested azoles as to the regulation the three functional gene groups was assessed at the ID_{10} of each compound individually. FLU dose-response curves (Figure 5) were used for comparison.

FLU exposure at its ID_{10} showed a greater response on the RA pathway (1.41) compared to the five remaining ID_{10} exposures. The other compounds, in the order of TDF>KTZ>DFZ>MCZ>PTZ, showed a decreasing magnitude of response on the RA pathway at their ID_{10} (Figure 6, A), quantified to 1.33, 1.24, 1.17, 1.13 and 1.08 fold, respectively. The commonly most responsive genes were *Cyp26a1*, *Dhrs3* and *Gbx2*. On the other hand, *Wnt5a* was the least responsive and *Mafb* was the most downregulated with the exception of a slightly but not statistically significant upregulated response to MCZ (1.23 fold change) (Figure 3). Exposure to FLU (1.3) and TDF (1.25) at their ID_{10} resulted in a similar response magnitude on general embryonic development related genes (Figure 6, B). The remaining compounds followed in the order of KTZ>MCZ≈DFZ>PTZ (1.21, 1.13, 1.14 and 1.09, respectively). The difference in response magnitude of this gene group between compounds was small, indicating that it mimics the morphological ID_{10} rather well. (Figure 6, B). Interestingly, *Tbx4* was most down regulated due to WEC exposure to either MCZ or PTZ. The most upregulated genes were the aforementioned RA-related genes, as well as *Lhx1*, *Hoxa1*, *Ngfr* and *Fgfbp3*.

In contrast to the above pathways, WEC exposure to DFZ (1.423) and KTZ (1.420) appeared to have a greater effect than FLU on the sterol biosynthesis pathway. TDF had an intermediate potency, and the least potent compounds were MCZ (1.10), FLU (1.07) and PTZ (1.07). (Figure 6, C; DFZ>KTZ>TDF>MCZ≈FLU≈PTZ). Both DFZ and KTZ upregulated *Msmo1*, *Hsd17b7* and *Sqle*. *Cyp51* was also upregulated by these two compounds, as well as by TDF. The commonly downregulated gene among the tested compounds was *Abca1*.

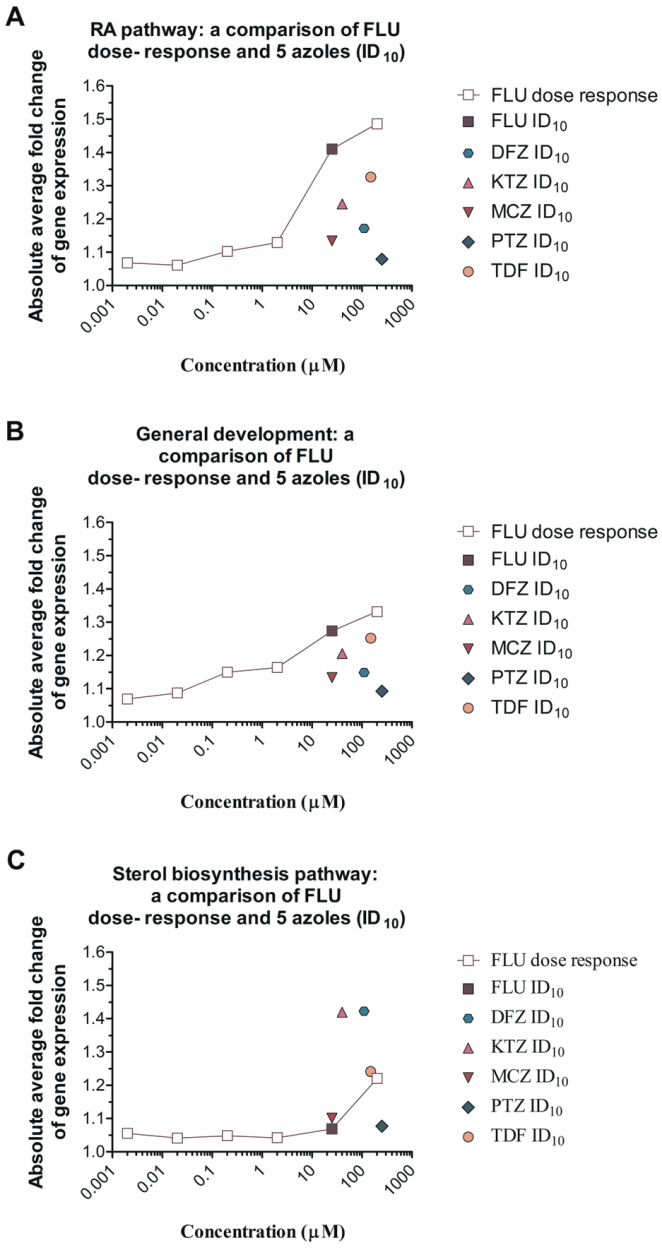


Figure 6: *Functional analysis of dose-dependent (ID_{10}) effects of tested azoles in rat WEC compared to the effects of FLU in a dose range per pathway. The response of the pathway*

is expressed as average fold change associated with RA pathway (A), general development (B) and sterol biosynthesis pathway (C) is compared to vehicle control (=1.0).

A comparison of gene expression responses within selected pathways between 4- and 24-h of exposure to FLU

Gene expression changes in response to FLU exposure in the RA and general embryonic development pathways varied in magnitude with exposure time. This was evident in the expression of *Cyp26a1*, *Cyp26b1*, *Hoxc10*, *Mafb* and *Wnt5a*, biomarkers of the RA pathway, (Figure 7, A). Similarly, genes in the general embryonic development pathway showed a varied response magnitude among genes, dependent on exposure duration. In the embryonic development pathway, *Ngfr* and *Lhx1* were upregulated in embryos exposed for 4 hours to almost twice the extent observed upon 24 hours of exposure. In contrast, *Pcsk9* and *Tbx4* were 2-fold more responsive after the prolonged exposure than after the short exposure (Figure 7, B).

In contrast, in the sterol biosynthesis pathway (Figure 7, C), the magnitude of gene expression of all genes was considerably higher in embryos exposed to FLU for 24 hours than in those exposed for 4 hours. The sterol-related genes, *Hmgcr* and *Mvd*, only showed regulation in embryos after prolonged (24-hour) FLU-treatment.

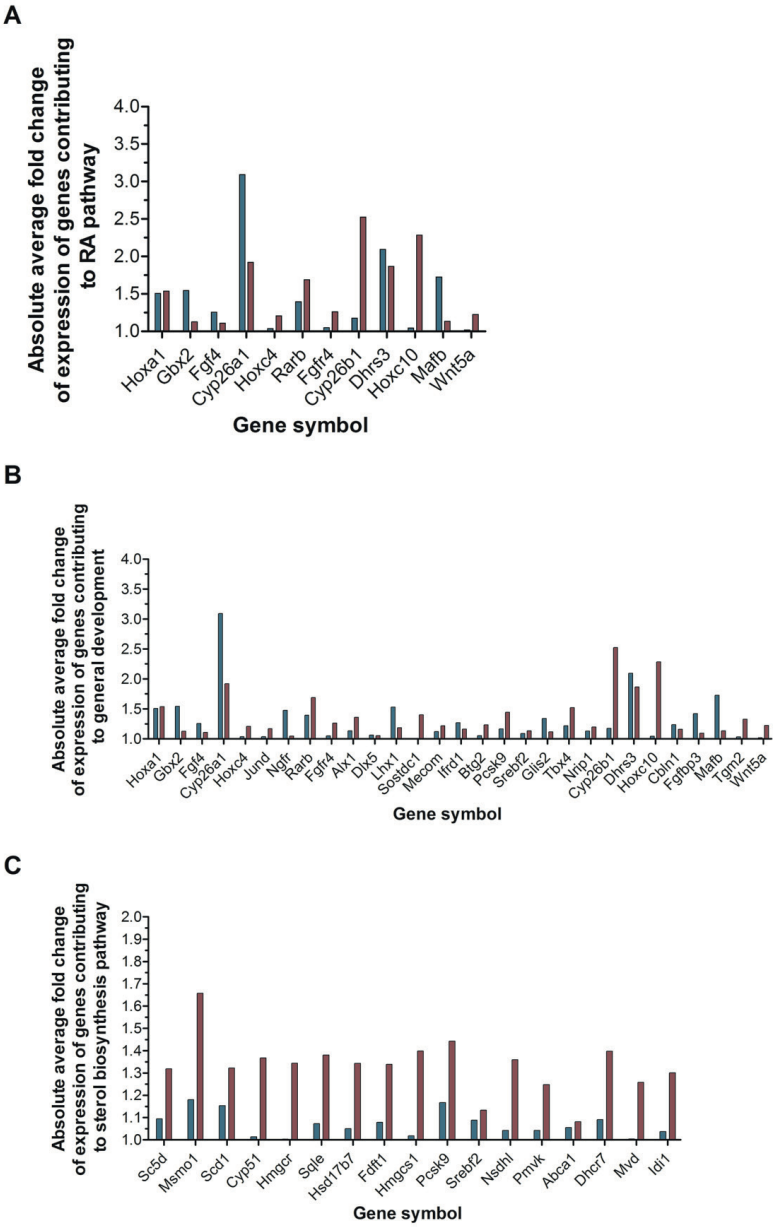


Figure 7: *A comparison of gene expression regulation in embryos exposed for 4 (blue) or 24 hours (red) to FLU at 25μM (ID_{10}) per functional pathway, RA pathway (A), general development (B) and sterol biosynthesis pathway (C).*

Comparison of in vitro with in vivo data

Data on the developmental toxic profile of the six tested azoles were obtained from literature *in vivo* studies. A potency raking was performed after calculating the BMD_{10} values, evaluating incidences of skeletal malformations and cleft palate. Table 4 contains an overview of the *in vivo* calculated BMD_{10} values and dLEL values, as well as *in vitro* results from the present study, including the ID_{10} values of morphological data (TMS) and response of genes participating in the RA pathway. It should be noted that for some compounds, e.g. miconazole, there is a limited availability of *in vivo* data. Additional data regarding the inhibition of *Cyp26a1* are included, which were kindly provided by BASF SE laboratories of Experimental Toxicology and Ecology.

In vivo exposure to FLU, KTZ and TDF caused cleft palate, while FLU was also inducing renal malformations, such as absence of renal papilla, a type of malformation generally not typical for azoles. Furthermore, rat *in vivo* exposure to DFZ and PTZ was associated with skeletal alterations, which included formation of supernumerary rudimentary ribs, extra ossification and deformation of thoracic vertebrae. FLU and KTZ were the most potent embryotoxicants *in vivo*, following TDF and MCZ. DFZ and PTZ were the compounds with less severe effects. Data derived from the EPA *ToxRef* database were in agreement with the aforementioned observations. *In vitro* studies on the inhibition of *Cyp26a1*, after exposure to the four out of six tested compounds, show that MCZ and TDF were the most potent compounds, while PTZ and KTZ inhibited the regulation of *Cyp26a1* at higher concentrations.

Table 4: Overview of *in vivo* and *in vitro* developmental toxicity data of the tested azoles.

Compound	*in vivo*		*in vitro*		
	BMD_{10} (µmol/kg)	**dLEL (µmol/kg)[54]**	**ID_{10} (TMS)**	**RA response (abs fold change)**	**IC_{50} *Cyp26a1* inhibition (µM)**
PTZ	917.8[43]	290.8	250	1.08	3.02
DFZ	596.5[42]	421.5	110	1.17	-
MCZ	258.3[41]	-	25	1.13	0.44
TDF	91.5[37, 38]	171.4	150	1.33	2.29
KTZ	20.1[39, 40]	-	40	1.25	12.02
FLU	9.1[36]	1.3	25	1.41	-

Discussion

In agreement with previous studies [25, 32, 51, 55], we observed a variety of concentration dependent embryotoxic responses in rat embryos, cultured and exposed *in vitro* to six azoles for 48 hours during GD 10-12. During this particular time window, major aspects of organogenesis and neurulation take place [11, 32, 33, 56]. Commonly observed malformations in *in vitro* systems exposed to embryotoxic azoles were axial defects, craniofacial malformations and impaired branchial arches formation [26, 57, 58]. KTZ and MCZ have been described to specifically affect heart function via dysregulating the K^+ and Ca^{2+} channels leading to heart-related syndromes [59].

In vivo developmental toxicity studies have been performed assessing the embryotoxic profile of the six tested compounds. As to cleft palate and skeletal malformations observed at gestation day 21 (GD21), FLU, KTZ and TDF were the most potent embryotoxic compounds, while MCZ, DFZ were less potent. PTZ induced embryotoxicity only at very high concentration, which are likely to be unattainable in *in vivo* situation, and thus in line with the very low embryotoxicity potential of this compound (Table 4). WEC morphology (Figure 1, Table 1, 2 & 4) assessed at GD12 indicated FLU, KTZ and MCZ as the most potent compounds, while TDF, DFZ and PTZ were less potent.

Following whole genome analysis in WEC exposed to each of the six azoles at their ID_{10} for effects on the TMS, we identified 87 genes significantly regulated by at least one of the tested compounds (ANOVA, $p<0.001$). Three gene sets (DAVID) were overrepresented among the regulated genes, related to the RA pathway, general embryonic development and the sterol biosynthesis pathway, respectively [24, 51]. Each of the gene sets showed some regulation for all compounds tested, likely reflecting non-toxic adaptive regulation at lower concentrations, given the absence of concomitant toxic effects (Figure 5). Such gene expression responses have been observed regularly in other systems as well [60, 61]. Above a certain exposure level the magnitude of regulation increased, in parallel with observed morphological effects, indicating that a threshold of adversity had been crossed [62].

We have additionally identified genes that were significantly regulated but they did not participate in the aforementioned pathways of interest. The genes with the highest fold change were *Zfp703*, *Txnip*, *Stambpl1*, *Lrrc75a*, *Cyp2u1* and *Fam101a*, which were regulated by at least one of the tested compounds. *Zfp703*, *Lrrc75a*, *Cyp2u1* and *Fam101a* do indirectly interact with RA in rat embryos. Their significant regulation identified in embryos exposed to FLU, KTZ and TDF. They have a crucial role in repressing transcription, oxidative stress or dysregulation of bone maturation related pathways. Additionally, *Txnip* is dysregulated after rat exposure to DFZ, which could be related to responses to oxidative

stress and negative regulation of cell division [63, 64]. These genes are of general interest for assessing the genomic regulation after embryonic exposure to xenobiotics.

The responsive general embryonic development gene set is involved in a variety of developmental processes and is highly dominated by RA related genes. The RA pathway has the crucial role of regulating the spatiotemporal balance of RA in the developing embryo, supporting normal growth and differentiation. Perturbations in the RA pathway may therefore be indicative of possible embryotoxicity, as they have been implicated in the toxicological mode of action of azoles [32].

The sterol biosynthesis pathway represents the pharmacological mode of action of azoles in mammalian species. Azoles have been designed to intervene with *Cyp51,* disturbing the conversion of lanosterol to ergosterol, which stabilizes the fungal cell wall. The interference with *Cyp51* and other sterol- related genes after treatment with azole compounds, indicate their efficacy concerning the pharmacological, crop protection purposes of their application. Thus, whilst the RA pathway is relevant for embryotoxicity, the sterol pathway is relevant for the intended mode of action of azoles.

The 4-hour concentration-response of FLU revealed a similar curve for TMS as for the RA pathway, whereas the sterol biosynthesis pathway showed strong responses at higher exposures only, corroborating that modulation of the RA pathway, and not of the sterol biosynthesis pathway, is primarily involved in the embryotoxic response.

Furthermore, at sub-embryotoxic concentrations, FLU exposure caused an upregulation of *Tbx4* followed by a downregulation with increasing concentration, whereas *Hoxa1* showed the opposite expression pattern. Also MCZ at its ID_{10} of 25µM showed upregulation of *Tbx4* and downregulation of both *Hoxa1* and *Hoxc4*, similarly to the gene expression profile of embryos exposed to FLU at sub-embryotoxic concentration of 0.2 µM, indicating potency differences between both compounds. *Tbx4* is regulated by *Hox* and *Wnt* gene families [65, 66], the expression of which is affected by local RA levels, which would explain the effect of FLU exposure on the regulation of *Tbx4*. The observed biphasic response is intriguing. One might speculate that *Tbx4* upregulation is countered by increasing toxic responses represented by extensive regulation of the RA pathway at higher concentrations. Both *Hoxa1* and *Tbx4* have a crucial role in embryonic development, with specific sites of action at the anterior-posterior patterning and limb formation, respectively [67, 68]. *Tbx4* is also as a transcription factor in the early lung mesoderm, promoting the co-ordination of *Fgf* (mainly *Fgf8*) and *Gli* proteins for the forthcoming lung development [69]. In the case of MCZ, the increased regulation of *Tbx4* at the ID_{10} could be also associated with the already described morphological effects on heart formation [70]. Their non-monotonic regulation patterns with

increasing exposure makes genes such as *Tbx4* and *Hoxa1* less useful as biomarkers of embryotoxic response.

As illustrated in Figure 6 (A, B, C), rat WEC exposure to FLU, TDF and KTZ at their ID_{10} revealed marked upregulation of genes related to general embryonic development and the RA pathway, showing most extensive upregulation of *Cyp26a1* and *Dhrs3* (Figure 3). The relative magnitude of the RA pathway regulation by the tested compounds is in line with their *in vivo* potency ranking (Table 4). Single genes within the RA pathway such as *Cyp26a1,* did not show this ranking (Table 4), indicating that whole pathway regulation may be a better indicator for embryotoxic potency than single genes [52].

Sterol-related gene expression regulation was most extensive in embryos exposed to DFZ and KTZ (Figure 6C). We observed the significant induction of *Cyp51*, *Msmo1*, *Hmgcr* and *Sqle*, which are indicators of increased pharmacological activity. *Cyp51* is the major biomarker of the pharmacological mode of action of our tested compounds. Among all, *Msmo1* was the highest regulated single gene. *Msmo1* role has been recently described as a determinant gene in fatty acid transcription, via interacting with the mammalian liver X receptors (LXRs). The LXRs are involved in the regulation of lipid and fatty acid metabolism and have an important role in central nervous system (CNS) development and, especially, in midbrain neurogenesis [71]. Additionally, LXR proteins could bind to retinoid X receptors (RXR), form heterodimers and control the regulation of gene expression. Interestingly, Gad *et al*., [72] have suggested that RA and lipid metabolism related-genes, such as *Msmo1*, *Cyp51*, and *Hsd17b*, might be indirectly associated, when they tested in bovine culture both under *in vivo* and *in vitro* experimental conditions.

The occurrence and type of malformations is dependent on the timing of exposure in pregnancy [62]. The same holds for gene expression regulation by chemical exposures [21, 32]. The embryo development gene set showed time-dependent higher as well as lower regulation of individual genes, whereas the sterol pathway consistently showed higher gene expression responses after longer exposure. In the sterol pathway, *Msmo1*, *Cyp51*, *Hmgr*, *Sqle* and *Pcsk9* were the most quantitatively pronounced genes at 24h- FLU exposed embryos, which are involved in the cascade of sterol related enzymatic reactions [73].

Furthermore, among the RA- related genes *Cyp26a1*, *Dhrs3*, *Mafb* and *Gbx2* were more upregulated in 4h than 24h FLU exposed embryos [74]. *Cyp26a1*, *Dhrs3* and *Mafb* play a role in determining RA levels in the embryo. On the other hand, *Cyp26b1* and *Hoxc10* were relatively highly upregulated at the later time point. These genes have a different expression pattern in the embryo as compared to e.g. *Cyp26a1.* Additionally, there was a higher regulation of *Ngfr*, *Lhx1* and *Fgfbp3* in 4-hour exposed embryos to FLU, compared to 24-hour of exposure. These genes are important contributors to general embryonic

development. *Ngfr* has a crucial role in the development of neurons in the embryonic brain, while it has been suggested as a mediator for activating thyroid hormone [75]. *Lhx1* has been suggested to co-operate with *Pax6* in mouse embryos for forming the anterior thalamus [76, 77]. Another study about the development of *Xenopus* and chick embryos has shown that *Lhx1* is a transcription factor for nephric duct formation during development, which is triggered by RA and is collaborating with *Hox* genes, *Pax2* and *Pax8* transcriptional factors for further activation of *Gata3* and the *Wnt* signalling pathway [78]. These observations illustrate the spatiotemporal differences in sensitivity of regulatory processes in embryogenesis, the perturbation of which underlies the specification of malformations. This aspect is likewise important when employing gene expression data for toxicity profiling.

Comparing the RA- with the sterol biosynthesis pathway, our data indicate that those compounds which more strongly affect the RA pathway, belong to the group of embryotoxic ones, whereas those who affect the sterol biosynthesis pathway, or those who have low activity on both pathways are less embryotoxic.

The data also show other differences in gene expression pattern that could be used to elucidate additional pathways involved in embryotoxicity. This analysis shows the usefulness of investigation of gene expression modulation to obtain mechanistic information pertinent to assess the toxicity versus functional efficacy of chemicals. The comparison of relatively simple markers such as *Cyp26a1* for the RA pathway (undesired or off target effects) and *Cyp51* for the sterol biosynthesis pathway (pharmacological or on-target effect) could potentially be used for the optimization in the development of new compounds.

Conflict of interest

The authors declare that there are no conflicts of interest.

Funding

This work was supported by BASF SE.

References

1. Scialli, A.R. and A.J. Guikema, *REACH and reproductive and developmental toxicology: still questions.* Syst Biol Reprod Med, 2012. **58**(1): p. 63-9.
2. van der Jagt K., M.S., Tørsløv J. & de Bruijn J. , *Alternative approaches can reduce the use of test animals under REACH* EUROPEAN COMMISSION DIRECTORATE GENERAL JRC JOINT RESEARCH CENTRE Institute for Health and Consumer Protection, http://home.kpn.nl/reach/downloads/reducingtheuseoftestanimalsunderreachihcprepor.pdf, 2004.
3. Augustine-Rauch, K., C.X. Zhang, and J.M. Panzica-Kelly, *In vitro developmental toxicology assays: A review of the state of the science of rodent and zebrafish whole embryo culture and embryonic stem cell assays.* Birth Defects Res C Embryo Today, 2010. **90**(2): p. 87-98.
4. Piersma, A.H., *Alternative methods for developmental toxicity testing.* Basic Clin Pharmacol Toxicol, 2006. **98**(5): p. 427-31.
5. Scialli, A.R., *The challenge of reproductive and developmental toxicology under REACH.* Regul Toxicol Pharmacol, 2008. **51**(2): p. 244-50.
6. Lee, H.Y., et al., *Alternative models in developmental toxicology.* Syst Biol Reprod Med, 2012. **58**(1): p. 10-22.
7. Spielmann, H., *The way forward in reproductive/developmental toxicity testing.* Altern Lab Anim, 2009. **37**(6): p. 641-56.
8. Genschow, E., et al., *The ECVAM international validation study on in vitro embryotoxicity tests: results of the definitive phase and evaluation of prediction models. European Centre for the Validation of Alternative Methods.* Altern Lab Anim, 2002. **30**(2): p. 151-76.
9. Piersma, A.H., *Validation of alternative methods for developmental toxicity testing.* Toxicol Lett, 2004. **149**(1-3): p. 147-53.
10. New, D.A., P.T. Coppola, and D.L. Cockroft, *Comparison of growth in vitro and in vivo of post-implantation rat embryos.* J Embryol Exp Morphol, 1976. **36**(1): p. 133-44.
11. Robinson, J.F., A. Verhoef, and A.H. Piersma, *Transcriptomic analysis of neurulation and early organogenesis in rat embryos: an in vivo and ex vivo comparison.* Toxicol Sci, 2012. **126**(1): p. 255-66.
12. Piersma, A.H., *Whole embryo culture and toxicity testing.* Toxicol In Vitro, 1993. **7**(6): p. 763-8.
13. Webster, W.S., P.D. Brown-Woodman, and H.E. Ritchie, *A review of the contribution of whole embryo culture to the determination of hazard and risk in teratogenicity testing.* Int J Dev Biol, 1997. **41**(2): p. 329-35.
14. Luijten, M., et al., *Application of a metabolizing system as an adjunct to the rat whole embryo culture.* Toxicol In Vitro, 2008. **22**(5): p. 1332-6.
15. Flick, B., et al., *Embryotoxic potential of N-methyl-pyrrolidone (NMP) and three of its metabolites using the rat whole embryo culture system.* Toxicol Appl Pharmacol, 2009. **237**(2): p. 154-67.
16. Ellis-Hutchings, R.G. and E.W. Carney, *Whole embryo culture: a "New" technique that enabled decades of mechanistic discoveries.* Birth Defects Res B Dev Reprod Toxicol, 2010. **89**(4): p. 304-12.
17. Piersma, A.H., et al., *Interlaboratory evaluation of embryotoxicity in the postimplantation rat embryo culture.* Reprod Toxicol, 1995. **9**(3): p. 275-80.
18. Sogorb, M.A., et al., *An integrated approach for detecting embryotoxicity and developmental toxicity of environmental contaminants using in vitro alternative methods.* Toxicol Lett, 2014. **230**(2): p. 356-67.
19. Robinson, J.F., J.L. Pennings, and A.H. Piersma, *A review of toxicogenomic approaches in developmental toxicology.* Methods Mol Biol, 2012. **889**: p. 347-71.
20. Robinson, J.F., M.J. Gormley, and S.J. Fisher, *A genomics-based framework for identifying biomarkers of human neurodevelopmental toxicity.* Reprod Toxicol, 2016. **60**: p. 1-10.
21. Balmer, N.V., et al., *From transient transcriptome responses to disturbed*

neurodevelopment: role of histone acetylation and methylation as epigenetic switch between reversible and irreversible drug effects. Arch Toxicol, 2014. **88**(7): p. 1451-68.

22. Daston, G.P. and J.M. Naciff, *Predicting developmental toxicity through toxicogenomics.* Birth Defects Res C Embryo Today, 2010. **90**(2): p. 110-7.
23. Hermsen, S.A., et al., *Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryotoxicity test and comparison with their in vivo potencies.* Toxicol In Vitro, 2011. **25**(3): p. 745-53.
24. Robinson, J.F., et al., *A comparison of gene expression responses in rat whole embryo culture and in vivo: time-dependent retinoic acid-induced teratogenic response.* Toxicol Sci, 2012. **126**(1): p. 242-54.
25. de Jong, E., et al., *Comparison of the mouse Embryonic Stem cell Test, the rat Whole Embryo Culture and the Zebrafish Embryotoxicity Test as alternative methods for developmental toxicity testing of six 1,2,4-triazoles.* Toxicol Appl Pharmacol, 2011. **253**(2): p. 103-11.
26. Menegola, E., et al., *Dysmorphogenic effects of some fungicides derived from the imidazole on rat embryos cultured in vitro.* Reprod Toxicol, 2006. **21**(1): p. 74-82.
27. Marotta, F. and G.M. Tiboni, *Molecular aspects of azoles-induced teratogenesis.* Expert Opin Drug Metab Toxicol, 2010. **6**(4): p. 461-82.
28. Zarn, J.A., B.J. Bruschweiler, and J.R. Schlatter, *Azole fungicides affect mammalian steroidogenesis by inhibiting sterol 14 alpha-demethylase and aromatase.* Environ Health Perspect, 2003. **111**(3): p. 255-61.
29. Cunningham, T.J. and G. Duester, *Mechanisms of retinoic acid signalling and its roles in organ and limb development.* Nat Rev Mol Cell Biol, 2015. **16**(2): p. 110-23.
30. McCaffery, P.J., et al., *Too much of a good thing: retinoic acid as an endogenous regulator of neural differentiation and exogenous teratogen.* Eur J Neurosci, 2003. **18**(3): p. 457-72.
31. Li, Z., et al., *Vitamin A deficiency induces congenital spinal deformities in rats.* PLoS One, 2012. **7**(10): p. e46565.
32. Luijten, M., et al., *Transcriptomics analysis of retinoic acid embryotoxicity in rat postimplantation whole embryo culture.* Reprod Toxicol, 2010. **30**(2): p. 333-40.
33. Dimopoulou, M., et al., *Flusilazole induces spatio-temporal expression patterns of retinoic acid-, differentiation- and sterol biosynthesis-related genes in the rat Whole Embryo Culture.* Reprod Toxicol, 2016.
34. Menegola, E., et al., *Postulated pathogenic pathway in triazole fungicide induced dysmorphogenic effects.* Reprod Toxicol, 2006. **22**(2): p. 186-95.
35. Farag, A.T. and H.H. Ibrahim, *Developmental toxic effects of antifungal flusilazole administered by gavage to mice.* Birth Defects Res B Dev Reprod Toxicol, 2007. **80**(1): p. 12-7.
36. Lamontia CL, S.R., Alvarez L., *Embryo-fetal toxicity and teratogenicity study of INH-6573-39 by gavage in the rat.* Unpublished reports No. HLR 444-83 and HLR 142-84 from Haskell Laboratory for Toxicology and Industrial Medicine, Wilmington, Delaware, USA. Submitted to WHO by E.I. du Pont de Nemours & Co., Inc., Wilmington, Delaware, USA. Summarized in JMPR Monograph—Flusilazole, Pesticide residues in food: 1995 evaluations Part II Toxicological & EnvironmentEnvironment. http://www.inchem.org/documents/jmpr/jmpmono/v95pr08.htm, 1984.
37. Machemer, L., *Evaluation of embryotoxic and teratogenic effects on rats following oral administration.* Report no. 6294, Bayer AG, Institut für Toxikologie, submitted by Bayer AG to WHO. (Unpublished), http://www.inchem.org/documents/jmpr/jmpmono/v81pr32.htm, 1976.
38. Unger, T.M., Van Goethem, D. & Shellenberger, *A teratological evaluation of Bayleton in mated female rats.* Midwest Research Institute, Report No. 324 to Mobay Chemical Corporation Agricultural Chemical Division submitted to WHO by Bayer AG. (Unpublished),

http://www.inchem.org/documents/jmpr/jmpmono/v83pr39.htm, 1982.

39. Nishikawa S., H.T., Muyazaki H., and Ohguro Y., *Reproduction studies of KW-144 in rats and rabbits.* Kiso To Rinsho (Clin Rep), 1984. **18**: p. 1433–1448.

40. Tachibana, M.N., Y. and Monro, A.M., *Toxicology of fluconazole in experimental animals.* In Fromtling, R.A. Recent trends in the discovery, development, and evaluation of antifungal agents, JR Prous, Barcelona, 1987: p. 93-102.

41. Ito, C., Y. Shibutanil, K. Inoue, K. Nakano and H. Ohnishi, *Toxicologocal Studies of Miconazole (II) Teratological Studies of Miconazole in Rats* IYAKUHIN KENKYU, 1976. **7**(3): p. 367-376.

42. Lochry, E.A., *Developmental toxicity study of CGA-169374 technical (FL-851406) administreted orally via gavage to Crl:COBS CD (SD) BR presumed pregnant rats.* Unpublished report No. 203-005 from Novartis Crop Protection AG., Basel, Switzerland and Argus Research Lab. Inc., Horsham, USA, Submitted to WHO by SYgenta Crop Protection AG, Basel, Switzerland. http://apps.who.int/iris/bitstream/10665/44064/1/9789241665230_eng.pdf?ua=1, 1987.

43. Stahl, B., *JAU 6476 - Developmental toxicity study in rats after oral administration. Unpublished report No. M-012279-01-1 from Bayer AG, Wuppertal, Germany. Submitted to WHO by Bayer CropScience AG, Germany.* https://www.google.nl/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&ved=0ahUKEwjcjNCx5-HPAhWDrxoKHebSDzYQFgghMAE&url=http%3A%2F%2Fapps.who.int%2Fpesticide-residues-jmpr-database%2FDocument%2F110&usg=AFQjCNGpjQOE2xfb0QbWPWwA5-avbrC8kQ&sig2=ZFM7G3fgK4EdIA0e_zX0hQ&cad=rja. 1997.

44. Brown, N.A. and S. Fabro, *Quantitation of rat embryonic development in vitro: a morphological scoring system.* Teratology, 1981. **24**(1): p. 65-78.

45. Robinson, J.F., et al., *Embryotoxicant-specific transcriptomic responses in rat postimplantation whole-embryo culture.* Toxicol Sci, 2010. **118**(2): p. 675-85.

46. Slob, W., *Dose-response modeling of continuous endpoints.* Toxicological Science, 2002. **66**(2): p. 298-312.

47. Eijssen, L.M., et al., *User-friendly solutions for microarray quality control and pre-processing on ArrayAnalysis.org.* Nucleic Acids Res, 2013. **41**(Web Server issue): p. W71-6.

48. Irizarry, R.A., et al., *Exploration, normalization, and summaries of high density oligonucleotide array probe level data.* Biostatistics, 2003. **4**(2): p. 249-64.

49. Dai, M., et al., *Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data.* Nucleic Acids Res, 2005. **33**(20): p. e175.

50. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.* Nat Protoc, 2009. **4**(1): p. 44-57.

51. Robinson, J.F., et al., *Triazole induced concentration-related gene signatures in rat whole embryo culture.* Reprod Toxicol, 2012. **34**(2): p. 275-83.

52. Tonk, E.C., J.L. Pennings, and A.H. Piersma, *An adverse outcome pathway framework for neural tube and axial defects mediated by modulation of retinoic acid homeostasis.* Reprod Toxicol, 2015. **55**: p. 104-13.

53. Smoot, M.E., et al., *Cytoscape 2.8: new features for data integration and network visualization.* Bioinformatics, 2011. **27**(3): p. 431-2.

54. USEPA, *Animal Toxicity Studies: Effects and Endpoints (Toxicity Reference Database - ToxRefDB files).* Retrieved from ftp://newftp.epa.gov/comptox/High_Throughput_Screening_Data/Animal_Tox_Data/ on June 2016, 2016.

55. Menegola, E., et al., *Craniofacial and axial skeletal defects induced by the fungicide triadimefon in the mouse.* Birth Defects Res B Dev Reprod Toxicol, 2005. **74**(2): p. 185-95.

56. Mitiku, N. and J.C. Baker, *Genomic analysis of gastrulation and*

organogenesis in the mouse. Dev Cell, 2007. **13**(6): p. 897-907.

57. Giavini, E. and E. Menegola, *Are azole fungicides a teratogenic risk for human conceptus?* Toxicol Lett, 2010. **198**(2): p. 106-11.
58. Tiboni, G.M., F. Marotta, and A.P. Castigliego, *Teratogenic effects in mouse fetuses subjected to the concurrent in utero exposure to miconazole and metronidazole.* Reprod Toxicol, 2008. **26**(3-4): p. 254-61.
59. Sung, D.J., et al., *Blockade of K+ and Ca2+ channels by azole antifungal agents in neonatal rat ventricular myocytes.* Biol Pharm Bull, 2012. **35**(9): p. 1469-75.
60. Hermsen, S.A., et al., *Triazole-induced gene expression changes in the zebrafish embryo.* Reprod Toxicol, 2012. **34**(2): p. 216-24.
61. Schulpen, S.H., et al., *A statistical approach towards the derivation of predictive gene sets for potency ranking of chemicals in the mouse embryonic stem cell test.* Toxicol Lett, 2014. **225**(3): p. 342-9.
62. Piersma, A.H., et al., *Reproductive toxicants have a threshold of adversity.* Crit Rev Toxicol, 2011. **41**(6): p. 545-54.
63. *Database resources of the National Center for Biotechnology Information.* Nucleic Acids Res, 2013. **41**(Database issue): p. D8-d20.
64. Shimoyama, M., et al., *The Rat Genome Database 2015: genomic, phenotypic and environmental variations and disease.* Nucleic Acids Res, 2015. **43**(Database issue): p. D743-50.
65. Barham, G., Clarke,Nicholas,M. P., *Genetic Regulation of Embryological Limb Development with Relation to Congenital Limb Deformity in Humans.* Journal of children's orthopaedics, 2009. **2**(1): p. 1-9.
66. Diez del Corral, R., et al., *Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension.* Neuron, 2003. **40**(1): p. 65-79.
67. Naiche, L.A., et al., *Identity and fate of Tbx4-expressing cells reveal developmental cell fate decisions in the allantois, limb, and external genitalia.* Dev Dyn, 2011. **240**(10): p. 2290-300.
68. Mallo, M. and C.R. Alonso, *The regulation of Hox gene expression during animal development.* Development, 2013. **140**(19): p. 3951-63.
69. Sakiyama, J., A. Yamagishi, and A. Kuroiwa, *Tbx4-Fgf10 system controls lung bud formation during chicken embryonic development.* Development, 2003. **130**(7): p. 1225-34.
70. Naiche, L.A. and V.E. Papaioannou, *Loss of Tbx4 blocks hindlimb development and*

affects vascularization and fusion of the allantois. Development, 2003. **130**(12): p. 2681-93.

71. Pinto, C.L., et al., *Lxr regulates lipid metabolic and visual perception pathways during zebrafish development.* Mol Cell Endocrinol, 2016. **419**: p. 29-43.

72. Gad, A., et al., *Molecular mechanisms and pathways involved in bovine embryonic genome activation and their regulation by alternative in vivo and in vitro culture conditions.* Biol Reprod, 2012. **87**(4): p. 100.

73. Larsen, L., et al., *Expression and localization of microRNAs in perinatal rat pancreas: role of miR-21 in regulation of cholesterol metabolism.* PLoS One, 2011. **6**(10): p. e25997.

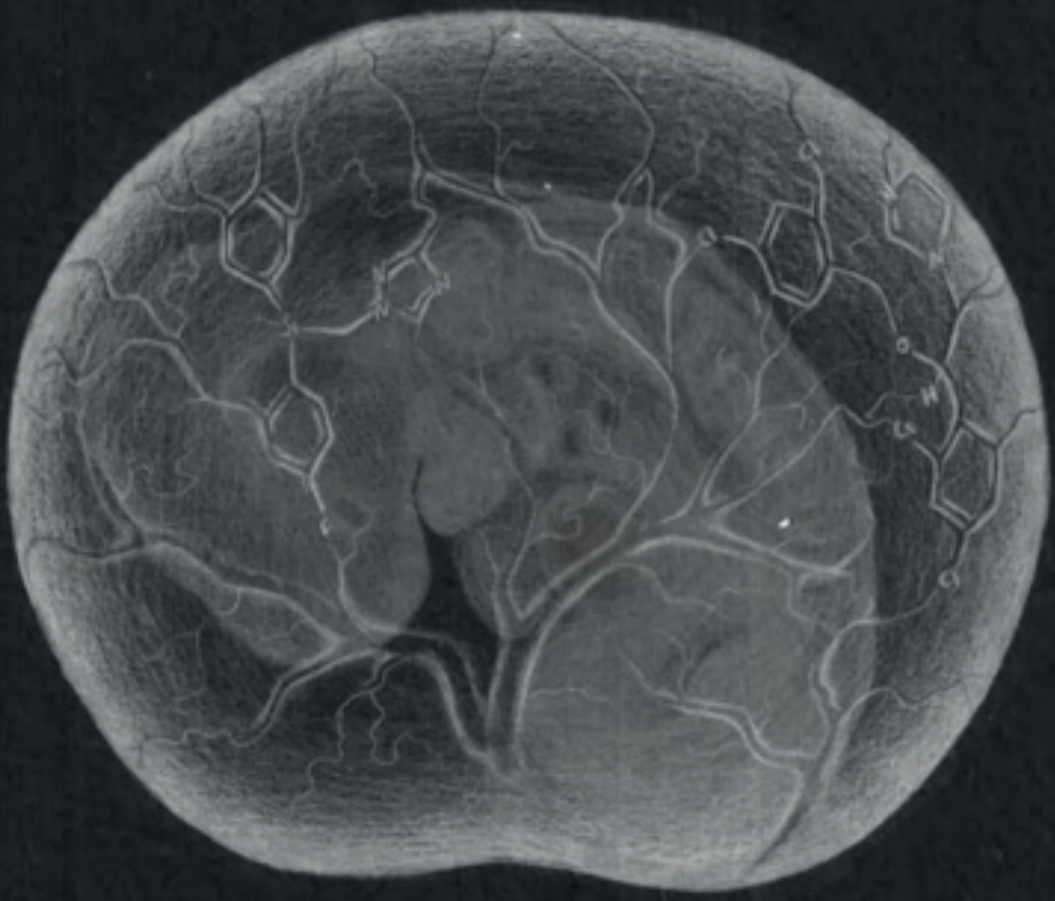
74. MacLean, G., et al., *Cloning of a novel retinoic-acid metabolizing cytochrome P450, Cyp26B1, and comparative expression analysis with Cyp26A1 during early murine development.* Mech Dev, 2001. **107**(1-2): p. 195-201.

75. Porterfield, S.P., *Thyroidal Dysfunction and Environmental Chemicals: Potential Impact on Brain Development.* Environmental Health Perspectives, 2000. **108**: p. 433-438.

76. Hevner, R.F., E. Miyashita-Lin, and J.L. Rubenstein, *Cortical and thalamic axon pathfinding defects in Tbr1, Gbx2, and Pax6 mutant mice: evidence that cortical and thalamic axons interact and guide each other.* J Comp Neurol, 2002. **447**(1): p. 8-17.

77. Pratt, T., et al., *A role for Pax6 in the normal development of dorsal thalamus and its cortical connections.* Development, 2000. **127**(23): p. 5167-78.

78. Costantini, F. and R. Kopan, *Patterning a complex organ: branching morphogenesis and nephron segmentation in kidney development.* Dev Cell, 2010. **18**(5): p. 698-712.



Chapter 4

A transcriptomic approach for evaluating the relative potency and mechanism of action of azoles in the rat Whole Embryo Culture

Myrto Dimopoulou, Aart Verhoef, Jeroen L.A. Pennings, Bennard van Ravenzwaay, Ivonne M.C.M. Rietjens and Aldert H. Piersma

Toxicology (2017) 392; 96-105

Abstract

We evaluated the effect of six azoles on embryonic development in the rat whole embryo culture (WEC). Using the total morphological scoring system (TMS), we calculated the ID_{10} concentration (effective dose for 10% decrease in TMS). For evaluating gene specific responses, we combined previously and newly collected transcriptomics data of rat WEC exposed to a total of twelve azoles at their ID_{10} for 4 hours. Results revealed shared expressions responses in genes involved in the retinoic acid (RA) and sterol biosynthesis pathways, which are respectively representatives of developmental toxicity and targeted fungicidal action of the azoles. Azoles with more pronounced effects on the regulation of RA-associated genes were generally characterized as more potent embryotoxicants. Overall, compounds with strong sterol biosynthesis related responses and low RA related responses were considered as more favourable candidates, as they specifically regulated genes related to a desired target response. Among the identified sterol associated genes, we detected that methylsterol monooxygenase 1 (*Msmo1*) was more sensitively induced compared to *Cyp51*, a classical biomarker of this pathway. Therefore, we suggest that *Msmo1* could be a better biomarker for screening the fungicidal value of azoles. In summary, we conclude that the embryonic regulation of RA and sterol metabolic pathways could be indicators for ranking azoles as embryotoxicants and determining their drug efficacy.

Keywords: Whole embryo culture; Toxicogenomics; Embryonic development; Azoles; Retinoic Acid; Sterol biosynthesis

Introduction

Regulatory guidelines for the risk assessment of chemicals require relatively high numbers of experimental animals for reproductive and developmental toxicity testing [1]. To reduce, refine and replace the use of laboratory animals, a variety of alternative assays has been developed over the past decades, including simple cell-line assays, organ cultures or more complicated whole embryo culture techniques and organs-on-a chip [2, 3].

An advanced *in vitro* model that mimics *in vivo* organogenesis and embryonic development is the rat whole embryo culture (WEC) technique [4, 5]. It is a widely used technique for screening embryotoxicants by monitoring both neurulation and organogenesis during gestational days (GD) 10 to 12 [6]. A variety of morphological endpoints is combined in the Total Morphological Score (TMS) [4]. Applying the TMS in rat WEC, effects of chemicals on the embryonic growth and development can be studied both qualitatively and quantitatively. WEC also enables the implementation of toxicogenomic-based approaches for mechanistic evaluation of the embryotoxic profile of xenobiotics. Gene signatures can predate and predict morphological consequences of toxic stimuli [7-11]. Furthermore, transcriptomics can be applied to identify biomarkers for detecting specific embryotoxic responses [9].

Azoles are antifungal agents for clinical and agricultural use. They have been designed to affect the *Cyp51* enzyme, which catalyses the conversion of lanosterol to ergosterol on the fungal cell membrane, and leads to cell death when affected [12]. In mammalian systems, *Cyp51* is less sensitive to azoles, but still critical for the sterol biosynthesis pathway. Moreover, azoles can induce many toxic responses in mammals by disturbing P450-mediated pathways and interfering with retinoic acid (RA) homeostasis [7, 13-15]. RA is crucial for maintaining balanced embryonic growth and differentiation, and *Cyp26a1* is its key regulatory metabolic enzyme, catalysing the first step in the degradation of RA, [16, 17]. Previous *in vivo* and *in vitro* studies suggest that when rat embryos were exposed to either RA or azoles, similar teratogenic outcomes were observed, including craniofacial and axial defects [5, 8, 16, 18]. Therefore, RA modulation may play a role in the developmental toxicity due to azole exposure.

In our previous study [7], we combined the WEC technique with transcriptomic analysis for determining the effects of six azoles. Gene expression signatures of embryos exposed to the six tested azoles suggested that a RA-associated gene set corresponded with the toxicological mode of action while a sterol biosynthesis-related gene set represented the fungicidal activity of the azole compounds. In the present study, we assessed the relative embryotoxic potencies of six additional compounds - three known and three novel azoles - by performing a global gene expression profiling of these azoles. Subsequently, the gene expression data of all twelve compounds were evaluated in one combined analysis,

focussing on the RA and sterol biosynthesis pathways. We aimed to define biomarkers related to the aforementioned pathways, as promising molecular endpoints for classifying the desired fungicidal as well as the embryotoxic responses of azoles, and correlating the latter with available *in vivo* embryotoxicity data.

Materials and Methods

Animal care

As described in our previous WEC studies [7, 14], all the animal studies were approved and performed at the National Institute of Public Health and the Environment (RIVM) in concordance with European regulations. Wistar rats (HsdCpd:WU) (Harlan, The Netherlands) were housed at the RIVM Animal Care facility in a climate-controlled room with a 12h light cycle (04:00-16:00 dark). Water and food were provided ad libitum. After acclimating for 2 weeks, virgin female rats were housed with male rats for a 3-hour mating period (9:00-12:00, described as GD 0). Mated dams were afterwards individually housed. Rats were daily monitored for their general health condition during the period of the present study.

Rat Whole Embryo Culture

Following previous studies [4, 7, 8, 10, 14], on GD 10, between 9:00 and 12:00 a.m., dams were euthanized by intracardiac injection of T61^{R} (Intervet, The Netherlands). Rat embryos were immediately separated from the uterus. The peripheral trophoblastic cell zone and parietal yolk sac membrane were removed under the microscope leaving both the visceral yolk sac and ectoplacental cone intact. Embryos with 1-5 somites were further cultured, while only embryos with 2-4 somites were used for gene expression studies [8]. Embryos were separately cultured in flasks with 2 mL culture medium, containing 90% pregnant bovine serum and 10% rat serum (Biochrom, Berlin, Germany), diluted with 14% Hank's solution (Gibco) and supplemented with 1.6 mg/mL D-glucose and 75 μg/mL L-methionine (Sigma-Aldrich, Zwijndrecht, The Netherlands). The culture flasks were placed in rotating incubators, completely protected from light exposure and with stable internal temperature of 37.7^{o}C. A mixture of gas was supplied twice daily for 30 seconds, with increasing concentration of oxygen: on the first day (GD10) at 9:00 and 16:00 (5% O_2, 5% CO_2, 90% N_2), on the second day (GD11) at 9:00 and 16:00 (20% O_2, 5% CO_2, 75% N_2) and on the third day (GD12) at 9:00 (40% O_2, 5% CO_2, 55% N_2).

Morphological assessment and statistical analysis of individual endpoints

Embryos were cultured for 48 h (GD 10-12) and morphologically assessed according to the TMS system [19]. Twenty morphological endpoints were included in this morphological assessment, which were sub-divided into two basic groups. These represented growth parameters (including yolk sac diameter, crown-rump length, head diameter and number of somites) and developmental/functional parameters, such as yolk sac and allantoic blood

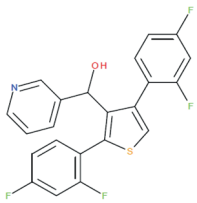
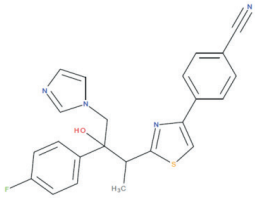
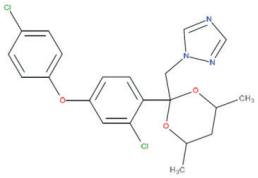
circulation, heart formation and heart beating, embryo- turning, caudal neural tube, optic and otic system, fore- and hind- limb, branchial arches, mandibular and maxillary process and the shape and size of somites. The TMS is a quantitative system for identifying any possible specific and selective embryotoxic effect of the tested compounds in rat embryos. Therefore, the sum of scores for each of the morphological endpoints was calculated for detecting any morphological alteration and for comparing with the time-matched controls. Within each exposure group, including also the vehicle control (DMSO), 8 rat embryos were evaluated. For normalizing the data and eliminating daily variation, the GD10 embryos within the same exposure group were derived from dams sacrificed on different days. Statistical analysis was performed using the parametric (Student's *t*-test) and non-parametric (Mann-Whitney) (unpaired), two-sided, and with 95% confidence intervals. Due to high agreement between these approaches, the significance values deriving from the Student's *t*-test are shown here. Images of the examined embryos (exposed for 48 hours to either DMSO or tested compounds) were obtained using an Olympus SZX9 camera at ×20 magnification and Olympus DP software.

Test compounds and exposure concentrations

This study combines data of six known azole compounds from our previous publication [7] and additional data of three known and three new azoles derived from the present study. For the present study, the following three known and three novel azoles were tested in rat WEC for 48 hours (0-48h) in a range of concentrations with the lowest concentration inducing no morphological effect to the highest being the maximal achievable concentration in culture. The three known azoles were: fenarimol (FEN; CAS#60168-88-9, purity 99.9 %, Sigma-Aldrich, Zwijndrecht, The Netherlands); propiconazole (PRO; CAS#60207-90-1, purity 99.1 %, Sigma-Aldrich, Zwijndrecht, The Netherlands); and tebuconazole (TEB; CAS#107534-96-3, purity 99.4 %, Sigma-Aldrich, Zwijndrecht) at 20, 60 200 and 600 μM. BASF SE (Ludwigshafen, Germany) kindly provided the three novel azole-compounds (with purity > 95 %) and their chemical information is summarised in Table 1. B595 and B600 were tested at 60, 200 and 600 μM; and B599 at 2, 6, 20 and 60 μM. All the compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Zwijndrecht, The Netherlands), and all embryos were exposed to a final DMSO concentration of 0.1%. As has been previously described, 0.1% DMSO did not significantly alter the morphology (4 and 48h) and has limited effects on gene expression after 4h of exposure [7, 10]. The concentration at which rat WEC were exposed to conduct the gene expression analysis was in the same line of concept with our previous study [7] and calculated after completing the morphological assessment of rat embryos (48 hours). Next, we calculated the concentration which results to 10% reduction of

the control TMS (ID_{10}) with both PROAST [20] and Graphpad software (www.graphpad.com). For microarray analysis, rat WEC were exposed for 4 hours (0-4h) to the tested compounds at their ID_{10} values: FEN at 140µM, PRO at 220µM, TEB at 115µM, B595 at 180µM, B599 at 5µM, and B600 at 110µM, as derived from the concentration response curves on TMS.

Table 1: Chemical information of the three novel azoles tested in the present study.

Code	Structure	Molecular weight (g/mol)
B595		415.4
B599		418.5
B600		434.3

Whole embryo RNA isolation

For transcriptomics, 4-hour cultured embryos were quickly scored on the basis of their somite number, their position in the yolk sac, neural tube developmental stage, crown-rump length and head diameter. They were then isolated from the yolk sac and ectoplacental cone, placed in 200µL RNAlater (Ambion, Austin, Texas), stored for one week at 4°C, and then stored for further processing at -80°C. After the embryos were thawed on ice, they were separately homogenized by passing them 10 times though a 1mL syringe with a 26G needle. RNA was further isolated by using the RNeasy Micro Plus RNA isolation kit (CAS number 74034, Qiagen, the Netherlands) and manufacturer's protocol. RNA was eluted with

14μM RNase-free H_2O and stored at -80^{o}C. Quantity and quality of the isolated RNA were measured with Nanodrop (Nanodrop Technologies Inc., Wilmington, Delaware) and 2100 BioAnalyzer (Agilent Technologies, Palo Alto, California). Samples with absorbance value between 1.9 and 2.2 (ratio 260mm/280mm) and RNA integrity number (RIN) higher than 8 were further used for performing the microarray analysis.

Microarray hybridization

RNA hybridization and microarray experimentations were performed by the Dutch Service and Support Provider (MAD) of the University of Amsterdam, the Netherlands. In agreement with our previous publication [7], for every sample, RNA was amplified, biotin-labelled and hybridized to Affymetrix GeneChip HT RG-230 PM Array Plates according to the provided protocols by Affymetrix (Santa Clara, CA). After staining, the HT Array plate was read by the Affymetrix GeneChip® HT Scanner and analyzed by the Affymetrix GeneChip® Operating Software. For performing the aforementioned steps, the GeneTitan® Hybridization, Wash, and Stain Kit for 3' IVT Arrays (cat no. 901530) was used. In total, 56 arrays were further analysed (8 embryos per exposure group, 6 tested compounds and 1 control group).

Microarray analysis and data processing

The quality control (QC) and the normalization of the microarray data were performed using the Affymetrix array QC pipeline at ArrayAnalysis.org webpage (www.arrayanalysis.org) [21], designed by the Department of Bioinformatics in Maastricht University. Due to normal expected biological differences between the two studies ([7] and present) and, consequently, to eliminate any experimental-specific gene responses, the raw data were separately normalized with their appropriate control for each study and accordingly processed. Raw microarray data were inspected for their quality by assessing the 3´/5´ ratios for β-actin and GAPDH, RNA degradation, background intensity, signal quality and the probe-set homogeneity with NUSE (Normalized Unscaled Standard Error) and RLE (Relative Log Expression). The Affymetrix CEL files were further normalized by using the Robust Multichip Average (RMA) algorithm [22] and the Brainarray custom CDF version 19 probe set annotation (http://brainarray.mbni.med.umich.edu/Brainarray/default.asp) [23]. In total, 13,877 probe sets, each corresponding to an Entrez Gene ID, were further evaluated by performing a statistical analysis in R (www.R-project.org) and Microsoft Excel. Raw and normalized data were deposited in NCBI GEO (www.ncbi.nlm.nih.gov/geo/) under accession number GSE102082.

Identification of significantly altered genes

Normalized data were log transformed. For each exposure condition, gene expression data were compared to the appropriate control (each study has a separate control group), for calculating absolute average fold changes of individual gene expression. Differentially expressed genes were identified by using ANOVA, using a p-value < 0.001 and a False Discovery Rate (FDR) of 10%, as stringency criteria. The statistical criteria were set similar to earlier published studies from our laboratory, and they partly determined the number of genes differentially expressed. The 53 genes, which were differentially expressed in at least one of the eight rat WEC samples from their respective exposure groups, were combined for further analysis. Gene expression responses were visualized using a heatmap combined with hierarchical clustering (Euclidean distance, Ward linkage) as well as Principal Component Analysis (PCA). Each bar in the heatmap represents the average of the gene expression in the experimental group compared to the respective control group of each study.

Functional interpretation analysis of differentially expressed genes

Following the concept of our previous study [7], functional annotation and overrepresentation analysis were performed using DAVID (https://david.ncifcrf.gov/) [24] and literature data [5, 17, 25]. Here, we additionally applied the gene sets already identified from our previous study, which included genes participating in RA pathway, general development and the sterol biosynthesis pathway. Furthermore, we indicated three additional pathways that importantly identified genes belong to apoptosis, neural differentiation, and vessel formation. The combined gene expression data were summarized to absolute average fold changes per pathway. Next, the absolute average fold changes of genes of interest or of the whole pathway per exposure group were plotted against the compound concentration used. Finally, the absolute fold change of gene expression per RA and sterol biosynthesis pathways versus the used ID_{10} concentrations and the relative *in vivo* potencies of the tested compounds in rat embryos were plotted in a 3D plot using R.

In vivo data analysis

In addition to previously derived *in vivo* data [7], a literature overview was performed to determine the *in vivo* developmental toxic profile of the three known azoles. Applying the same criteria concerning the species, chemical exposure during specific GD and scheme of dosing range, we selected studies performed in rats orally exposed to the tested compounds during either GD6-15 or GD7-16 at multiple dose regimes. Studies with at least one control

group and two dose groups were selected to allow analysis using the Benchmark Dose (BMD) approach. The BMD values were calculated based on the evidence of adverse skeletal changes or cleft palate formation, both selected as sensitive endpoints of *in vivo* developmental toxicity and specific for the tested group of chemicals. For some of the tested compounds, other morphological endpoints were considered for calculating the BMD values, dependent on the specificity of the malformations observed. A concentration-response curve was fitted to the data to determine the BMD for the selected benchmark response (BMR) for each tested azole. The BMD was defined as 10% additional incidence of adverse skeletal changes, cleft palate or any other relevant morphological alteration (BMD_{10}). The BMD_{10} of each compound was calculated with BMD and PROAST software [20] using dichotomous concentration-response models (quantal data). Among the several models that were fitted, the selection of the best model was determined based on the goodness of fit (p-value>0.05). The *in vivo* prenatal developmental toxicity data for the three new azoles were provided by BASF. For the three novel compounds, given the available data, we proceeded with a qualitative *in vivo* potency ranking concept, which was adjusted and applied in our study, including also the known compounds. For implementing this approach of *in vivo* analysis, the profiles of the tested compounds were characterized as potent, moderate and weak or non-potent.

Results

Relative potency of azoles causing morphological alterations in rat WEC

All azoles induced some form of developmental toxicity in a concentration-dependent manner in WEC (Figure 1, Table 2). All newly tested compounds showed statistically significant effects on TMS at concentrations higher than 60 µM, except B599, which affected TMS at 20 µM (Table 2). Caudal neural tube and somite formation were the most sensitive parameters for all compounds, except PRO. ID_{10} concentrations on TMS were calculated for all the tested compounds from Figure 1, after combining the current and our previous study [7]. The decreasing potency ranking of the tested azoles was as follows: B599 > FLU ~ MCZ > KTZ > DFZ ~ B600 > TEB > FEN > TDF > B595 > PRO > PTZ with ID_{10}s of 5, 25, 40, 110, 115, 140, 150, 180, 220 and 250 µM, respectively.

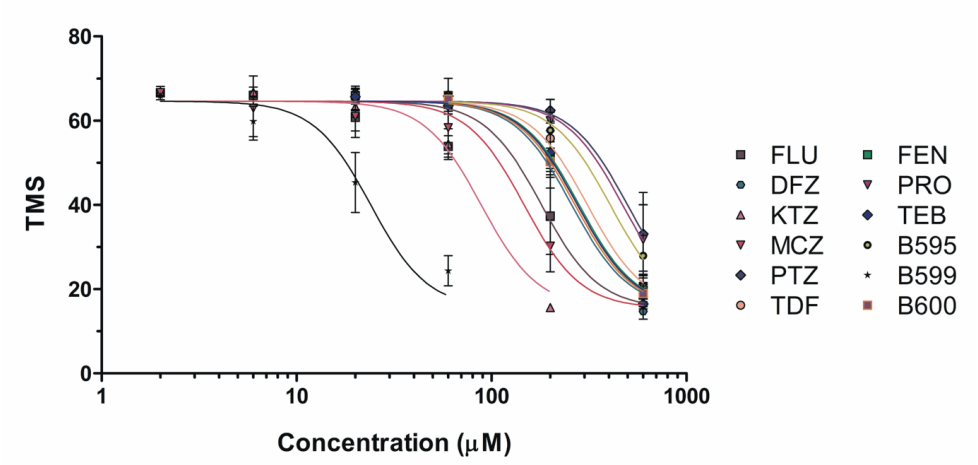


***Figure 1**: Total Morphological Score (TMS) concentration-responses of twelve azoles in the rat WEC after 48 hours of exposure. Each point represents a mean ± SD (N=8). The curves for the six compounds in the left side list were reproduced from [7].*

Table 2: Overview of morphological effects of the tested azoles in the rat WEC assay.

Compound	Concentration (µM)	TMS	CRL (mm)	S_{48h}– S_{0h}	FORE	MID	HIND	CAUD	OTIC	OPTIC	BRAN	MAND-MAX	SOM	HEART
DMSO	0	65.8±10.7	4±0.11	25±0.83	-	-	-	-	-	-	-	-	-	-
FEN	20	65.9±1.74	4.1±0.09	24±0.92	-	-	-	-	-	-	-	-	-	-
	60	63.8±1.62	4.0±0.27	24±1.28	-	-	-	-	-	-	-	-	-	-
	200	51.7±7.71***	3.7±0.19	20±2.00***	-	-	-	**	*	-	*	*	**	-
	600	19.8±3.35****	2.3±0.52***	#	****	****	****	***	****	****	****	****	****	****
PRO	20	65.2±1.71	4.0±0.16	25±0.92	-	-	-	-	-	-	-	-	-	-
	60	64.4±1.75	4.1±0.09	24±0.92	-	-	-	-	-	-	-	-	-	-
	200	60.0±5.04*	3.8±0.18	23±1.13*	-	-	-	-	-	-	*	-	-	-
	600	31.8±8.24****	3.1±0.32***	#	****	****	****	****	***	****	****	**	****	****
TEB	20	65.7±1.07	4.2±0.14	24±0.52	-	-	-	-	-	-	-	-	-	-
	60	63.6±2.03	4.0±0.28	23±0.93	-	-	-	-	-	-	-	-	-	-
	200	52.4±5.22**	3.7±0.16	19±2.00*	-	-	-	**	-	-	*	-	**	-
	600	15.3±20.5****	1.7±0.10*	#	****	****	****	***	****	****	****	****	****	****
B595	60	66.2±0.84	3.9±0.17	24±1.19	-	-	-	-	-	-	-	-	-	-
	200	57.7±4.24*	3.7±0.19*	22±1.93*	-	-	-	*	-	-	-	-	**	-
	600	27.9±12.12****	2.9±0.58****	14±4.96****	****	****	****	***	***	***	****	***	****	****
B599	2	65.9±0.92	4.0±0.19	24±0.00	-	-	-	-	-	-	-	-	-	-
	6	59.8±3.60	4.0±0.14	23±0.71	-	-	-	-	-	-	-	-	-	-
	20	45.3±7.10**	3.7±0.16	18±1.28***	**	-	-	***	**	-	**	-	**	-
	60	24.4±3.57****	2.7±0.23***	12±3.78****	****	***	****	***	****	****	****	***	****	****
B600	60	65.1±1.32	3.9±0.37	24±0.64	-	-	-	-	-	-	-	-	-	-
	200	50.3±1.60*	3.5±0.23*	23±0.92*	-	-	*	**	-	-	*	*	-	*
	600	19.0±3.67****	1.9±0.50****	#	****	****	****	***	****	****	****	****	****	****

Each number represents a mean ± SD (N=8, Student's *t*-test: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$). CRL: crown-rump length; S_{48h}- S_{0h}: number of somites that formed during the culture period of rat WEC; FORE: forebrain; MID: midbrain; HIND: hindbrain; CAUD: caudal neural tube; OTIC: otic system; OPTIC: optic system; BRAN: branchial arches; MAND-MAX: mandibular and maxillary process; SOM: quality of somites and HEART: heart; "#": could not be measured.

Significantly regulated genes across twelve azoles

For studying the effect of the tested azoles on the transcriptome, embryos were exposed for 4 hours on GD10 (0-4 hours of culture) to the ID_{10} concentration of each compound, as calculated from Figure 1. Somite formation was unaffected directly after all 4-hour exposures, indicating the absence of developmental delays at that stage (Figure 2).

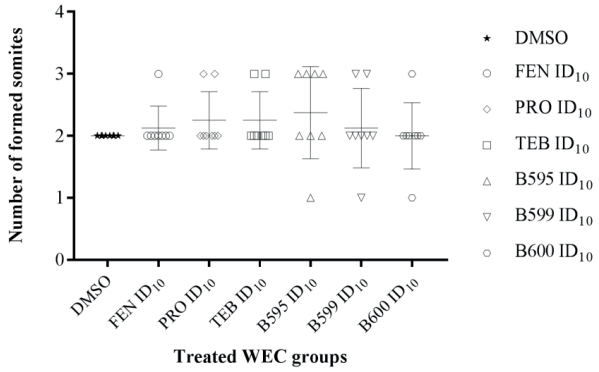


Figure 2*: Somitogenesis in rat embryos exposed for 4 hours to six azoles at their ID_{10} concentration, collected for whole transcriptome analysis. Individual data with mean ± SD are plotted (N=8 embryos per group).*

For analysing the gene expression data, we compared each exposure group with the appropriate concurrent vehicle control and we applied the same stringency criteria as mentioned previously (p-value < 0.001 and FDR of 10%) [7]. The combined data analysis revealed 53 genes that were statistically significantly regulated by at least one of the twelve azoles. As shown in Figure 3, embryonic exposure to KTZ and DFZ caused the highest number of statistically significant regulation of genes. On the other hand, MCZ and PTZ did not show statistically significantly regulated genes under the stringency criteria applied.

The hierarchical clustering of the expression data of the 53 genes is illustrated as a heatmap (Figure 4). Pathway analysis using DAVID revealed enrichment of genes involved in six pathways or processes; RA metabolism, general development, sterol biosynthesis, apoptosis, neural differentiation and vessel formation (Figure 4, right panel). For some of the genes, an overlap was observed among pathways. For example, *Cyp26a1* appears both in the RA pathway and in the general development pathway.

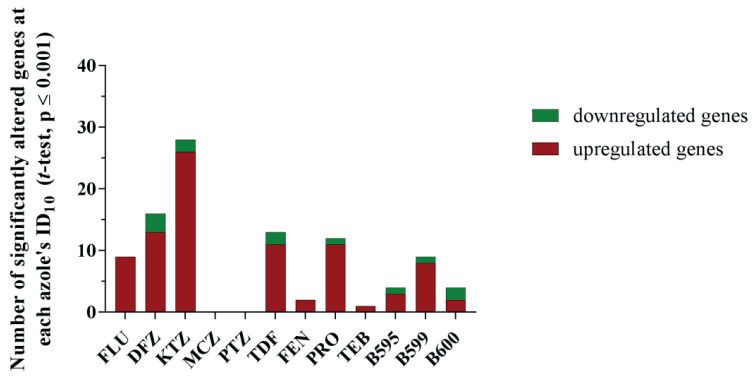


***Figure 3**: Number of genes statistically significantly regulated by each azole at the ID_{10} on TMS among the tested azoles (p-value<0.001 and FDR 10%).*

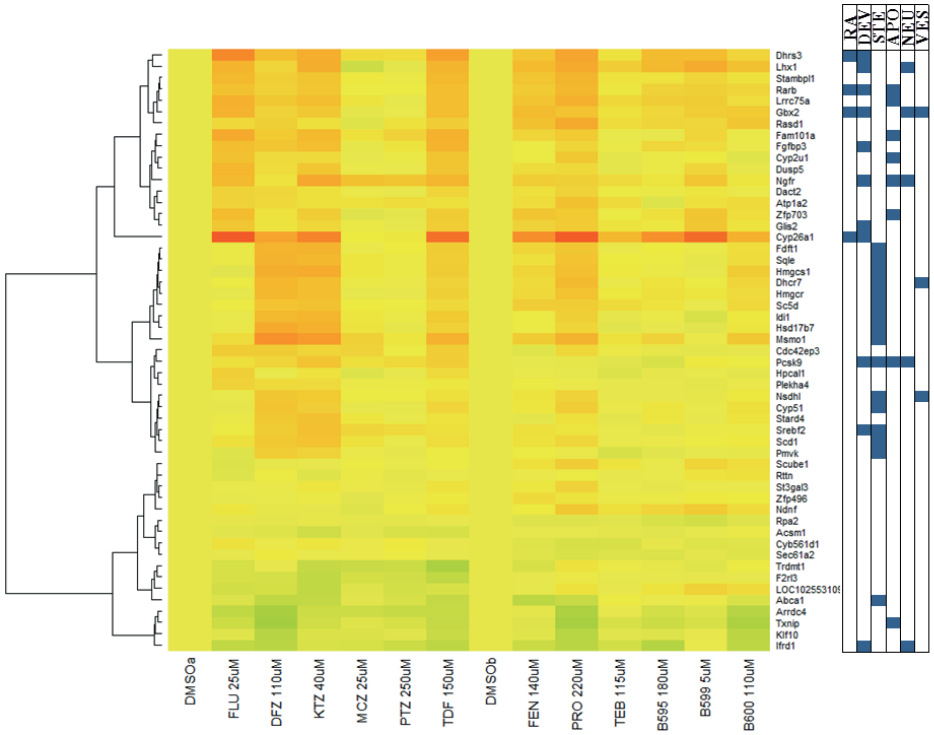


***Figure 4**: Hierarchical clustering of the average gene expression change in rat WEC by twelve azoles (N=8, p-value<0.001 and FDR 10%), with which 53 genes were identified as statistically significantly regulated by at least one of the compounds. Right panel: gene functionality in six pathways: RA (RA), general development (DEV), sterol biosynthesis*

(STE), apoptosis (APO), neural differentiation (NEU) and vessel formation (VES). Colors indicate changes to vehicle. Red, up-regulation; green, down-regulation; yellow, unchanged.

Quantitative gene expression changes in the RA and sterol biosynthesis pathways

Within the six functional gene groups that were identified, the RA and sterol biosynthesis pathways were further analysed. As illustrated in Figure 5, the RA pathway showed a higher magnitude of regulation compared to the sterol biosynthesis pathway in embryos exposed to most compounds, excluding DFZ, MCZ and PTZ. DFZ induced regulation of both pathways to the same extent. MCZ and PTZ revealed a lack of response of both pathways under the significance thresholds applied.

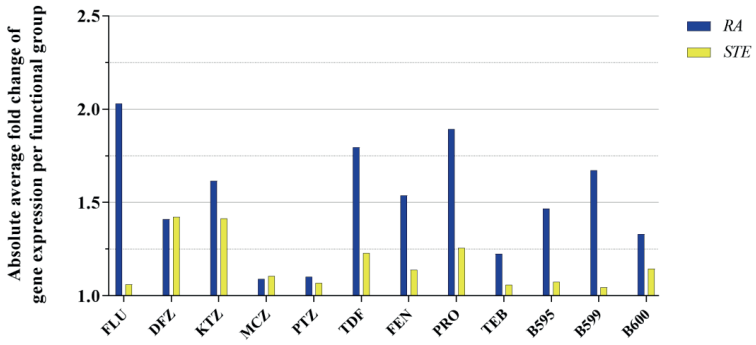


Figure 5*: Quantitative gene expression changes, related to the RA and sterol biosynthesis pathways, of twelve azoles in the rat WEC.*

Gene expression changes observed throughout the sterol biosynthesis pathway

The sterol biosynthesis pathway in mammalian systems consists of a cascade of enzymatic reactions initiated by fatty acid degradation. As in fungi, lanosterol is further converted to intermediate moieties, which are substrates for *Cyp51*, *Msmo1* and *Nsdhl* for synthesizing cholesterol (Figure 6A).

We numbered the enzymes included on the microarray in the order of appearance in the sterol biosynthesis pathway (Figure 6A) and plotted their gene expression changes by the different azoles (Figure 6B). *Msmo1* showed the highest gene expression regulation after exposure to the tested compounds, except for PTZ, TEB and B599 (Figure 6B). The greatest effect on the regulation of *Msmo1* was observed in rat embryos exposed to DFZ (1.96), KTZ (1.82) and TDF (1.55). The remaining genes were regulated in a relatively similar expression ratio, with the exception of *Dhcr7* in the case of PRO, which reached almost the same level of expression of *Msmo1* (Figure 6B), at a fold change of 1.5.

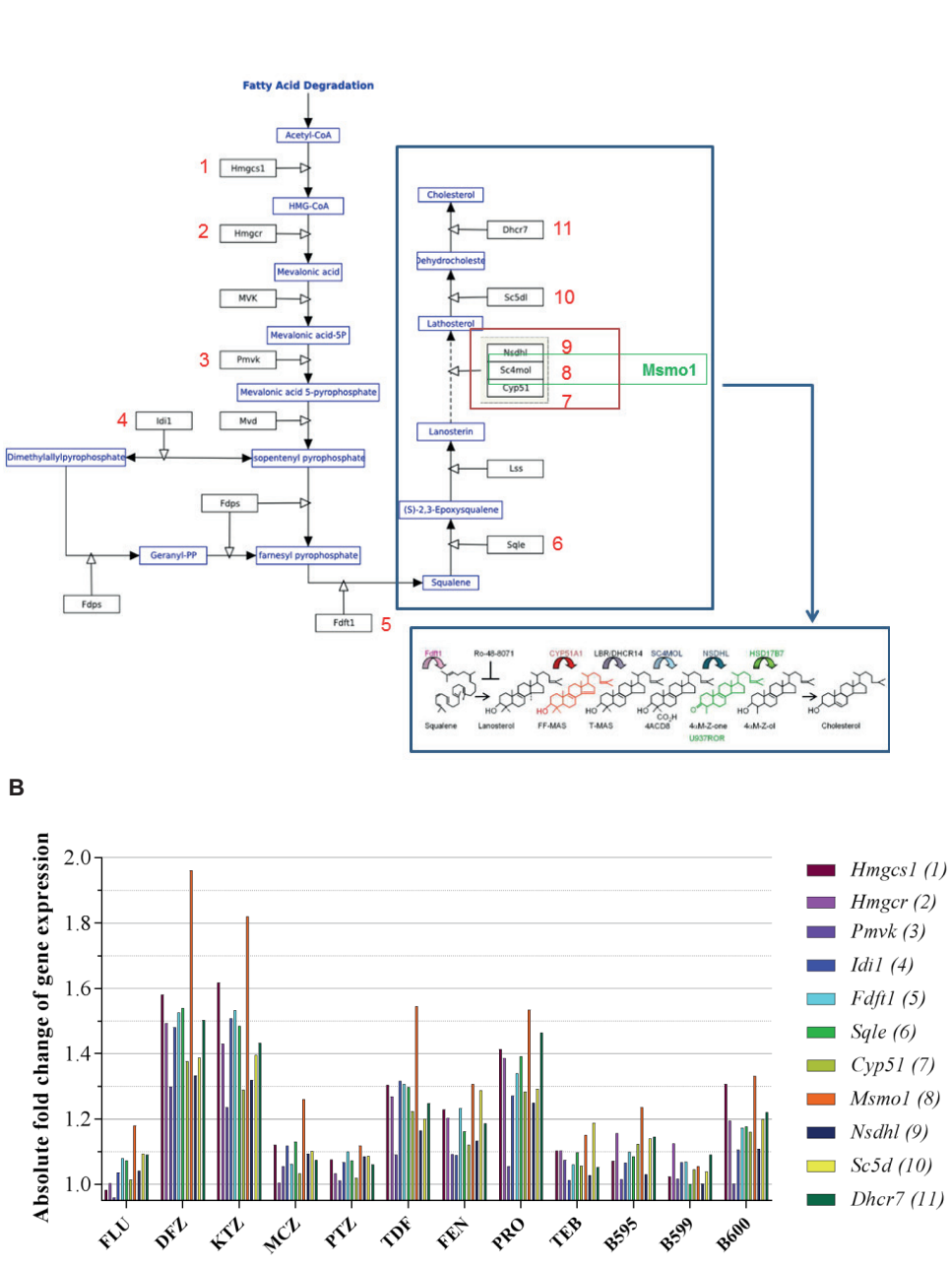


***Figure 6**: **A**. The sterol biosynthesis pathway in the Rattus norvegicus, including the main intermediate moieties and the contributing enzymes adapted from www.wikipathways.org [26] and [27]. **B**. The quantitative regulation of the genes that participate in the sterol biosynthesis pathway in rat WEC exposed to twelve azoles.*

A general comparison of in vivo and in vitro data

In vivo studies on rat embryos, in which the developmental toxic profile of the twelve azoles was tested, were further analysed and the BMD_{10} value of each compound was calculated (Table 3). With these data, we performed a potency ranking based on the calculated BMD_{10} concentration, which was based on an overall assessment of doses-dependent embryotoxic effects. The BMD_{10} was derived based on the most sensitive endpoint, which might differ between compounds. Abnormalities might include skeletal defects, cleft palate, and absence of renal papilla or hydronephrosis. For the three novel compounds B595, B599 and B600, *in vivo* prenatal developmental toxicity data were provided by BASF SE laboratories. The potency ranking of these compounds was qualitatively performed based on limited dose-response information [28] and resulted in the following order: B599 > B600 > B595. Based on the *in vivo* qualitative and quantitative (where applicable) data, we allocated the twelve tested compounds into one of three developmental toxicity potency groups. The most potent compounds were B599, FLU and KTZ. The moderately embryotoxic compounds *in vivo* were B600, FEN, MCZ, TDF and TEB, while the weak or non-potent compounds were B595, DFZ, PRO and PTZ (Table 3). Table 3 contains also our *in vitro* data of the twelve azoles, including the ID_{10} concentrations based on TMS.

Figure 7 shows a comparison of RA pathway regulation (x-axis), ID_{10} in WEC (y-axis) and sterol biosynthesis pathway regulation (z-axis) with *in vivo* potency groups (Table 3, bar colour). B599, FLU and KTZ, the potent developmental toxicants profile both *in vivo* (red bars) and *in vitro* (low ID_{10} in the WEC assay), tended to have a more pronounced effect on regulation of the RA pathway (Figure 7). The compounds with moderate developmental toxic profile (yellow bars) showed a more limited effect on the RA and sterol biosynthesis pathways. MCZ was classified as a moderate compound in the *in vivo* situation, which was not in agreement with the morphological assessment of embryos in the WEC assay. Additionally, the transcriptomic data revealed an absence of gene-responses in embryos exposed to this azole (Figure 3). These data were similar to the transcriptome data obtained from embryos exposed to PTZ, which was selected as a non-toxic compound for our study. In contrast, PRO, a weak embryotoxicant *in vivo* and *in vitro*, presented a strong RA-related profile, similar to TDF. DFZ and KTZ conceded a comparable regulation of the sterol biosynthesis pathway, but DFZ did not significantly disturb the RA-related genes. For the remainder of the compounds, we found mixed responses, with a stronger regulation of the RA pathway than of the sterol biosynthesis pathway (Figure 7).

Table 3: Overview of *in vivo* and *in vitro* developmental toxicity data of twelve azoles.

Compound	*in vitro* WEC	*in vivo*	
	ID_{10} (µM)	BMD_{10} (µmol/kg)	Potency Group
B599	5	-	Potent
FLU	25	9.1 [29]	Potent
MCZ	25	258.3 [30]	Moderate
KTZ	40	20.1 [31]	Potent
B600	110	-	Moderate
DFZ	110	596.5 [32]	Weak
TEB	115	275.8 [33]	Moderate
FEN	140	88.5 [34]	Moderate
TDF	150	91.5 [35]	Moderate
B595	180	-	Weak
PRO	220	386.7 [36]	Weak
PTZ	250	917.8 [37]	Weak

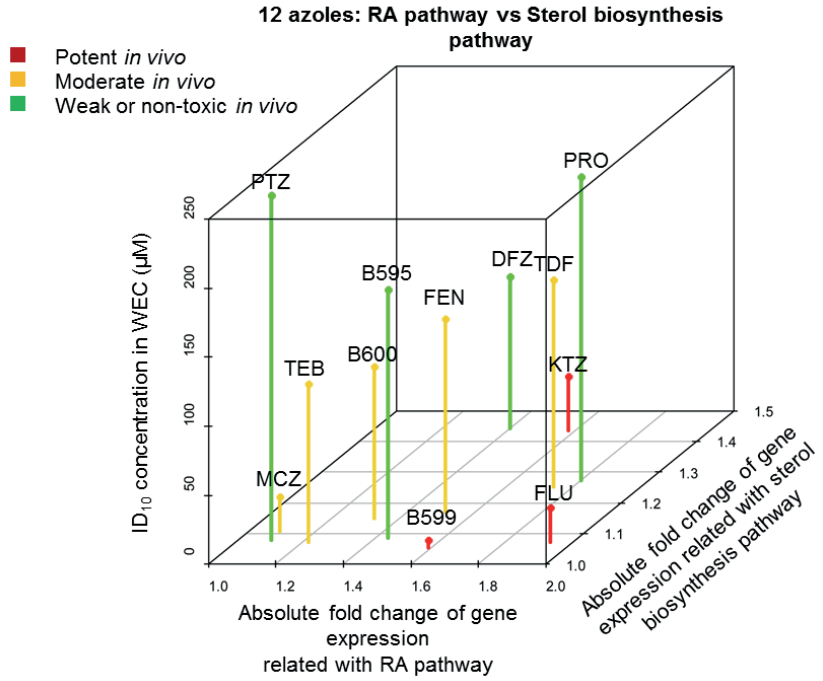


***Figure 7**: Correlation of in vivo and in vitro in the rat WEC data for twelve tested azoles. Bars with red, yellow and green colour indicate in a qualitative way the potent, moderate and weak or non-toxic in vivo profile of these azoles. The length of the bars represents the in vitro ID_{10} concentration (y-axis).*

Discussion

In the present study, azoles induced concentration dependent developmental toxic responses in rat WEC, including abnormalities in neural tube closure, formation of the branchial arches and development of the otic cup. Embryos exposed *in vivo* to the same azoles demonstrated commonly observed abnormalities for triazoles , including cleft palate [31] and skeletal abnormalities [30, 32, 33, 35-37], or hydronephrosis [34] and abnormalities in the urogenital system [29]. It should be noted that some of these abnormalities are induced *in vivo* at stages beyond the WEC developmental period. The pattern of abnormalities due to either *in vivo* or *in vitro* exposure to azoles is similar to that observed after exposure to RA [5, 8]. This observation is supportive of an involvement of the RA pathway in the developmental toxicity of azoles. Comparing *in vitro* ID_{10} with the *in vivo* BMD_{10} levels (Table 3), we concluded that potency ranking in the WEC was largely similar to the potency ranking in the *in vivo* situation.

We identified 53 genes statistically significantly regulated (ANOVA, p-value<0.001, FDR 10%) by at least one of the compounds, which were further categorized into six functional gene-groups. We further analysed the responses of genes associated with the sterol biosynthesis and RA pathways, due to their crucial role for determining the fungicidal mode of action (sterol biosynthesis pathway) and the embryotoxic potency (RA pathway) of the tested compounds.

Among the regulated sterol biosynthesis related genes, *Msmo1* showed the highest increase in expression, after embryonic exposure to KTZ and DFZ, as well as TDF and PRO. Despite the fact that mammalian systems are less sensitive than fungal systems to azoles [38], the expression of sterol related genes in both biological systems determines azoles' fungicidal activity. The observed significant induction of *Msmo1* (or its synonym, *Sc4mol*) was also identified in previous studies in the rat WEC [7, 25], as well as in the zebrafish test (ZET) [39] and Embryonic Stem Cell Test (EST) [40]. Additionally, considering that the expression pattern of all the individual sterol related genes was constant among the tested compounds (Figure 6B), we suggest that *Msmo1* could be a more sensitive biomarker compared to the already characterized biomarker *Cyp51* [12] for studying the fungicidal activity. However, for concluding about the extent of each gene's specific importance in the sterol biosynthesis pathway, studies on the level of the metabolome are needed. *Msmo1* is involved in an oxidation-reduction process, while it is also associated with malformations, such as microcephaly and congenital cataract, which could be linked with its extra role in the central nervous system development (CNS), and especially in the midbrain neurogenesis [41]. Pinto at al. [42] described that *Msmo1* transcription is activated by the liver X receptors (LXR), which are binding to the retinoid X receptors (RXR), a connection that could be further

associated with RA. Additionally, *Srebp* transcriptional factors regulate the cholesterol biosynthesis pathway in mammalian systems, via interacting with the binding sites of *Hmgcr* and *Fdft1* in the mevalonate arm in the beginning of the pathway [43]. *Srebp* interacts directly with LXR and therefore may indirectly regulate genes in the sterol biosynthesis pathway [42, 44].

Additionally, we observed that the potent *in vivo* and *in vitro* embryotoxicants, as well as the moderate TDF and the weak PRO, altered the expression of RA-related genes in a similar manner. The commonly highest upregulated gene was *Cyp26a1*, which is upregulated for metabolizing excess level of RA [45]. Therefore, we suggest that the overexpression of the RA pathway could be the underlying mechanism of induced developmental toxicity of azoles in the rat WEC. Consequently, the application of RA-related biomarkers is valuable for distinguishing highly potent embryotoxicants within the same class of chemicals.

MCZ, a compound with potent *in vitro* and moderate *in vivo* embryotoxic potency, lacked a statistically significant response on the level of transcriptome in our combined analysis. This suggests that transcriptomics may not be the optimal method to detect the embryotoxic mode of action of MCZ. Apoptosis, an additionally identified functional gene group, was extensively regulated by the azoles that showed the highest response of RA-related genes. Interestingly, similar to the strong *in vitro* embryotoxicants FLU, KTZ and B599, MCZ did show enhanced expression of *Ngfr*, an apoptosis related gene (Figure 4). *Ngfr* is associated with neuron differentiation in the brain region [46], while it has been also suggested to be mediator for thyroid hormone activation [47] and a negative regulator of angiogenesis [48]. Another apoptosis related strong effect was identified on the expression of *Fam101a*, which is localized in the midbrain and forebrain of 5-somite stage embryos [49, 50], while it is involved in the bone maturation and interacts with RA [51]. Furthermore, *Txnip*, a general biomarker of stress responses, is related with the dysregulation of cell division [52, 53]. The similarity of expression among genes of RA and apoptosis pathways could support our hypothesis that RA related responses are directly linked to developmental toxic responses and, therefore, could justify the consequent embryotoxicity of the corresponding azoles.

Moreover, *Lhx1*, the most pronounced expressed gene among the neural differentiation related genes, was remarkably affected in WEC exposed to B599, FLU, KTZ and TDF, which are among the most potent compounds. *Lhx1* has been also suggested to be indirectly associated with RA and RA-related morphological alterations. It is localized in the brain and has been shown to interact with development related genes and transcriptional factors [54], such as the *Hox* and *Pax* genes, and therefore, it could be indirectly correlated with the activation of the *Gata* and *Wnt* signalling pathways [55-57]. Karavanov *et al*. [58]

have also described its additional role in the kidney development during embryonic development and in later stages for maintaining the function of the ureteric bud.

Embryos exposed to azoles with high ID_{10} concentrations disclosed a notable downregulation of a set of genes, which could explain the sensitivity of the WEC system compared to the *in vivo* screening in ranking DFZ and PRO. The highest regulation of *Ifrd1*, which participates in neuron differentiation and general development pathways (Figure 4), could be associated with cellular stress in multicellular organisms according to Zhao *et al*. [59]. In those embryos, we also observed a significant accompanied downregulation of both *Arrdc4*, a protein that regulates the ubiquitin-protein transferase activity [60], and *Txnip* (Figure 4). *Txnip* is a member of the alpha arrestin protein family (to which *Arrdc4* belongs too), however the exact mechanism of collaboration of these two genes has not been elucidated yet [61].

To summarize, we investigated the potency ranking of twelve azoles in the rat WEC, the vast majority of which was in line with the *in vivo* potency ranking. We also studied the toxicological and fungicidal mode of action of the selected compounds on the level of transcriptome using the set of biomarkers that has been previously selected [7]. We concluded that the most potent embryotoxicants, both *in vivo* and *in vitro*, revealed an overexpression of genes that participated in RA related pathways, and were associated with apoptosis and stress responses. Moreover, we identified responses of genes that participated in the sterol biosynthesis pathway and, therefore, related to the fungicidal mode of action. We found that *Msmo1* was a more sensitive biomarker for screening the functional efficacy of azoles compared to *Cyp51*, which could improve the *in vitro* assessment of existing and future antifungal chemicals.

Funding Information

This work was supported by a collaborative project between Wageningen University (grant number 6153511466), RIVM and BASF SE.

References

1. van der Jagt K., M.S., Tørsløv J. & de Bruijn J. , *Alternative approaches can reduce the use of test animals under REACH* EUROPEAN COMMISSION DIRECTORATE GENERAL JRC JOINT RESEARCH CENTRE Institute for Health and Consumer Protection, http://home.kpn.nl/reach/downloads/reducingtheuseoftestanimalsunderreachihcprepor.pdf, 2004.
2. Augustine-Rauch, K., C.X. Zhang, and J.M. Panzica-Kelly, *In vitro developmental toxicology assays: A review of the state of the science of rodent and zebrafish whole embryo culture and embryonic stem cell assays.* Birth Defects Res C Embryo Today, 2010. **90**(2): p. 87-98.
3. Piersma, A.H., *Alternative methods for developmental toxicity testing.* Basic Clin Pharmacol Toxicol, 2006. **98**(5): p. 427-31.
4. Piersma, A.H., *Validation of alternative methods for developmental toxicity testing.* Toxicol Lett, 2004. **149**(1-3): p. 147-53.
5. Robinson, J.F., A. Verhoef, and A.H. Piersma, *Transcriptomic analysis of neurulation and early organogenesis in rat embryos: an in vivo and ex vivo comparison.* Toxicol Sci, 2012. **126**(1): p. 255-66.
6. New, D.A., P.T. Coppola, and D.L. Cockroft, *Comparison of growth in vitro and in vivo of post-implantation rat embryos.* J Embryol Exp Morphol, 1976. **36**(1): p. 133-44.
7. Dimopoulou, M., et al., *Embryotoxic and pharmacologic potency ranking of six azoles in the rat whole embryo culture by morphological and transcriptomic analysis.* Toxicol Appl Pharmacol, 2017. **322**: p. 15-26.
8. Luijten, M., et al., *Transcriptomics analysis of retinoic acid embryotoxicity in rat postimplantation whole embryo culture.* Reprod Toxicol, 2010. **30**(2): p. 333-40.
9. Robinson, J.F., J.L. Pennings, and A.H. Piersma, *A review of toxicogenomic approaches in developmental toxicology.* Methods Mol Biol, 2012. **889**: p. 347-71.
10. Robinson, J.F., et al., *Embryotoxicant-specific transcriptomic responses in rat postimplantation whole-embryo culture.* Toxicol Sci, 2010. **118**(2): p. 675-85.
11. Daston, G.P. and J.M. Naciff, *Predicting developmental toxicity through toxicogenomics.* Birth Defects Res C Embryo Today, 2010. **90**(2): p. 110-7.
12. Marotta, F. and G.M. Tiboni, *Molecular aspects of azoles-induced teratogenesis.* Expert Opin Drug Metab Toxicol, 2010. **6**(4): p. 461-82.
13. de Jong, E., et al., *Comparison of the mouse Embryonic Stem cell Test, the rat Whole Embryo Culture and the Zebrafish Embryotoxicity Test as alternative methods for developmental toxicity testing of six 1,2,4-triazoles.* Toxicol Appl Pharmacol, 2011. **253**(2): p. 103-11.
14. Dimopoulou, M., et al., *Flusilazole induces spatio-temporal expression patterns of retinoic acid-, differentiation- and sterol biosynthesis-related genes in the rat Whole Embryo Culture.* Reprod Toxicol, 2016. **64**: p. 77-85.
15. Menegola, E., et al., *Dysmorphogenic effects of some fungicides derived from the imidazole on rat embryos cultured in vitro.* Reprod Toxicol, 2006. **21**(1): p. 74-82.
16. Piersma, A.H., E.V. Hessel, and Y.C. Staal, *Retinoic acid in developmental toxicology: Teratogen, morphogen and biomarker.* Reproductive Toxicology.
17. Tonk, E.C.M., J.L.A. Pennings, and A.H. Piersma, *An adverse outcome pathway framework for neural tube and axial defects mediated by modulation of retinoic acid homeostasis.* Reproductive Toxicology, 2015. **55**: p. 104-113.
18. Cunningham, T.J. and G. Duester, *Mechanisms of retinoic acid signalling and its roles in organ and limb development.* Nat Rev Mol Cell Biol, 2015. **16**(2): p. 110-23.
19. Brown, N.A. and S. Fabro, *Quantitation of rat embryonic development in vitro: a morphological*

scoring system. Teratology, 1981. **24**(1): p. 65-78.

20. Slob, W., *Dose-response modeling of continuous endpoints.* Toxicological Science, 2002. **66**(2): p. 298-312.
21. Eijssen, L.M., et al., *User-friendly solutions for microarray quality control and pre-processing on ArrayAnalysis.org.* Nucleic Acids Res, 2013. **41**(Web Server issue): p. W71-6.
22. Irizarry, R.A., et al., *Exploration, normalization, and summaries of high density oligonucleotide array probe level data.* Biostatistics, 2003. **4**(2): p. 249-64.
23. Dai, M., et al., *Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data.* Nucleic Acids Res, 2005. **33**(20): p. e175.
24. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.* Nat Protoc, 2009. **4**(1): p. 44-57.
25. Robinson, J.F., et al., *Triazole induced concentration-related gene signatures in rat whole embryo culture.* Reprod Toxicol, 2012. **34**(2): p. 275-83.
26. Kutmon, M., et al., *WikiPathways: capturing the full diversity of pathway knowledge.* Nucleic Acids Research, 2016. **44**(D1): p. D488-D494.
27. Santori, F.R., et al., *Identification of natural RORgamma ligands that regulate the development of lymphoid cells.* Cell Metab, 2015. **21**(2): p. 286-97.
28. Li, H., et al., *Extended evaluation on the ES-D3 cell differentiation assay combined with the BeWo transport model, to predict relative developmental toxicity of triazole compounds.* Arch Toxicol, 2016. **90**(5): p. 1225-37.
29. Lamontia CL, S.R., Alvarez L., *Embryo-fetal toxicity and teratogenicity study of INH-6573-39 by gavage in the rat.* Unpublished reports No. HLR 444-83 and HLR 142-84 from Haskell Laboratory for Toxicology and Industrial Medicine, Wilmington, Delaware, USA. Submitted to WHO by E.I. du Pont de Nemours & Co., Inc., Wilmington, Delaware, USA. Summarized in JMPR Monograph—Flusilazole, Pesticide residues in food: 1995 evaluations Part II Toxicological & EnvironmentEnvironment. http://www.inchem.org/documents/jmpr/jmpmono/v95pr08.htm, 1984.
30. Ito, C., Y. Shibutanil, K. Inoue, K. Nakano and H. Ohnishi, *Toxicologocal Studies of Miconazole (II) Teratological Studies of Miconazole in Rats* IYAKUHIN KENKYU, 1976. **7**(3): p. 367-376.
31. Tachibana, M.N., Y. and Monro, A.M., *Toxicology of fluconazole in experimental animals.* In Fromtling, R.A. Recent trends in the discovery, development, and evaluation of antifungal agents, JR Prous, Barcelona, 1987: p. 93-102.
32. Lochry, E.A., *Developmental toxicity study of CGA-169374 technical (FL-851406) administreted orally via gavage to Crl:COBS CD (SD) BR presumed pregnant rats.* Unpublished report No. 203-005 from Novartis Crop Protection AG., Basel, Switzerland and Argus Research Lab. Inc., Horsham, USA, Submitted to WHO by SYgenta Crop Protection AG, Basel, Switzerland. http://apps.who.int/iris/bitstream/10665/44064/1/9789241665230_eng.pdf?ua=1, 1987.
33. Becker, H., Vogel, W. & Terrier, C., *Embryotoxicity study (including teratogenicity) with HWG 1608 technical in rat*, in *Unpublished report Ref. No. R 4451, prepared by Research & Consulting Co. AG (RCC), Itingen, Switzerland. Submitted to WHO by Bayern AG, Leverkusen, Germany*. 1988.
34. Hoffman, D.G., Owen, N.V. & Adams, E.R., *A teratology study with EL-222 in the rat.* Unpublished report No. R06279 form Lilly research Laboratories, USA. Submitted to WHO by DowElanco Europe, Wantage, Oxon, United Kingdom, 1980.
35. Unger, T.M., Van Goethem, D. & Shellenberger, *A teratological evaluation of Bayleton in mated female rats.* Midwest Research Institute, Report No. 324 to Mobay Chemical Corporation Agricultural Chemical Division submitted to WHO by Bayer AG. (Unpublished), http://www.inchem.org/documents/jmpr/jmpmono/v83pr39.htm, 1982.

36. Giknis, M.L.A., *A teratology (segment II) study in rats.* Unpublished report no.: 86004 from Ciba-Geigy Pharmaceutical division, New Jersey. Submitted to WHO by Ciba-Geigy Ltd., Basle, Switzerland 1987.

37. Stahl, B., *JAU 6476 - Developmental toxicity study in rats after oral administration. Unpublished report No. M-012279-01-1 from Bayer AG, Wuppertal, Germany. Submitted to WHO by Bayer CropScience AG, Germany.* https://www.google.nl/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&ved=0ahUKEwjcjNCx5-HPAhWDrxoKHebSDzYQFgghMAE&url=http%3A%2F%2Fapps.who.int%2Fpesticide-residues-jmpr-database%2FDocument%2F110&usg=AFQjCNGpjQOE2xfb0QbWPWwA5-avbrC8kQ&sig2=ZFM7G3fgK4EdIA0e_zX0hQ&cad=rja. 1997.

38. Trosken, E.R., et al., *Comparison of lanosterol-14 alpha-demethylase (CYP51) of human and Candida albicans for inhibition by different antifungal azoles.* Toxicology, 2006. **228**(1): p. 24-32.

39. Hermsen, S.A., et al., *Triazole-induced gene expression changes in the zebrafish embryo.* Reprod Toxicol, 2012. **34**(2): p. 216-24.

40. van Dartel, D.A., et al., *Concentration-dependent gene expression responses to flusilazole in embryonic stem cell differentiation cultures.* Toxicol Appl Pharmacol, 2011. **251**(2): p. 110-8.

41. He, M., et al., *Mutations in the human SC4MOL gene encoding a methyl sterol oxidase cause psoriasiform dermatitis, microcephaly, and developmental delay.* J Clin Invest, 2011. **121**(3): p. 976-84.

42. Pinto, C.L., et al., *Lxr regulates lipid metabolic and visual perception pathways during zebrafish development.* Mol Cell Endocrinol, 2016. **419**: p. 29-43.

43. Mazein, A., et al., *A comprehensive machine-readable view of the mammalian cholesterol biosynthesis pathway.* Biochem Pharmacol, 2013. **86**(1): p. 56-66.

44. Horton, J.D., *Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis.* Biochem Soc Trans, 2002. **30**(Pt 6): p. 1091-5.

45. Rhinn, M. and P. Dollé, *Retinoic acid signalling during development.* Development, 2012. **139**(5): p. 843-858.

46. Do, H.T., et al., *Nerve growth factor (NGF) and pro-NGF increase low-density lipoprotein (LDL) receptors in neuronal cells partly by different mechanisms: role of LDL in neurite outgrowth.* Journal of Neurochemistry, 2016. **136**(2): p. 306-315.

47. Porterfield, S.P., *Thyroidal Dysfunction and Environmental Chemicals: Potential Impact on Brain Development.* Environmental Health Perspectives, 2000. **108**: p. 433-438.

48. Parsi, S., et al., *Experimental Verification of a Predicted Intronic MicroRNA in Human NGFR Gene with a Potential Pro-Apoptotic Function.* PLOS ONE, 2012. **7**(4): p. e35561.

49. Hirano, M., et al., *cfm is a novel gene uniquely expressed in developing forebrain and midbrain, but its null mutant exhibits no obvious phenotype.* Gene Expr Patterns, 2005. **5**(3): p. 439-44.

50. Mizuhashi, K., et al., *Filamin-interacting proteins, Cfm1 and Cfm2, are essential for the formation of cartilaginous skeletal elements.* Hum Mol Genet, 2014. **23**(11): p. 2953-67.

51. NCBI, *Database resources of the National Center for Biotechnology Information.* Nucleic Acids Res, 2013. **41**(Database issue): p. D8-d20.

52. Dunn, L.L., et al., *THE EMERGING ROLE OF THE THIOREDOXIN SYSTEM IN ANGIOGENESIS.* Arteriosclerosis, thrombosis, and vascular biology, 2010. **30**(11): p. 2089-2098.

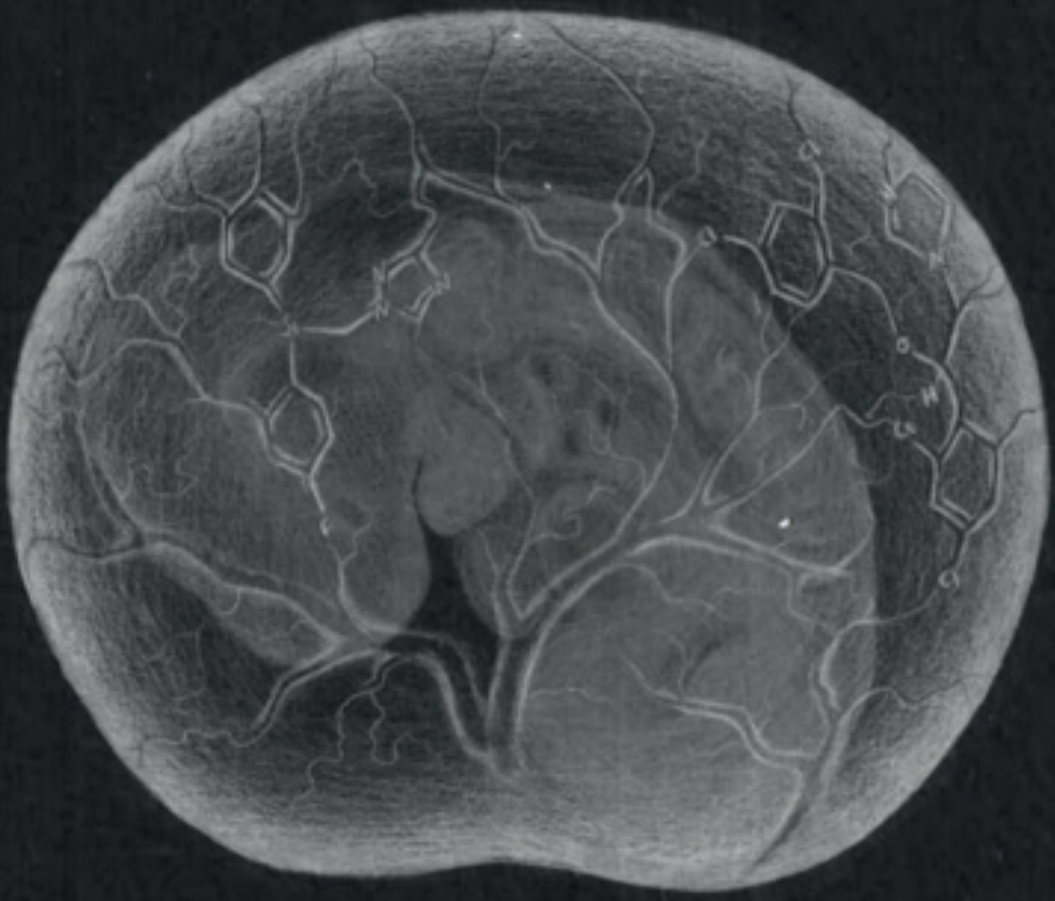
53. Patwari, P., et al., *The interaction of thioredoxin with Txnip. Evidence for formation of a mixed disulfide by disulfide exchange.* J Biol Chem, 2006. **281**(31): p. 21884-91.

54. Furuyama, T., et al., *Distribution of Rlim, an LIM homeodomain gene, in the rat brain.* Neurosci Lett, 1994. **170**(2): p. 266-8.

55. Costantini, F. and R. Kopan, *Patterning a complex organ: branching*

morphogenesis and nephron segmentation in kidney development. Dev Cell, 2010. **18**(5): p. 698-712.

56. Hevner, R.F., E. Miyashita-Lin, and J.L. Rubenstein, *Cortical and thalamic axon pathfinding defects in Tbr1, Gbx2, and Pax6 mutant mice: evidence that cortical and thalamic axons interact and guide each other.* J Comp Neurol, 2002. **447**(1): p. 8-17.
57. Pratt, T., et al., *A role for Pax6 in the normal development of dorsal thalamus and its cortical connections.* Development, 2000. **127**(23): p. 5167-78.
58. Karavanov, A.A., et al., *Expression pattern of the rat Lim-1 homeobox gene suggests a dual role during kidney development.* Int J Dev Biol, 1998. **42**(1): p. 61-6.
59. Zhao, C., et al., *Stress-sensitive regulation of IFRD1 mRNA decay is mediated by an upstream open reading frame.* J Biol Chem, 2010. **285**(12): p. 8552-62.
60. Mackenzie, K., et al., *Regulation of the divalent metal ion transporter via membrane budding.* 2016. **2**: p. 16011.
61. Fishilevich, S., et al., *GeneHancer: genome-wide integration of enhancers and target genes in GeneCards.* Database, 2017. **2017**: p. bax028-bax028.



Chapter 5

A comparison of the embryonic stem cell test and whole embryo culture assay combined with the BeWo placental passage model for predicting the embryotoxicity of azoles

Myrto Dimopoulou, Aart Verhoef, Caroline A. Gomes, Catharina W. van Dongen, Ivonne M.C.M. Rietjens, Aldert H. Piersma and Bennard van Ravenzwaay

Accepted for publication, Toxicology Letters

Abstract

In the present study, we show the value of combining toxico-dynamic and -kinetic *in vitro* approaches for embryotoxicity testing of azoles. We also report on the alterations in gene expression induced by azoles. Both the whole embryo culture (WEC) assay and the embryonic stem cells test (EST) predicted the *in vivo* potency ranking of the twelve tested azoles with moderate accuracy. Combining these results with relative placental transfer rates (Papp values) as determined in the BeWo cell culture model, increased the predictability of both WEC and EST, with R^2 values increasing from 0.51 to 0.87 and from 0.35 to 0.60, respectively. The comparison of these *in vitro* systems correlated well (R^2 = 0.67), correctly identifying the strong and weak embryotoxicants. Evaluating specific gene responses related with the toxicological and fungicidal mode of action of the tested azoles in WEC and EST, we observed that the differential regulation of *Dhrs3* and *Msmo1* reached higher magnitudes in both systems compared to *Cyp26a1* and *Cyp51*. Establishing sensitive biomarkers across all the *in vitro* systems for studying the underlying mechanism of action of chemicals, such as azoles, is valuable for comparing alternative *in vitro* models and for improving insight in the mechanism of developmental toxicity of chemicals.

Key words: Whole embryo culture; stem cell test; placental transfer; azoles; biomarkers; embryotoxicity

Introduction

The risk assessment of chemicals is still highly dependent on the use of experimental animals [1, 2]. Additionally, complying with the European chemical safety legislation (REACH), the number of experimental animals will dramatically increase, reaching an estimated 22 million vertebrates [3, 4]. Reproductive and developmental toxicology requires the highest percentage of experimental animals, which may reach an estimated 60% [5, 6]. Over the past decades, awareness has risen about the necessity of developing alternative approaches to animal testing for reducing, refining and replacing the animal testing [7]. A number of *in vitro* alternative assays has been developed for screening developmental toxicants, including cell lines, single organs and whole embryo cultures. The European Centre for the Validation of Alternative Methods (EVCAM) has already validated the Embryonic Stem Cell test (EST), the limb bud micromass test and the rat Whole Embryo Culture (WEC) assay as alternative *in vitro* tests for studying the developmental toxicity of xenobiotics [3, 8, 9].

Among these alternative tests for screening developmental toxicity, only the EST does not require the use of animals or animal-derived tissue [9, 10]. Murine pluripotent embryonic stem cells can be isolated, cultured and further differentiated to a variety of cell-types, such as cardiomyocytes, neural, red blood cells and others [11, 12]. One requirement for the differentiation is the formation of multicellular aggregates, the embryonic bodies (EBs), which allows the induction of cells of endo-, meso- and ectoderm after continuous *in vitro* culture, mimicking the egg-cylinder stage of an *in vivo* 5-day old embryo [13]. However, the EST is a simplified method, as only one morphological endpoint, the assessment of contracting cardiomyocytes following the 10-day differentiation protocol, is considered. Additionally, it lacks both the complexity and programmed pattern formation of a whole organism and has not any metabolic capacity [3, 9]. The rat WEC is a more complex model, which mimics quite well the *in vivo* situation [14, 15]. In the WEC assay a variety of morphological endpoints, quantitatively summarized as a total morphological score, is evaluated for concluding about any possible effect on the embryonic growth and development [16, 17]. This model covers gestational day (GD) 10 to 12, when neurulation and organogenesis occur. This critical time window gives an important advantage for closely monitoring the developmental toxicity in the rat WEC, but on the other hand is one of the restrictions of the system as it is not applicable for other time windows of development [3, 18].

One common disadvantage of EST and WEC is the different exposure situation compared to *in vivo* [19]. The placenta is an important organ during the pregnancy, required for the transportation of nutrients, oxygen and hormones from the mother to the fetus, as well as for removing waste products from the embryonic side [20, 21]. Xenobiotics are differently

transferred across the placenta according to their physicochemical properties [22]. Therefore, its evaluation would allow better prediction of to which extent the embryo or fetus will be exposed to possible embryotoxicants [19, 23, 24]. The *ex vivo* placental perfusion model is a human-based system for studying the transport of xenobiotics across the placenta. However, this model is laborious and dependent on the availability of fresh placentas that are donated from mothers after deliveries [25]. An alternative to the *ex vivo* placental perfusion model is the BeWo transport assay, which is easy, fast and cheap. The BeWo b30 cell line, human choriocarcinoma derived cells, grow on permeable membranes from transwell inserts and form a confluent cell layer (the BeWo layer) [25-27]. Using the transwell system, it is possible to mimic the *in vivo* situation, by dividing the well in the basolateral and apical compartments, which are related to the maternal and embryonic sides, respectively [28]. By exposing the apical compartment to the xenobiotic under assessment, its transportation to the basolateral compartment can be measured.

The assessment of embryotoxic profiles of xenobiotics in the EST and the rat WEC, in combination with the BeWo model, enables the evaluation of the morphological effects at concentrations that have been corrected for the placental transfer [29, 30]. Furthermore, because gene expression responses precede the corresponding morphological changes and, consequently, could predict them, gene expression studies could be further implemented here for elucidating the mode of toxicological action of the testing compounds [31-33]. Consequently, the combination of more *in vitro* assays with multi-target readouts may improve the predictions of embryotoxic responses, distinguishing classes of chemicals or identifying unique signatures within the same chemical group [34-36].

Azoles are antifungal agents with agricultural and clinical use [37]. They have been designed to interact with the sterol biosynthesis pathway and further inhibit the fungal *Cyp51*, which is the catalyst for converting lanosterol to ergosterol, disturbing the fungal cell-membrane integrity [37, 38]. Some of the azoles have previously shown embryotoxicity in *in vivo* systems, mainly introducing abnormalities related with craniofacial alterations and skeletal dysmorphogenesis [29, 39-43]. The main cause of their increased embryotoxicity has been postulated to be their interaction with retinoic acid (RA) -related enzymes, such as *Cyp26a1* and *Dhrs3*, which are modulators of the RA homeostasis in mammalian systems [40, 44-46]. RA has been shown to be a morphogen in vertebrate embryogenesis [45, 47].

In the present study, twelve azoles were tested in the EST and the obtained results were compared to WEC assay results, which have been reported in our previous study [48]. Furthermore, their placental transport rates, obtained with the BeWo model, were combined with the effective concentrations in the EST and WEC, to assess if an improvement of the correlation between *in vitro and in vivo* developmental toxicity (potency ranking) could be

achieved. We also compared the expression of four genes, two biomarkers of the toxicological (*Cyp26a1* and *Dhrs3*) and two of the intended fungicidal (*Cyp51* and *Msmo1*) mode of action of azoles.

Materials and Methods

Chemicals

Nine azoles compounds were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands); flusilazole (FLU; CAS# 85509-19-9); difenoconazole (DFZ; CAS# 119446-68-3); ketoconazole (KTZ; CAS# 65277-42-1); prothioconazole (PTZ; CAS# 178928-70-6); triadimefon (TDF; CAS# 43121-43-3); fenarimol (FEN; CAS#60168-88-9); propiconazole (PRO; CAS#60207-90-1); and tebuconazole (TEB; CAS#107534-96-3). Miconazole (MCZ, CAS# 22916-47-8) was purchased from Sigma-Aldrich and Alfa Aesar (Germany). BASF SE (Ludwigshafen, Germany) kindly provided three novel azoles, encoded as B595, B599 and B600, of which the chemical structures are shown in Figure 1. All the compounds, tested in the EST, were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Zwijndrecht, The Netherlands) at 0.25 % v/v final concentration in the culture medium. Antipyrine (CAS# 60-80-0) and amoxicillin (CAS# 26787-78-0) were purchased from Sigma (Germany) and were used as controls for high and low permeability in the BeWo transport tests, respectively. They were dissolved in DMSO at a maximum 0.5 % v/v final concentration in HBSS (Biochrom, Germany) in cell culture. Fluorescein (CAS# 2321-07-5, Sigma, Germany) was used as paracellular transfer control in the BeWo model and was dissolved in HBSS.

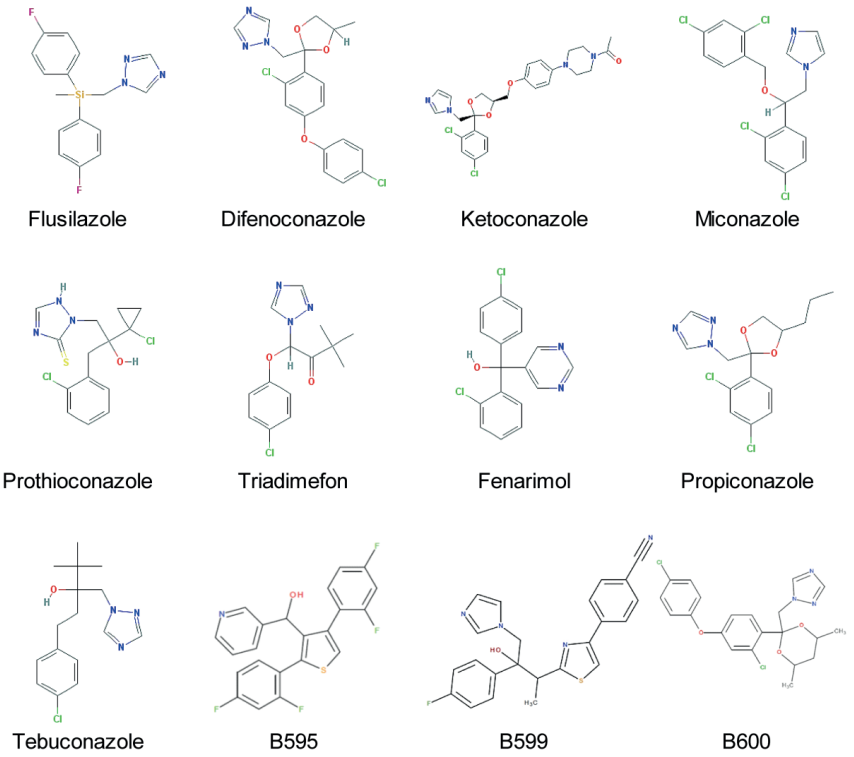


***Figure 1**: Chemical structures of the twelve azoles under assessment*

Embryonic stem cell test (EST)

Pluripotent stem cell culture

Murine D3 embryonic stem (ES-D3) cells (ATCC, Rockville, MD) were cultured in polystyrene cell culture petri dishes of 35mm diameter (Corning) in humidified atmosphere at 37°C and 5% CO_2 and they were routinely subcultured every 2-3 days. They were maintained in culture medium, which consisted of Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 20% fetal bovine serum (Greiner Bio-One), 1% non-essential amino acids (Gibco), 1% penicillin/streptomycin (Gibco), 2mM L-glutamine (Gibco) and 0.1mM β-mercaptoethanol (Sigma-Aldrich). To maintain the pluripotency of the ES-D3 cells, murine leukemia inhibitor factor (mLIF; ESGRO, Millipore, the Netherlands) was directly added to the culture petri dish at a final concentration of 1000 units/mL.

Cardiomyocyte differentiation assay

Cardiac differentiation of ES-D3 cells was performed according to the protocol [12] using the previously described culture medium without containing mLIF. In brief, stem cell suspensions of $15x10^4$ cells/mL were further diluted to $3.75x10^4$ cells/mL and placed on ice before starting the hanging drops protocol. Next, 20 µL of stem cell suspension was placed on the inner part of the lid of a 100 mm diameter petri dish (Greiner Bio-One) containing 5 mL phosphate buffered saline (PBS, Gibco), Ca^{2+} and Mg^{2+} free, resulting to hanging drops. Each petri dish contained 56 drops, and each drop consisted of 750 cells. The dishes were cultured for three days at 37°C and 5% CO_2, during which the ES-D3 proliferated and formed cell-aggregates, called embryonic bodies (EBs). On day three, EBs were transferred to a 60mm-diameter bacterial petri dish (Greiner Bio-One), containing 5 mL culture medium without mLIF, and were further cultured under the same conditions for two days. After, each individual EB was transferred to a single well in a 24-well plate (TPP, Trasadingen, Switzerland), containing 1mL culture medium without mLIF. After five days, the cardiac differentiation was microscopically determined by examining whether the EBs had turn into contracting myocardial cells. The plates were scored as positive or negative based on the presence or absence of contracting myocardial cells, respectively. For each test compound, the chemical exposure started at the differentiation day 3 and refreshed at day 5 when EBs were transferred in a new plate-setup. Except for KTZ, all the tested compounds were tested at a range of concentration from 0.01 µM to 300 µM. The highest concentration at which KTZ tested was 100 µM. For each compound, three independent experiments were performed (biological replicates), while each experimental day the experiments were performed in duplicates (technical replicates). Additionally, solvent and medium control cultures were separately observed for each of the tested compounds. Tests were accepted when 21 out of 24 EBs exposed to the solvent control (0.25% v/v DMSO) were contracting. The number of positive EBs was expressed as fraction of total control (24 EBs).

Cell viability assay

ES-D3 cells were seeded in a 96-well plate (Greiner Bio-One) diluted to $1x10^4$ cells/mL (500 cells per well) in culture medium containing mLIF and incubated at 37°C and 5% CO_2 to allow cell adherence. Afterwards, 150 µL culture medium was added to full-fill each well up to 200 µL volume. After continuous incubation for three days, the culture medium was removed and replaced with freshly made exposure medium (200 µL), supplemented with mLIF and containing the corresponding test compound and concentration for each exposure. On day 5, 100 µL of the supernatant medium was removed and 20 µL of CellTiter-Blue (G8081, Promega, Leiden, the Netherlands) was added in each of the wells and incubated

for two more hours. The assay measures the metabolic capacity of viable cells exposed to the tested chemicals to reduce resazurin (blue) into resorufin (pink), which is highly fluorescent. The non-viable cells lose this metabolic capacity and, therefore, their do not generate a fluorescent signal. The fluorescence was read using SpectraMax® M2 spectrofluorometer (Molecular Devices, Berkshire, United Kingdom) at 544 nm and 590 nm (excitation and emission, respectively). Two independent experiments, in six technical replicates each, were performed for each of the tested compounds. Solvent (0.25% v/v DMSO), positive (0.1 µg/mL 5-FU) and negative (500 µg/mL Penicillin) controls were included in every experiment in six replicates.

Concentration-response curves and determination of both ID_{50} and IC_{50}

Results from the EST were analysed and concentration-response curves were fitted using the PROAST software [49]. Based on the fit of the curve and with 95% confidence intervals, we calculated the concentration (ID_{50}) at which the number of contracting cells were reduced to 50% of the control (0.25% v/v DMSO). Following the same rationale, we also calculated the IC_{50} concentration at which exposed cells resulted to 50% decrease in cell viability by analysing the data obtained from the corresponding assays. The calculated ID_{50} concentration for each of the tested compounds was further applied for performing the gene expression studies.

EST exposure to azoles for gene expression analysis

The experimentally calculated ID_{50} concentrations for each of the tested azoles were: 23.5 µM FLU, 13.6 µM DFZ, 13.5 µM KTZ, 20.5 µM MCZ, 122 µM PTZ, 31.8 µM TDF, 77.5 µM FEN, 78.6 µM PRO, 57.8 µM TEB, 36.7 µM B595, 7.2 µM B599 and 31.8 µM B600. Following the cardiomyocyte differentiation protocol, we exposed the EBs on day 3 and we collected them after 24 hours of exposure (day 4) in eppendorf tubes, containing 800 µL RNAprotect (Qiagen, Cat. # 76526), which were further stored at -20 ^{o}C prior to RNA extraction. Concurrent solvent (0.25 % v/v DMSO) and culture medium controls were exposed and collected similarly to the exposed cultures. All the exposure groups consisted of 8 replicates and the control groups contained 12 replicates. Additionally, extra plates containing EBs (three independent experiments in duplicates) exposed to the tested azoles at their ID_{50} were further cultured, following the 10-day EST differentiation protocol. Then, they were microscopically examined to determine whether the efficiency of the applied concentration for inhibiting the cardiomyocyte contraction is indeed 50%. Three independent

experiments, in technical duplicate each, were performed, including also solvent and culture medium controls.

RNA isolation

Before starting the RNA isolation, samples were thawed on ice. RNA was further isolated using the RNeasy Mini-extraction kit (Qiagen, Cat. # 74104), following manufacturer's protocol. In brief, tubes containing the EBs were spun down and excess volume of RNAprotect was discarded. Then, we added the RLT buffer and by gently pipetting the EBs were broken down into smaller pieces. We treated the samples with QIAshredder (Qiagen, Cat. # 79654) for better homogenization and therefore, for increasing the RNA yields. Then, the lysates were transferred into RNeasy spin columns for purifying the RNA extracts. We additionally treated the extracts with the RNase-Free DNase set (Qiagen, Cat. # 79254), for achieving better purification. Quantity and quality of RNA yields were determined with Nanodrop (Nanodrop Technologies Inc., Wilmington, Delaware) and 2100 BioAnalyzer (Aligent Technologies, Amstelveen, the Netherlands). Samples with absorbance values between 1.9 and 2.2 (ratio 260 nm/280 nm) and RNA integrity number (RIN) higher than 8 were further used for performing the gene expression study.

Gene expression with Real-Time PCR

For performing the RT-PCR analysis, cDNA was synthesized by using the high-capacity cDNA archive kit containing random hexamer primers (Applied Biosystems, Foster City, CA, Cat. # 4368814), according to manufacturer's instructions. The quantification of the mRNA of the genes of interest was measured with TagMan gene expression assays (Applied Biosystems) on a 7500 Fast Real-Time PCR system. We followed the two-step PCR protocol provided by the manufacturer, which included the following thermal cycling conditions: 95^{o}C for 20s for the first cycle, followed by 40 cycles of 95^{o}C for 3 s and 60^{o}C for 30 s. The measured mRNA markers were *Cyp26a1* (Applied Biosystems, Cat. # Mm00514486_m1), *Dhrs3* (Applied Biosystems, Cat. # Mm00488080_m1), *Cyp51* (Applied Biosystems, Cat. # Mm00490968_m1) and *Msmo1* (Applied Biosystems, Cat. # Mm00499390_m1). The used housekeeping genes were Hprt1 (Applied Biosystems, Cat. # Mm03024075_m1) for *Cyp26a1* and *Dhrs3*, and Polr2a (Applied Biosystems, Cat. # Mm00839502_m1) for *Cyp51* and *Msmo1*. The No Template Control (NTC) and No Reverse Transcriptase (no RT) control were included in every RT-PCR run for assessing the reliability of the produced results. The mRNA expression was normalized to the value of Hprt1 or

Polr2a for each of the reactions according to the comparative Ct method (ΔΔCt) and the obtained results were relatively expressed in fold induction of gene expression.

Gene expression analysis-data processing

The parametric Student's t-test two-sided with $p < 0.05$ approach was used for determining the statistical significance on the expression of the selected genes in EST exposed to chemicals compared to this exposed to the solvent control (DMSO). All the exposure and control (DMSO and medium) groups consisted of 8 and 12 samples, respectively.

BeWo Transport Model

BeWo b30 culture

The BeWo b30 culture and transport experiments were performed as described in Li *et al*. [29, 30] with slight adaptations. The BeWo b30 cell line was purchased from AddexBio (Cat. # C0030002, Lot. # 7985832; San Diego, USA). It was confirmed to be bacteria, yeast and mycoplasma negative (certificate of analysis from AddexBio). For maintaining and culturing the BeWo cell line, Dulbecco's Modified Eagle's Medium (DMEM) (Cat. # FG0445), HBSS without phenol red with $Ca^{2+}Mg^{2+}$ (Cat. # L2035), Fetal Bovine Serum (FBS) Superior (Cat. # S0615, Lot. # 0114F), Penicillin/Streptomycin solution (Cat. # A2213), Trypsin / EDTA 0.05 % / 0.002 % (Cat. # L2143) and PBS without $Ca^{2+}Mg^{2}$ (Cat. # L1825) were purchased from Biochrom, Germany. Sodium Pyruvate (Cat. # 11360-039) was purchased from Gibco Life Sciences, Germany. BeWo b30 cells (passages 24-36) were routinely subcultured, 3 to 4 times per week, and maintained in polystyrene cell-culture flasks (TRP, Switzerland) in culture medium consisted of DMEM supplemented with 10% (v/v) FBS, 1% (v/v) Penicillin/Streptomycin solution and 1% (v/v) Sodium Pyruvate under a humidified atmosphere of 5% CO_2 at 37°C. The cells were harvested after their treatment with 0.05% trypsin-EDTA solution. For their subculture (passage), they were seeded in a new cell culture flask at a density of 2 to 2.5 x 10^6 cells/flask and incubated at 37°C and 5% CO_2. For transport experiment, they were transferred to 12-well plates with transwell polycarbonate membranes (12 mm diameter, 0.4 µM pore size; Cat. # 3401, Corning Costar, USA) pre-coated with human placental collagen type IV (Cat. #5533, Sigma-Aldrich), where they were seeded at a density of 1 x 10^5 cell/cm^2 in a 0.5 mL volume (apical compartment), while the basolateral compartment contained 1.5 mL culture medium. The culture conditions were the same as during the simple cell maintenance (37°C and 5% CO_2). The medium in both compartments was daily changed until day 6 of post-seeding, when the transfer experiments were performed.

BeWo transport experiments

For the Bewo transport model, literature data obtained from Li *et al*. [29, 30] and additional experimental data generated by BASF SE (Germany) were used in this study. The BeWo experimental data were generated following the methodology described in these literature studies with slight adaptations. Before starting the transfer experiments, the wells were equilibrated in HBSS in both compartments for 30 minutes in the incubator and transepithelial electrical resistance (TEER) values were measured using a voltmeter (EVOM X, Cat. No. 72564, World Precision Instruments, USA) with an EVOM electrode set (STx2, World Precision Instruments, USA). The plates were placed under a hot plate at 37°C to minimize temperature effects. TEER values were corrected for collagen-coated wells without the presence of cells, and transformed in Ω x cm^2, by multiplying the measured values in Ω per the insert area (1.12 cm^2). Only wells showing a TEER value ≥ 44 Ω x cm^2) were used for transfer experiments. Fresh stock solutions of the compounds were made (10 mM FLU, DFZ, MCZ, TDF, amoxicillin and antipyrine in DMSO; 1 mM fluorescein in HBSS) and working solutions were made by diluting in HBSS 200x for FLU, DFZ, TDF, amoxicillin and antipyrine, and a dilution of 1000x for MCZ. The resulting exposure concentrations were 50 µM for FLU, DFZ, TDF, amoxicillin and antipyrine, and 10 µM for MCZ, with a maximum concentration of 0.5% DMSO in any case. These chosen concentrations were based on the preparatory cell viability experiment results, which showed that the cell viability of 3-day BeWo cells was not affected after exposure to 50 µM of FLU, DFZ and TDF. However, due to decreased cell viability at the concentration of 20 µM of MCZ (75.9 ± 3.0 % viability), the BeWo transport experiments for MCZ were performed at 10 µM. Fluorescein was diluted 50 times in HBSS leading to an exposure solution of 20 µM, which was added at the performed experiments as a paracellular control (restricted transfer in the presence of BeWo cells). During the substance preparation and transfer experiments, the test compounds were protected from light exposure due to their increased sensitivity (especially for MCZ and fluorescein). Starting the transport experiments, 0.5 mL of the exposure solutions of each of the test substances and controls were added into the apical compartment and 1.5 mL of HBSS (transport buffer) to the basolateral compartment. Directly after exposure, 0.2 mL samples of the exposure solutions (in triplicate) were collected in 96 deep well plates (Thermo Fisher Scientific, Germany). After 15, 30, 60 and 90 min of plate incubation in a humidified atmosphere with 5 % CO_2 at 37 ^oC, samples of 0.2 mL were collected from the basolateral compartment and replaced by an equal volume of HBSS. At the end of the transport experiments, an additional sample of 0.2 mL was collected form the apical compartment, for calculating the recovered amount of every tested compound. In each transport experiment, antipyrine and amoxicillin were included as controls of high and low

permeability of the BeWo layers, respectively. Collected samples were stored at -20 °C for further determining their transport rates by LC-MS analysis, conducted at the contract research organization Pharmacelsus GmbH (Germany).

In the end of the transport experiments, the transwells were washed twice with HBSS and equilibrated for 30 min in the incubator, with 0.5 mL HBSS in the apical, and 1.5 mL HBSS in the basolateral compartment. TEER was measured again using the previously described method. Hereafter, 0.5 mL of MTT working solution and 1.5 mL of culture medium were added to the apical and basolateral compartments, respectively. After incubation of 30 minutes at 37 ^{o}C, MTT (apical compartment) was replaced by 800 µL 95% DMSO in HBSS. After 30 s of shaking, 150 µL of samples from the apical compartment were collected and divided in triplicate in a 96-well plate (TRP, Switzerland). The absorbance was measured at wavelengths 570 and 690 nm using spectrophotometer (Wallac Equipment).

Ultra-High Performance Liquid Chromatography (UHPLC) Analysis

Samples were analysed using the Accela 1250 ultra-high performance liquid chromatography (UHPLC) to quantify the amount of the tested azoles that were transferred from the apical to basolateral compartment and, based on these data, to calculate their transport rates and relative Papp values. Samples with 3 µL injection volume were separated on an analytical column (Accucore PFP, 2.6 µm, 50 cm x 2.1 mm) with a pre-column (C6-Phenyl, 4 cm x 2 mm, Phenomenex, Germany). The UHPLC was performed in the gradient mode using acetonitrile + 0.2% heptafluoro butyric acid (HFBA), as the organic phase, and 0.1% formic acid (FA) in nanopure water, as aqueous phase. Eluents were pumped (Dionex UltiMate 3000 RS pump) with a flow rate of 0.6 ml/min. Measurements applying the Orbitrap™ technology with the Q-Exactive plus mass spectrometer (MS) were used to analyze the samples. As MS tune file, a generic tune file was used. The $[M+H]^+$ ion of the diisooctyl phthalate (m/z391.28429), which is ubiquitously present in the solvent system, was used as a lock mass for internal mass calibration. The MS was operated in the positive full scan mode, the accurate masses of the monitoring ions ±5 mDa were used for test item and internal standard peak integration. Further analyzer settings were as follows: maximum injection time 150 ms, sheath gas 40, aux gas 10, sweep gas 2, spray voltage 3.8 kV, capillary temperature 350°C and heater 350°C. For analyzing the LC-MS data, the operating software Xcalibur 4.0.27.19 was used.

Analysis of BeWo transport data

For each of the test compounds, the concentration of the samples collected at 0, 15, 30, 60 and 90 min from the basolateral, as well as from a sample from the apical compartment at 90 min were determined and converted to nmol. For each compound, the linear appearance rate in the basolateral compartment was calculated, used for estimating the apparent permeability (Papp) coefficients, according to the following formula:

$$Papp\ coefficient\ \left(\frac{cm}{s}\right) = \frac{\frac{\Delta Q}{\Delta t}}{A \times Co}$$

For calculating the amount of the test compound ΔQ (nmol), transported to the basolateral chamber after certain duration of the transport experiment, Δt (s), we applied the following formula: ΔQ at t_{x+1} = [amount determined at t_{x+1} (μM) x 1.5 mL] + [amount removed at t_x (μM) x 0.2 mL]. A (1.12 cm^2) is the insert surface area and Co is the determined actual exposure concentration (μM). The relative Papp values were calculated by dividing the Papp coefficient values of each test compound with the Papp coefficient value of antipyrine (reference compound). The recovery of each test compounds was also calculated by adding the amount (nmol) of the compound in the apical and basolateral compartment at t_{90}, adjusted with the actual exposure amount (nmol) in the apical compartment (0.5 mL), which was set at 100%.

In vivo data analysis

As previously described, we collected literature data regarding the *in vivo* developmental toxic profile of the twelve tested azoles [48]. In brief, we selected studies performed in rats, which were orally exposed to the tested compounds during either GD 6-15 or GD 7-16 at multiple dose regimes. Studies with at least one control group and two dose groups were selected to allow analysis using the Benchmark Dose (BMD) approach. The BMD values were calculated based on the evidence of sensitive endpoints of *in vivo* developmental toxicity. A concentration-response curve was fitted to the data to determine the BMD for the selected benchmark response (BMR) for each tested azole. The BMD was defined as 10% additional incidence of developmental toxicity (BMD_{10}) with BMD and PROAST software [49], using dichotomous concentration-response models (quantal data). Among the several models that were fitted, the selection of the best model was determined based on the goodness of fit (*p*-value>0.05). The *in vivo* prenatal developmental toxicity data for the three new azoles were provided by BASF. For the three novel compounds, given the available data, we proceeded with a qualitative *in vivo* potency ranking concept, which was adjusted and applied in our study, including also the known compounds. For implementing this

approach of *in vivo* analysis, the profiles of the tested compounds were characterized as potent, moderate and weak or non-potent.

Correlation tests

To determine the relationship between the different alternative assays (EST and WEC), with or without their combination with the BeWo transfer model, we plotted their calculated ID_{50} against ID_{10} values, respectively. The ID_{10} concentrations in the rat WEC were obtained from our previous study [48]. Then, we fitted a line based on minimizing the sum of residuals of the horizontal and vertical distances of the data to the line, to which fit is characterized by the coefficient of determination R^2, representing the fraction of variation. For evaluating whether any of the alternative *in vitro* assays could better predict the *in vivo* developmental toxic profile of the tested azoles, we plotted the *in vivo* BMD10 concentrations versus the calculated ID_{50} or ID_{10} in the case of the EST or WEC, respectively. Additionally, we corrected the calculated ID_{50} and ID_{10} values with the relative Papp values to evaluate whether their correlations with the *in vivo* BMD_{10} values may be improved.

Moreover, we investigated how the two *in vitro* systems (WEC and EST) could predict the expression of 4 genes of interest, which could be further associated with the induced developmental toxic profile in rat embryos due to exposure to the tested azoles. The expression of the selected genes were obtained from our previous transcriptomics analysis [48].

Results

Effect of azoles in inhibiting the differentiation of ES-D3 cells

All azoles induced a concentration-dependent inhibition of cardiomyocyte differentiation in the EST (Figure 2). Except for MCZ and B595, all the ID_{50} values were observed at concentrations much lower than those that caused cytotoxic effects on the cells, assuring that the effects did not occur due to cytotoxicity, but because of differentiation inhibition by the test compound. B599, KTZ and DFZ were the most potent azoles, showing ID_{50} values in a range of 7 to 14 µM. ID_{50}s of MCZ, FLU, TDF, B600 and B595 were between 20 and 37 µM. TEB, FEN and PRO were moderately strong embryotoxicants in the EST, revealing ID_{50}s ranging between 55 and 80 µM. PTZ was the weakest azole with an ID_{50} at 122 µM.

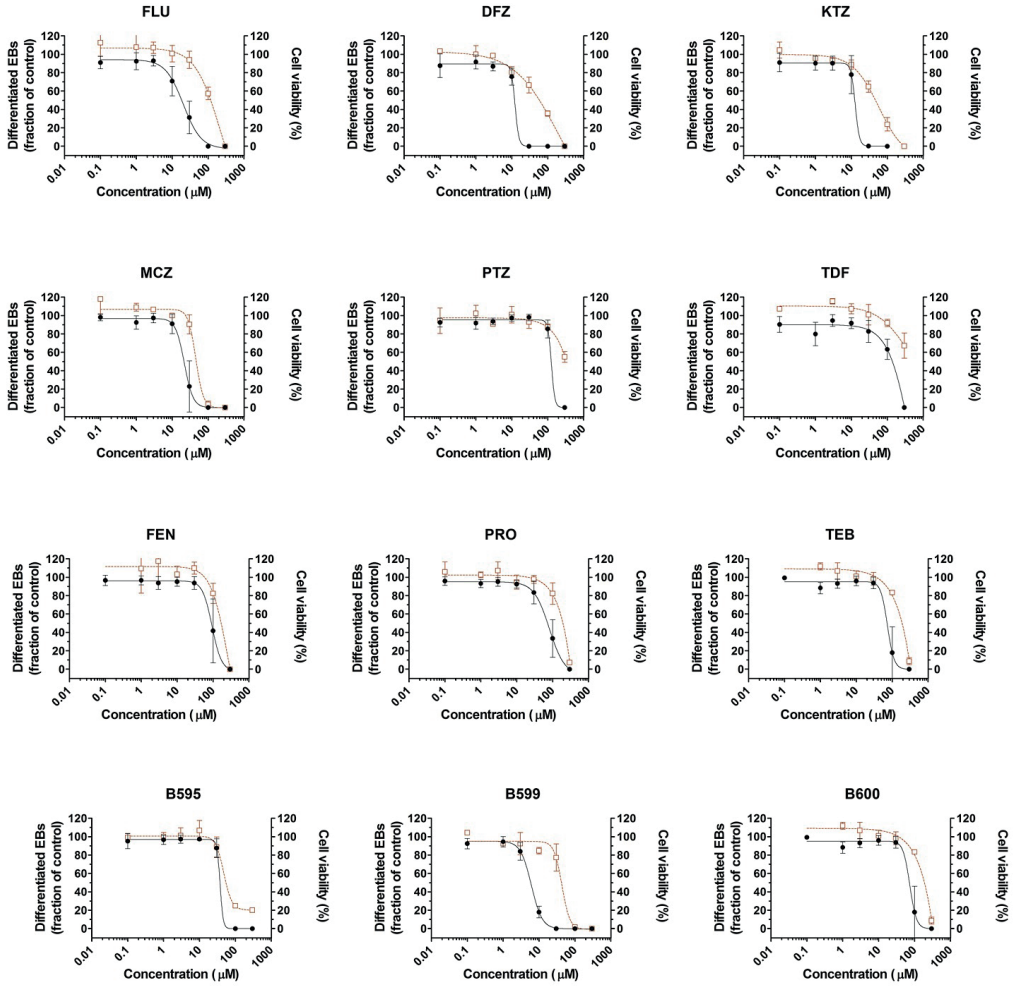


***Figure 2**: Concentration-dependent effects of the twelve azoles in the EST, illustrated with the black cycles (●) and black line, representing three independent experiments in technical duplicates. Concentration-dependent effects of the twelve azoles on viability of the ES-D3 cells, illustrated with the red open squares (□) and red dashed line, representing two independent experiments in six technical replicates.*

In vitro transport of compounds in the BeWo model

Table 1 shows the relative Papp values of twelve azoles as well as antipyrine (high permeability control) and amoxicillin (low permeability control) as used in the present study.

Before starting the transport experiments and at the end of them, the TEER values were measured for controlling the membrane integrity, showing no statistically significant change in the average of two biologically independent experiments (each performed in triplicate). The additional restriction of fluorescein transport (paracellular control) was considered as an extra parameter for reassuring the validity of the obtained results (data not shown).

The transport experiments were initiated by adding in the apical compartment 50 μM for FLU, DFZ, TDF and both controls, and 10 μM for MCZ due to its cytotoxicity at 20 and 50 μM (pilot experiment, data not shown).

Figure 3 illustrates the amount of tested compounds detected in the basolateral compartment at different sampling times in the transport experiment. The high transwell passage of antipyrine (positive control), and the limited transport of amoxicillin (negative control), showed the validity of the performed experiments. Up to 60 min, the concentration of the azoles to the basolateral compartment increased in a linear fashion with time and, therefore, the data obtained after 60 min of incubation were used for calculating the Papp coefficient and relative Papp values (Table 1).

MCZ did not show linear transfer to the basolateral compartment through the BeWo layer, which might be related to problems with the chemical analysis, so that hardly any transport to the basolateral compartment through the BeWo layer was detected (Figure 3), leading to unreliable Papp coefficient and relative Papp values (Table 1). We decided to exclude MCZ for further comparative analyses.

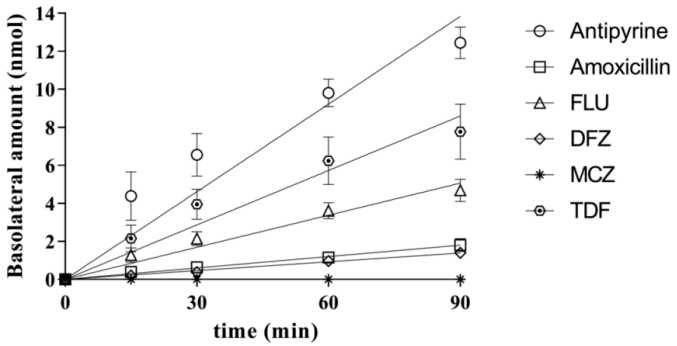


***Figure 3**: The transferred amount into the basolateral compartment over time for the four tested azoles in the BeWo model, in addition to the high (antipyrine) and low (amoxicillin) permeability controls. The initial concentration was 50 µM (25 nmol) for all the tested compounds including the controls, except for MCZ, which was tested at 10 µM (5 nmol). Data represent the mean ± SD of two biological replicates, each of which was performed in technical triplicate.*

***Table 1**: Papp coefficient and relative Papp values of twelve azoles (mean ± SD) and the permeability controls in the BeWo model. Four of the tested azoles (including the controls) were tested in the laboratories of BASF (this paper) and the other eight were obtained from Li et al., as indicated.*

compound	Papp coefficient (10^{-6} cm/s)[a]	Relative Papp value
FLU[b]	24.25 ± 3.3	0.53
DFZ[b]	9.46 ± 2.1	0.21
KTZ[c]	31 ± 2.4	0.79
MCZ[b]	0.87 ± 0.7	0.02
PTZ[c]	16 ± 2.7	0.40
TDF[b]	36.28 ± 8.1	0.79
FEN[c]	21 ± 3.2	0.55
PRO[c]	27 ± 3.8	0.70
TEB[c]	33 ± 4.3	0.86
B595[d]	3.0 ± 0.4	0.08
B599[d]	18.4 ± 1.1	0.47
B600[d]	7.9 ± 1.0	0.20
Amoxicillin[b]	6.75 ± 1.1	0.15
Antipyrine[b]	46.00 ± 3.9	1.00

a: Mean ± SD
b: Experimental data from this paper
c: Obtained from Li et al.[29]
d: Obtained from Li et al.[30]

Correlations of in vivo and in vitro developmental toxicity of azoles

The BMD_{10} value of each compound was calculated from *in vivo* prenatal developmental toxicity studies performed with each of the nine azoles (Table 2), considering abnormalities in skeleton, cleft palate formation, absence of renal papilla and hydronephrosis. For the three coded compounds provided by BASF SE, we concluded upon a qualitative potency

ranking, due to limitations on the available *in vivo* data, characterizing B599, B600 and B595 as a potent, moderate and weak azole, respectively.

Table 2: Overview of *in vivo* developmental toxicity data of nine azoles.

Compound	*In vivo* BMD_{10} (µmol/kg bw)
FLU	9.1 [50]
DFZ	596.5 [51]
KTZ	20.1 [52]
MCZ	258.3 [53]
PTZ	917.8 [54]
TDF	91.5 [55]
FEN	88.5 [56]
PRO	386.7 [57]
TEB	275.8 [58]

For deciding upon the developmental toxicity data derived from both the rat WEC and EST in combination with the BeWo model, we corrected their ID_{10} (WEC) [48] and ID_{50} concentrations (EST) by dividing them with their respective relative Papp values.

Figure 4A and 4B show the moderately strong correlations between the ID concentrations of twelve and eleven (excluding MCZ) azoles in WEC versus EST, with close to identical R^2 values of 0.67 and 0.66. As shown in figure 5 and 6, the potency rankings were similar showing that in both *in vitro* models B599, KTZ and FLU were the most potent, while B600, PTZ and B595 were the weakest azoles.

For correlating the *in vivo* BMD_{10} concentrations with the obtained *in vitro* WEC and EST ID values, we proceeded with eight compounds (MCZ was excluded as well as the three coded compounds, for which limited *in vivo* dose-response information was available). For these eight azoles, their *in vivo* BMD_{10} concentrations were better correlated with the ID_{10} concentrations in the rat WEC when they were corrected with the BeWo relative Papp values (R^2 = 0.87) compared to the non-corrected values (R^2 = 0.51) (Figure 5). Similarly in Figure 6, the corrected ID_{50} concentrations of the eight azoles tested in the EST were better

correlated with the *in vivo* BMD_{10}, resulting in an R^2 = 0.60, compared to the poorer correlation coefficient of 0.35 that the non-corrected ID_{50} showed.

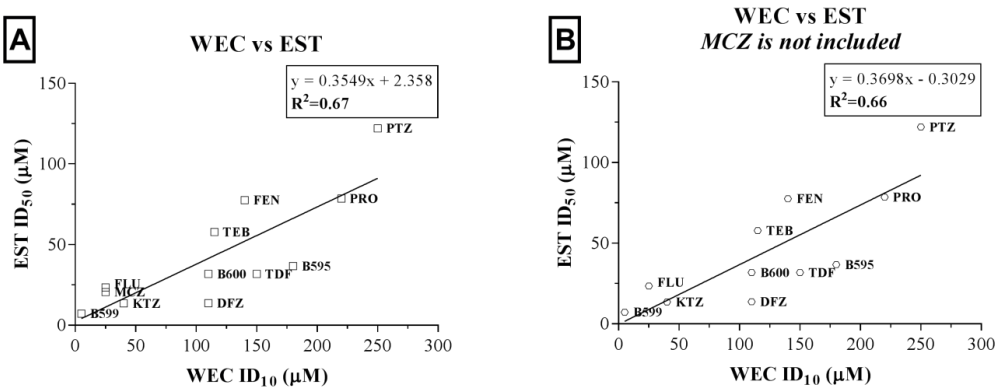


***Figure 4**: Correlation between the ID values of (A) twelve and (B) eleven azoles tested in the WEC and EST, respectively.*

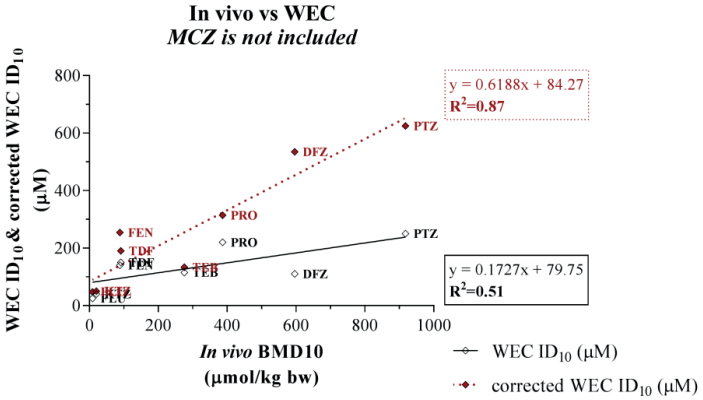


***Figure 5**: Correlation of the in vivo BMD_{10} (µmol/kg bw) with ID_{10} (µM, ◊) and ID_{10} as corrected with relative Papp values (µM, ♦), determined in the BeWo model, for eight azoles tested in the WEC, excluding MCZ and the three coded azoles obtained from BASF SE.*

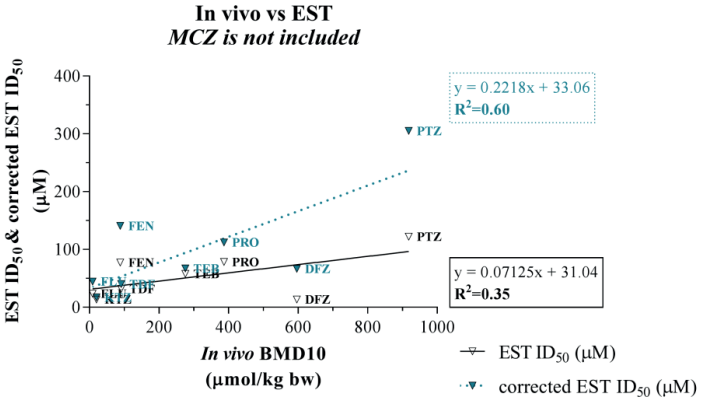


***Figure 6**: Correlation of the in vivo BMD_{10} (µmol/kg bw) with ID_{50} (µM, ▽) and ID_{50} as corrected with relative Papp values (µM, ▼), determined in the BeWo model, for eight azoles tested in the EST, excluding MCZ and the three coded azoles obtained from BASF SE.*

Quantitative comparison of gene expression changes in the EST

In a previous study in WEC [48], we showed that early embryotoxic responses to azoles were associated with the regulation of RA- regulating genes; *Cyp26a1* and *Dhrs3*. Furthermore, in the same study, we observed that the sterol-mediated biosynthesis pathway genes, *Cyp51* and *Msmo1*, also showed an extensive response to these azoles. Now, for comparison, we quantified the expression of the aforementioned four genes in the EST, exposed to the same azoles. As illustrated in Figure 7, the expression of *Cyp26a1* in EST exposed to any of the tested compounds at their ID_{50}s tended to be supressed, while the expression *Dhrs3* generally showed upregulation, which was significant after exposure to FLU, MCZ and B599 with a 4.3, 3.2 and 3.3 fold change, respectively. The fold change induction of *Cyp51* in the EST ranged from 1.5 to 2.5. Additionally, the expression of *Msmo1* showed similar changes as *Cyp51*, with exposures to FLU, MCZ, TEB and PRO, reaching fold changes of 2.4, 2.6, 3.0 and 2.5, respectively. Lower fold induction of both *Cyp51* and *Msmo1* expression was observed after exposure to the ID_{50} of PTZ, TDF and B595. Except for exposure to FEN and TEB, the upregulation of the RA related gene *Dhrs3* was higher compared to the sterol related genes, *Cyp51* and *Msmo1*.

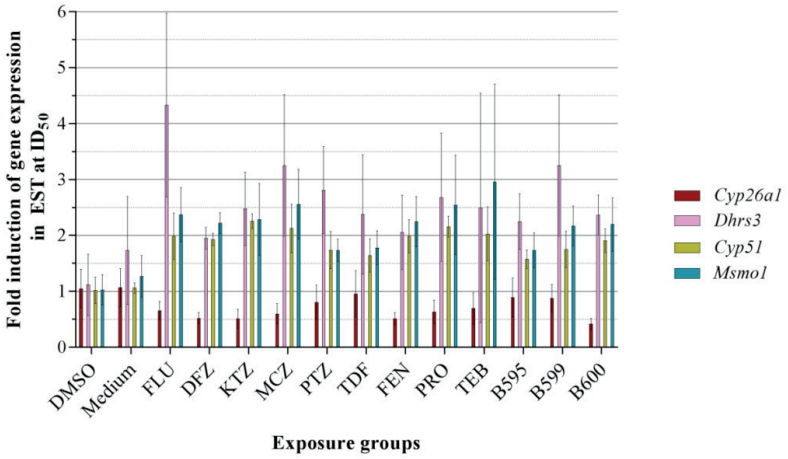


***Figure 7**: Fold induction of four genes, Cyp26a1, Dhrs3, Cyp51 and Msmo1 in the EST exposed to ID_{50} concentrations of each of the twelve azoles. Each bar represents a mean ± SD (N=8 for tested compounds and N=12 for solvent control and medium control).*

Discussion

In the present study, we assessed the concentration-dependent embryotoxicity of twelve azoles in the murine EST, by testing the inhibition of contracting cardiomyocyte differentiation (Figure 2). We performed a cross-model comparison, using results from our previous study [48], in which the same azoles were tested in the rat WEC. The potencies of the twelve azoles tested in the rat WEC versus the murine EST, based on the calculated ID_{10} and ID_{50} concentrations respectively, were reasonably well correlated (R^2 = 0.67). This was in agreement with the correlation that de Jong *et al*. have previously reported using six azoles, among which two were in common with those in our study, similarly tested in the WEC and EST [42]. Both these *in vitro* models correctly detected the strong embryotoxicants, B599, FLU, KTZ and MCZ, as well as the weakest azoles PRO and PTZ. Considering the *in vivo* potencies of the tested azoles, their correlations with the potency ranking in the WEC and EST assays led to moderate and rather weak relationships, represented with an R^2 = 0.51 and R^2 = 0.35, respectively. The better association of *in vivo* data with those derived from the WEC model may reflect the biological complexity of WEC compared to EST [3, 9]. The rat WEC contains intact embryos, which may respond to both external and internal signals in a more comprehensive manner [59-61], compared to stem cells, which is a less complex multicellular system [11] and, therefore, their responses to any stimuli are likely more specific and probably restricted. For example, while examining the embryotoxic effects in rat WEC exposed to a chemical, we assessed a large number of morphological endpoints, including growth and differentiation parameters. On the other hand, the chemical assessment in the EST is based on microscopical observation of contracting cardiomyocytes only, which led to qualitative conclusions that could be less detailed compared to WEC [3]. Moreover, the limited applicability domain of EST and the limitations on the time window of each of the *in vitro* tests, may explain the differences in *in vitro* potency ranking of azoles between the WEC and EST, as well as their deviation from their *in vivo* embryotoxic potency [9, 62].

The co-implementation of the BeWo model, for correcting the ID_{10} and ID_{50} concentrations in WEC and EST led to considerable improvements in the predictability of both *in vitro* models of *in vivo* developmental toxicity, showing R^2 of 0.87 and 0.60, respectively. Previous studies showed that the BeWo model is quite well associated with the *ex vivo* placental model and, subsequently, it is an adequate model for initial screening the placental transfer rates of chemicals [21, 25, 28]. The BeWo layer is a reductionistic model of the intact placenta, but it shows the importance of combining *in vitro* assays for designing batteries of alternative testing approaches [26, 27]. The estimated relative Papp values showed that, although azoles belong to the same chemical group, their placental transfer rates diverge. In

experiments in which the integrity of the BeWo layer was shown to be intact, increased relative Papp values characterized increased transport of the chemical. However, attention should be given to those compounds that showed a low Papp value, such as B595, because they may have accumulated into the BeWo cells and, therefore, lead to false negative conclusions [30]. The elevated intracellular accumulation of B595 could be also linked to increased placental toxicity, which may be related to the increased *in vivo* post-implantation losses [30]. Furthermore, in this study, we showed that MCZ did not pass through the BeWo layer to the basolateral compartment (Figure 3), but also even directly after its addition to the apical compartment it could not be detected (low recovery rate). MCZ might be bound to the polycarbonate membrane of the transwell plate or accumulated in the BeWo cells. Another explanation could be the metabolism of the parent compound, to MCZ-metabolites. Similarly to the human placenta, the BeWo cell layer has enzymatic activity, reflected by the expression of *Cyp* enzymes, *Cyp1a1*, *Cyp3a4* and *Cyp2c9* [63]. The last two enzymes are also responsible for drug metabolism in the liver [64]. Despite the fact that metabolism is a realistic aspect in *in vivo* and *in vitro* (when is applied) systems, the assumption of metabolism of MCZ in the BeWo cells might be unlikely because of short duration between exposure and sampling. Because of the poor ability of BeWo model to predict the placental transport rate of MCZ, we excluded MCZ from further analyses.

Here, we also observed that the estimated relative placental transfer rates were dependent on the molecular weight of the tested azoles, showing a very good correlation characterized with an R^2 of 0.70 (data not shown). KTZ was excluded from this association, because while it has the highest molecular weight (531.43 g/mol) of the selected tested azoles, its relative transport rate was estimated to 0.79, which was among the highest calculated values. In general, chemicals with low molecular weight are expected to pass easier through the *in vivo* placental barrier, while increased lipophilicity may additionally support their transport [25, 65]. Similarly to the *in vivo* placental transfer, the transport of xenobiotics in the human *ex vivo* perfusion placental system is dependent on physicochemical properties [25], which may also affect the transport of chemicals through the BeWo cells due to high agreement between these two systems [27]. Therefore, supplementary research on the physicochemical and molecular properties of the tested compounds could further elucidate the mechanism of BeWo model transfer and explain the observed behaviour of both MCZ and KTZ in the present study. Furthermore, the characterization of possible interactions between the compounds and membrane transporters could give additional information for the permeability of chemicals through the BeWo layer [29].

The RA concentration in mammals is regulated by *Dhrs3* and *Cyp26a1* expression, by synthesizing and metabolizing it, respectively, to maintain its balance for optimal embryonic

growth and differentiation [44, 45, 47, 66]. Therefore, regulation of RA-related gene expression may be linked to embryotoxic responses [46, 60]. In line with the functionality of these genes, in EST the strong embryotoxicants *in vitro*; FLU, MCZ and B599, upregulated *Dhrs3*. and downregulated *Cyp26a1*. In WEC, both these genes were upregulated. The rat WEC does have a more complex biological structure, organized as a whole network of more sophisticated responses, similar to *in vivo* embryos [60, 61]. Developmental effects are often localized to specific parts of embryos. Similarly, as illustrated in our previous study [44], the expression of *Cyp26a1* is relatively high in the brain region and in the tail end of the embryo. Subsequently, by homogenization for quantifying gene expression changes in whole embryos, the dilution of the observed effects is inevitable, which may mask region-specific effects in the embryo. Therefore, EST may be a more straightforward *in vitro* screening system for detecting explicit RA related effects on the gene regulation level, due to its biological simplicity.

In the present research, we also observed that the effects on the regulation of the selected sterol-related genes in the EST followed the same pattern of expression as in the rat WEC. However, as in a previous study [40], the level of induction was greater in the case of gene expression studies in the EST, which may indicate differences in sensitivity between the systems and, subsequently, quick gene-mediated responses to chemical exposure. Both the selected genes (*Cyp51* and *Msmo1*) are participating in the same step in the mammalian sterol biosynthesis pathway [37, 67]. However, the higher induction of *Msmo1* in screening sterol-mediated responses in both WEC and EST, supports our previous suggestion of replacing *Cyp51* by *Msmo1* for further compound testing as a major sterol-related biomarker for assessing relevant effects of chemicals in *in vitro* systems [48].

In conclusion, *Dhrs3* and *Msmo1* could be sensitive biomarkers for screening RA- and sterol- mediated responses, respectively, in both WEC and EST. Supportive results were observed in transcriptomics studies in the rat WEC, zebrafish embryo test (ZET) and EST after exposure to azoles, in which the sensitivity of these genes suggested that they could be valuable candidate-biomarkers for screening the mode of embryotoxicity of azoles [40-43].

In summary, we observed that the potency ranking of the *in vitro* developmental toxicity of the chemical class of azoles in the WEC and EST correlated quite well. Additionally, we showed that the prediction of *in vitro* developmental toxicity of azoles was notably improved when both WEC and EST were combined with the BeWo model, incorporating simple *in vitro* placental kinetics. Elucidating the mechanisms of action of azoles in the EST, we revealed that *Dhrs3* and *Msmo1* were expressed within a similar pattern compared to their expression in the rat WEC. Considering the relatively high response magnitude of *Dhrs3*, as well as the detection of *Cyp26a1* suppression in the EST, we suggested that EST might be an adequate

in vitro system to screen direct RA-mediated effects on the level of the genome after exposure to azoles. However, answering intricate questions regarding the complexity of toxicological responses in more complex biological systems, WEC may be more informative. Overall, defining universal gene-biomarkers among the available *in vitro* systems may facilitate a better cross-model comparison and improve the *in vitro* - *in vivo* extrapolations for better predicting developmental toxicants.

Acknowledgements

We thank Conny van Oostrom and Hennie Hodemaekers for their technical support.

Funding information

This work was supported by a collaborative project between Wageningen University (grant number 6153511466), RIVM and BASF SE.

References

1. Augustine-Rauch, K., C.X. Zhang, and J.M. Panzica-Kelly, *In vitro developmental toxicology assays: A review of the state of the science of rodent and zebrafish whole embryo culture and embryonic stem cell assays.* Birth Defects Res C Embryo Today, 2010. **90**(2): p. 87-98.
2. van der Jagt K., M.S., Tørsløv J. & de Bruijn J. , *Alternative approaches can reduce the use of test animals under REACH* EUROPEAN COMMISSION DIRECTORATE GENERAL JRC JOINT RESEARCH CENTRE Institute for Health and Consumer Protection, http://home.kpn.nl/reach/downloads/reducingtheuseoftestanimalsunderreachihcprepor.pdf, 2004.
3. Piersma, A.H., *Alternative methods for developmental toxicity testing.* Basic Clin Pharmacol Toxicol, 2006. **98**(5): p. 427-31.
4. Scialli, A.R., *The challenge of reproductive and developmental toxicology under REACH.* Regul Toxicol Pharmacol, 2008. **51**(2): p. 244-50.
5. Rovida, C. and T. Hartung, *Re-evaluation of animal numbers and costs for in vivo tests to accomplish REACH legislation requirements for chemicals - a report by the transatlantic think tank for toxicology (t(4)).* Altex, 2009. **26**(3): p. 187-208.
6. Scialli, A.R. and A.J. Guikema, *REACH and reproductive and developmental toxicology: still questions.* Syst Biol Reprod Med, 2012. **58**(1): p. 63-9.
7. Adler, S., et al., *Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010.* Arch Toxicol, 2011. **85**(5): p. 367-485.
8. Lee, H.Y., et al., *Alternative models in developmental toxicology.* Syst Biol Reprod Med, 2012. **58**(1): p. 10-22.
9. Sogorb, M.A., et al., *An integrated approach for detecting embryotoxicity and developmental toxicity of environmental contaminants using in vitro alternative methods.* Toxicol Lett, 2014. **230**(2): p. 356-67.
10. Spielmann, H., *The way forward in reproductive/developmental toxicity testing.* Altern Lab Anim, 2009. **37**(6): p. 641-56.
11. Spielmann, H., et al., *The embryonic stem cell test (EST), an in vitro embryotoxicity test using two permanent mouse cell lines: 3T3 fibroblasts and embryonic stem cells.* In Vitro Toxicology, 1997. **10**: p. 119-127.
12. Genschow, E., et al., *Validation of the embryonic stem cell test in the international ECVAM validation study on three in vitro embryotoxicity tests.* Altern Lab Anim, 2004. **32**(3): p. 209-44.
13. Doetschman, T.C., et al., *The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium.* J Embryol Exp Morphol, 1985. **87**: p. 27-45.
14. Webster, W.S., P.D. Brown-Woodman, and H.E. Ritchie, *A review of the contribution of whole embryo culture to the determination of hazard and risk in teratogenicity testing.* Int J Dev Biol, 1997. **41**(2): p. 329-35.
15. Ellis-Hutchings, R.G. and E.W. Carney, *Whole embryo culture: a "New" technique that enabled decades of mechanistic discoveries.* Birth Defects Research Part B: Developmental and Reproductive Toxicology, 2010. **89**(4): p. 304-312.
16. New, D.A.T., *WHOLE-EMBRYO CULTURE AND THE STUDY OF MAMMALIAN EMBRYOS DURING ORGANOGENESIS.* Biological Reviews, 1978. **53**(1): p. 81-122.
17. Brown, N.A. and S. Fabro, *Quantitation of rat embryonic development in vitro: a morphological scoring system.* Teratology, 1981. **24**(1): p. 65-78.
18. Piersma, A.H., et al., *Validation of the postimplantation rat whole-embryo culture test in the international ECVAM validation study on three in vitro embryotoxicity tests.* Altern Lab Anim, 2004. **32**(3): p. 275-307.
19. Blaauboer, B.J., *Biokinetic modeling and in vitro-in vivo extrapolations.* J Toxicol Environ Health B Crit Rev, 2010. **13**(2-4): p. 242-52.

20. Benirschke , K., P. Kaufmann, and R. Baergen, *Pathology of the Human Placenta.* Springer New York, 2006.
21. Prouillac, C. and S. Lecoeur, *The role of the placenta in fetal exposure to xenobiotics: importance of membrane transporters and human models for transfer studies.* Drug Metab Dispos, 2010. **38**(10): p. 1623-35.
22. Pacifici, G.M. and R. Nottoli, *Placental transfer of drugs administered to the mother.* Clin Pharmacokinet, 1995. **28**(3): p. 235-69.
23. Strikwold, M., et al., *Combining in vitro embryotoxicity data with physiologically based kinetic (PBK) modelling to define in vivo dose-response curves for developmental toxicity of phenol in rat and human.* Arch Toxicol, 2013. **87**(9): p. 1709-23.
24. Myllynen, P., M. Pasanen, and O. Pelkonen, *Human placenta: a human organ for developmental toxicology research and biomonitoring.* Placenta, 2005. **26**(5): p. 361-71.
25. Poulsen, M.S., et al., *Modeling placental transport: Correlation of in vitro BeWo cell permeability and ex vivo human placental perfusion.* Toxicology in Vitro, 2009. **23**(7): p. 1380-1386.
26. Bode, C.J., et al., *In Vitro Models for Studying Trophoblast Transcellular Transport.* Methods in molecular medicine, 2006. **122**: p. 225-239.
27. Mitra, P. and K.L. Audus, *In Vitro Models and Multidrug Resistance Mechanisms of the Placental Barrier*, in *Drug Absorption Studies: In Situ, In Vitro and In Silico Models*, C. Ehrhardt and K.-J. Kim, Editors. 2008, Springer US: Boston, MA. p. 368-396.
28. Li, H., et al., *Assessment of an in vitro transport model using BeWo b30 cells to predict placental transfer of compounds.* Arch Toxicol, 2013. **87**(9): p. 1661-9.
29. Li, H., et al., *Use of the ES-D3 cell differentiation assay, combined with the BeWo transport model, to predict relative in vivo developmental toxicity of antifungal compounds.* Toxicology in Vitro, 2015. **29**(2): p. 320-328.
30. Li, H., et al., *Extended evaluation on the ES-D3 cell differentiation assay combined with the BeWo transport model, to predict relative developmental toxicity of triazole compounds.* Archives of Toxicology, 2016. **90**(5): p. 1225-1237.
31. Robinson, J.F., J.L. Pennings, and A.H. Piersma, *A review of toxicogenomic approaches in developmental toxicology.* Methods Mol Biol, 2012. **889**: p. 347-71.
32. Robinson, J.F. and A.H. Piersma, *Toxicogenomic approaches in developmental toxicology testing.* Methods Mol Biol, 2013. **947**: p. 451-73.
33. Daston, G.P. and J.M. Naciff, *Predicting developmental toxicity through toxicogenomics.* Birth Defects Res C Embryo Today, 2010. **90**(2): p. 110-7.
34. Adeleye, Y., et al., *Implementing Toxicity Testing in the 21st Century (TT21C): Making safety decisions using toxicity pathways, and progress in a prototype risk assessment.* Toxicology, 2015. **332**: p. 102-11.
35. Krewski, D., et al., *Toxicity testing in the 21st century: a vision and a strategy.* J Toxicol Environ Health B Crit Rev, 2010. **13**(2-4): p. 51-138.
36. Piersma, A.H., et al., *Evaluation of an alternative in vitro test battery for detecting reproductive toxicants.* Reprod Toxicol, 2013. **38**: p. 53-64.
37. Marotta, F. and G.M. Tiboni, *Molecular aspects of azoles-induced teratogenesis.* Expert Opin Drug Metab Toxicol, 2010. **6**(4): p. 461-82.
38. Georgopapadakou, N.H., *Antifungals: mechanism of action and resistance, established and novel drugs.* Curr Opin Microbiol, 1998. **1**(5): p. 547-57.
39. Dimopoulou, M., et al., *Embryotoxic and pharmacologic potency ranking of six azoles in the rat whole embryo culture by morphological and transcriptomic analysis.* Toxicol Appl Pharmacol, 2017. **322**: p. 15-26.
40. Robinson, J.F., et al., *A comparison of gene expression responses in rat whole embryo culture and in vivo: time-dependent retinoic acid-induced teratogenic response.* Toxicol Sci, 2012. **126**(1): p. 242-54.
41. Hermsen, S.A., et al., *Triazole-induced gene expression changes in the zebrafish embryo.* Reprod Toxicol, 2012. **34**(2): p. 216-24.

42. de Jong, E., et al., *Comparison of the mouse Embryonic Stem cell Test, the rat Whole Embryo Culture and the Zebrafish Embryotoxicity Test as alternative methods for developmental toxicity testing of six 1,2,4-triazoles.* Toxicol Appl Pharmacol, 2011. **253**(2): p. 103-11.

43. van Dartel, D.A.M., et al., *Concentration-dependent gene expression responses to flusilazole in embryonic stem cell differentiation cultures.* Toxicology and Applied Pharmacology, 2011. **251**(2): p. 110-118.

44. Dimopoulou, M., et al., *Flusilazole induces spatio-temporal expression patterns of retinoic acid-, differentiation- and sterol biosynthesis-related genes in the rat Whole Embryo Culture.* Reprod Toxicol, 2016. **64**: p. 77-85.

45. Piersma, A.H., E.V. Hessel, and Y.C. Staal, *Retinoic acid in developmental toxicology: Teratogen, morphogen and biomarker.* Reprod Toxicol, 2017. **72**: p. 53-61.

46. Luijten, M., et al., *Transcriptomics analysis of retinoic acid embryotoxicity in rat postimplantation whole embryo culture.* Reprod Toxicol, 2010. **30**(2): p. 333-40.

47. Tonk, E.C., J.L. Pennings, and A.H. Piersma, *An adverse outcome pathway framework for neural tube and axial defects mediated by modulation of retinoic acid homeostasis.* Reprod Toxicol, 2015. **55**: p. 104-13.

48. Dimopoulou, M., et al., *A transcriptomic approach for evaluating the relative potency and mechanism of action of azoles in the rat Whole Embryo Culture.* Toxicology, 2017.

49. Slob, W., *Dose-response modeling of continuous endpoints.* Toxicological Science, 2002. **66**(2): p. 298-312.

50. Lamontia CL, S.R., Alvarez L., *Embryo-fetal toxicity and teratogenicity study of INH-6573-39 by gavage in the rat.* Unpublished reports No. HLR 444-83 and HLR 142-84 from Haskell Laboratory for Toxicology and Industrial Medicine, Wilmington, Delaware, USA. Submitted to WHO by E.I. du Pont de Nemours & Co., Inc., Wilmington, Delaware, USA. Summarized in JMPR Monograph—Flusilazole, Pesticide residues in food: 1995 evaluations Part II Toxicological & EnvironmentEnvironment. http://www.inchem.org/documents/jmpr/jmpmono/v95pr08.htm, 1984.

51. Lochry, E.A., *Developmental toxicity study of CGA-169374 technical (FL-851406) administreted orally via gavage to Crl:COBS CD (SD) BR presumed pregnant rats.* Unpublished report No. 203-005 from Novartis Crop Protection AG., Basel, Switzerland and Argus Research Lab. Inc., Horsham, USA, Submitted to WHO by SYgenta Crop Protection AG, Basel, Switzerland. http://apps.who.int/iris/bitstream/10665/44064/1/9789241665230_eng.pdf?ua=1, 1987.

52. Tachibana, M.N., Y. and Monro, A.M., *Toxicology of fluconazole in experimental animals.* In Fromtling, R.A. Recent trends in the discovery, development, and evaluation of antifungal agents, JR Prous, Barcelona, 1987: p. 93-102.

53. Ito, C., Y. Shibutanil, K. Inoue, K. Nakano and H. Ohnishi, *Toxicologocal Studies of Miconazole (II) Teratological Studies of Miconazole in Rats* IYAKUHIN KENKYU, 1976. **7**(3): p. 367-376.

54. Stahl, B., *JAU 6476 - Developmental toxicity study in rats after oral administration. Unpublished report No. M-012279-01-1 from Bayer AG, Wuppertal, Germany. Submitted to WHO by Bayer CropScience AG, Germany.* *https://www.google.nl/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&ved=0ahUKEwjcjNCx5-HPAhWDrxoKHebSDzYQFgghMAE&url=http%3A%2F%2Fapps.who.int%2Fpesticide-residues-jmpr-database%2FDocument%2F110&usg=AFQjCNGpjQOE2xfb0QbWPWwA5-avbrC8kQ&sig2=ZFM7G3fgK4EdIA0e_zX0hQ&cad=rja.* 1997.

55. Unger, T.M., Van Goethem, D. & Shellenberger, *A teratological evaluation of Bayleton in mated female rats.* Midwest Research Institute, Report No. 324 to Mobay Chemical Corporation Agricultural Chemical Division submitted to WHO by Bayer AG. (Unpublished),

http://www.inchem.org/documents/jmpr/jmpmono/v83pr39.htm, 1982.

56. Hoffman, D.G., Owen, N.V. & Adams, E.R., *A teratology study with EL-222 in the rat.* Unpublished report No. R06279 form Lilly research Laboratories, USA. Submitted to WHO by DowElanco Europe, Wantage, Oxon, United Kingdom, 1980.

57. Giknis, M.L.A., *A teratology (segment II) study in rats.* Unpublished report no.: 86004 from Ciba-Geigy Pharmaceutical division, New Jersey. Submitted to WHO by Ciba-Geigy Ltd., Basle, Switzerland 1987.

58. Becker, H., Vogel, W. & Terrier, C., *Embryotoxicity study (including teratogenicity) with HWG 1608 technical in rat*, in *Unpublished report Ref. No. R 4451, prepared by Research & Consulting Co. AG (RCC), Itingen, Switzerland. Submitted to WHO by Bayern AG, Leverkusen, Germany*. 1988.

59. Robinson, J.F., et al., *Embryotoxicant-specific transcriptomic responses in rat postimplantation whole-embryo culture.* Toxicol Sci, 2010. **118**(2): p. 675-85.

60. Robinson, J.F., A. Verhoef, and A.H. Piersma, *Transcriptomic analysis of neurulation and early organogenesis in rat embryos: an in vivo and ex vivo comparison.* Toxicol Sci, 2012. **126**(1): p. 255-66.

61. Fang, H., et al., *Transcriptome analysis of early organogenesis in human embryos.* Dev Cell, 2010. **19**(1): p. 174-84.

62. Piersma, A.H., et al., *A critical appraisal of the process of regulatory implementation of novel in vivo and in vitro methods for chemical hazard and risk assessment.* Crit Rev Toxicol, 2014. **44**(10): p. 876-94.

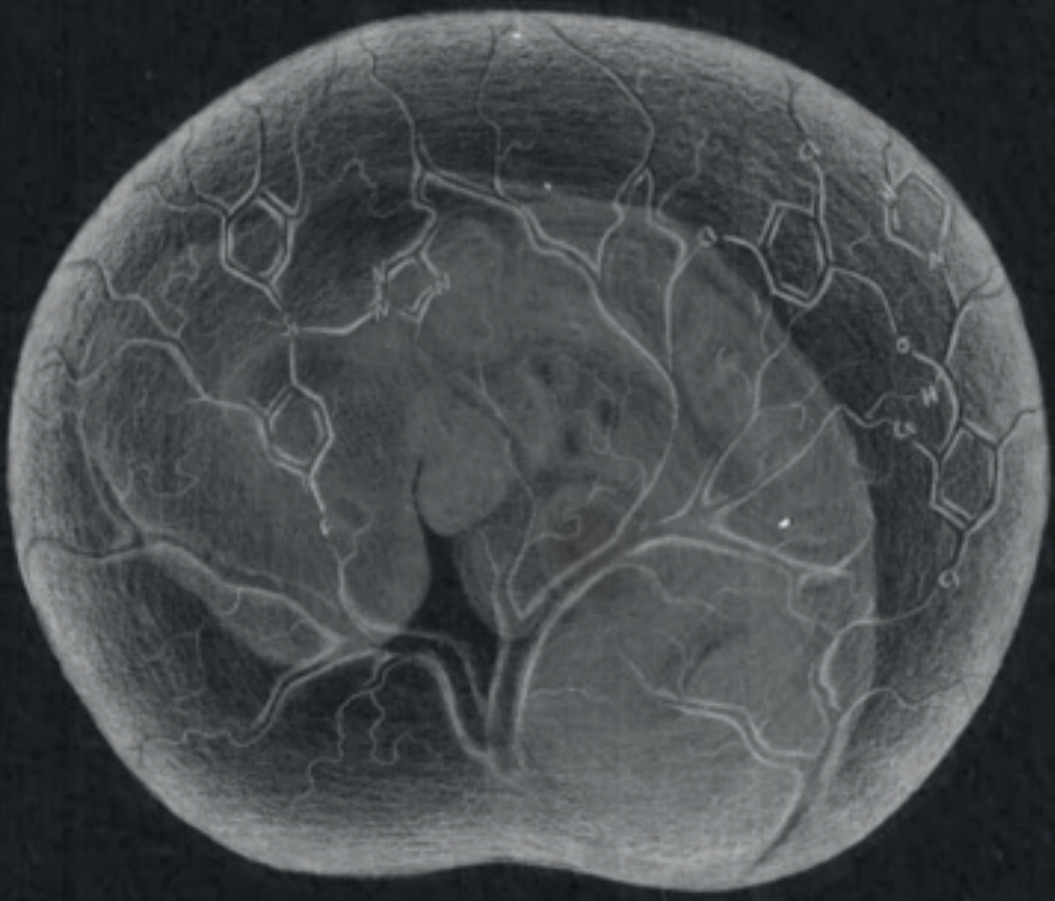
63. Myllynen, P. and K. Vähäkangas, *Placental transfer and metabolism: An overview of the experimental models utilizing human placental tissue.* Toxicology in Vitro, 2013. **27**(1): p. 507-512.

64. Dvorak, Z., *Drug-drug interactions by azole antifungals: Beyond a dogma of CYP3A4 enzyme activity inhibition.* Toxicol Lett, 2011. **202**(2): p. 129-32.

65. Sastry, B.V.R., *Techniques to study human placental transport.* Advanced Drug Delivery Reviews, 1999. **38**(1): p. 17-39.

66. Metzler, M.A. and L.L. Sandell, *Enzymatic Metabolism of Vitamin A in Developing Vertebrate Embryos.* Nutrients, 2016. **8**(12): p. 812.

67. Santori, F.R., et al., *Identification of natural RORgamma ligands that regulate the development of lymphoid cells.* Cell Metab, 2015. **21**(2): p. 286-97.



Chapter 6

General discussion and future perspectives

❖ General discussion

Alternatives for animal testing

In **chapters 2, 3, 4 and 5** of this thesis, emphasis is given on implementing alternative approaches for testing the developmental toxicity of pharmaceutical and chemical substances for reducing the number of experimental animals [1, 2], while targeting to mimic the *in vivo* situation and, therefore, to improve the predictions for humans. In **chapter 5**, we showed that the rat WEC and murine EST sufficiently correlated with R^2=0.67 in predicting the embryotoxic responses of the twelve azoles. Beside the good correlation of predictions in the *in vitro* assays, there was also a high agreement between the *in vivo* and *in vitro* data (**Chapter 4 and 5**), either obtained from the rat WEC (R^2=0.87) or murine EST (R^2=0.60), when the correlated data were combined with the relative placental transport rates of azoles. Moreover, increased accuracy was detected in both *in vitro* assays corrected with BeWo model data concerning their predictability of *in vivo* potent and weak or non-potent azoles. This confirms the importance of applying a battery of complementary *in vitro* assays for succeeding in mimicking the complexity of the *in vivo* situation and, subsequently, improving the *in vivo* to *in vitro* correlation [3-6]. On the other hand, interspecies differences in both toxicokinetics as well as toxicodynamics should always be taken into consideration during the risk assessment of chemicals in order to avoid future tragedies, such as the thalidomide incidence back in 1960s [4]. The available animal-based models predict the human developmental toxicity with 60-80% success, demonstrating a partly successful approach for screening and categorizing developmental toxicants. This illustrates the potential of alternatives to animal approaches for predicting developmental toxicity of chemicals in a faster and less costly way, highlighting priorities of further testing for eliminating the risk for humans and the environment [6].

The implementation of embryonic stem cell tests in developmental toxicology is currently possibly the simplest non-animal assay for screening embryotoxicants, with reduced time and cost providing experimental advantages [7-9]. In **chapter 5**, for studying the developmental specific effects of azoles in inhibiting the differentiation phase of ESCs, we selected to expose the system to the tested compounds from day 3 onwards [10-12]. The selection of the appropriate time window is a crucial factor for elucidating the functional processes of the ECs, as well as for contributing to the prediction models of developmental toxicology (for example based on *in vivo* and *in vitro* correlations described in **chapter 5**). The time window of the 10-day protocol of the EST includes both the proliferation and differentiation phases [6, 8]. The starting point of the EST mimics stem cells in a 3.5-day old embryo (mouse). On day 3, the EBs demonstrate the egg-cylinder stage of a 5-day old embryo, including the three germ layers of meso-, ecto- and endoderm, from which other

types of cells and organs are further structured and organized. It is emphasized that until day 3 of the EST differentiation protocol, the proliferation phase dominates, while from day 3 onwards the differentiation gradually increases [13]. However, restrictions regarding the applicability domain of the EST and the sensitivity of the time window, at which the test is performed, could have a significant effect on the read-out of the murine EST itself [2, 3]. Despite the careful selection of the exposure time window in **chapter 5**, the morphological assessment of differentiated ESCs into beating cardiomyocytes is the only microscopically qualitative way for concluding on the developmental toxicity of compounds in the EST, which could also lead to subjective classifications [4]. Knowing that effects on the level of genome precede the manifestations of toxicity, by studying the regulation of gene expression in a biologically simple system, such as the EST, we may conclude upon the underlying mechanism of toxicity.

As presented in **chapters 2, 3 and 4**, the application of more complicated *in vitro* systems, such as the rat WEC, may eliminate some of the restrictions of EST, which follow from its simplicity (referring to its applicability domain and sensitivity of time window), and may enhance possible descriptions of morphogenetic relationships in the cellular or organ networks that normally exist in a whole organism [3, 4, 6, 14, 15]. The quantitative assessment (TMS system) of rat embryos on GD 12 may better predict the effects of chemicals during this developmental time window, as it is closer to the *in vivo* situation [16-18], while the applicability domain of this developmental toxicity test is increased compared to the strictly defined domain for the cardiac EST [6]. Another advantage of the rat WEC is the constant monitoring and evaluation of the 48-hour developmental phase, which is comparable to a large extent with both the rat and human *in vivo* situation and it gives information not only for the success of the culture itself, but also for early embryotoxicity of the tested compounds [14, 19]. Despite the advantages or disadvantages of the rat WEC and murine EST, which can to some extent be eliminated when the assays are combined, the greatest restrictions of these alternatives are the absence of metabolism and the lack of the placenta (including the maternal side). The importance of the presence of the placenta is partly illustrated in **chapter 5**, showing a notable improvement in detecting possible embryotoxicants by additionally implementing the *in vitro* BeWo placental model combining it with the alternative developmental toxicity tests [4, 15, 20].

Limitations and improvements of the in vitro methodologies

The metabolism

In **chapter 4 and 5** of this thesis, we examined the developmental toxicity profile of twelve azoles in the rat WEC and EST. In either the *in vivo* or *in vitro* developmental toxicity studies,

the profiles of parent compounds are assessed concerning their possible embryotoxicity. Absorption, distribution, metabolism and excretion (ADME) information on chemicals, for which the toxic profile is evaluated, is highly important for concluding on the targeted effects or for understanding any possible adverse effects in tissues or whole organs. It is known that the induced developmental toxicity is not always due to the parent compound, since it could be also caused by the formed metabolites [20, 21]. On the other hand, the teratogenicity of some chemicals could be decreased due to the metabolism of the parent compound to less toxic products. In the *in vivo* systems, the embryonic enzymatic activity is almost absent. However, possible metabolites of parent compounds may end up in embryos either via placental transfer of maternally metabolized compounds or via metabolism of the compounds in the placenta itself.

Azoles are chemicals that are absorbed and rapidly metabolized in excretable products, which means that they will not bioaccumulate in the biological system. It has been suggested that in fish the azole metabolites are two orders of magnitude less toxic than the azole-parent compounds [22]. In mammals, azoles are strong inhibitors of several Cyp450 enzymes, especially of the drug related phase I metabolic enzymes *Cyp2c9*, *Cyp2c19* and *Cyp3a4* and the steroidogenesis enzymes *Cyp17*, *Cyp19* and *Cyp51* [23, 24]. Azole-related inhibition of Cyp450 enzymes, which disturbs the metabolism of other drugs, may lead to toxic effects, which are associated with the accumulation (overdosing) of the non-azole drugs, but not because of azoles themselves [23]. Furthermore, the azole-mediated modulation of phase III transporters, such as the multidrug transporter P-glycoprotein *Abcb1*, which is active during liver regeneration and hepatocarcinogenesis, may increase the azole-liver uptake and, therefore, affect the sterol metabolism, causing increased (maternal) toxicity [24, 25]. Additionally, it has been also found that in invertebrates the increased toxicity of azoles is probably because of the appearance of toxic metabolites and alterations in the phase II metabolic enzyme glutathione-S-transferase [22]. Overall, it can be concluded that the developmental toxicity of azoles is induced by the parent compound and is not the result of metabolism. However, attention should be given to some azoles, such as triadimefon, for which a metabolite (triadimenol) is recognised and registered as an individual azole, as well as prothioconazole, which is a non-embryotoxicant (NOAEL= 80 mg/kg bw/day), while its metabolite desthio-prothioconazole is quite potent (NOAEL=1 mg/kg bw/day) [26, 27].

For avoiding both false negative and false positive characterization of the tested compounds in *in vitro* models, metabolism can be introduced by adding extra *in vitro* systems, rich in enzymatic activity, such as microsomes, postmitochondrial supernatants and hepatocytes, to the *in vitro* cultures [28-30]. For example, some proteratogenic compounds, such as alpha-

cyclophosphamide, were correctly classified when tested in *in vitro* systems including metabolic activity, otherwise their classification was false negative [21, 29, 31, 32]. However, some previous attempts showed that when adding metabolic activity to the *in vitro* systems for developmental toxicity, undesirable results were observed [29]. First, some metabolic systems alone were too cytotoxic for the cultured system. Another issue when the enzymatic activity is added for correlating the *in vivo* with the *in vivo* results is that the formed metabolites are not the same among the species (human versus rat) while their concentrations vary dependent on the applied species or on the embryonic stage [20, 33]. As a practical solution, it has been suggested that the separate assessment of the parent compound and its known metabolites in the same embryonic system (dependent on the species) could provide the information needed for building the underlying pharmacokinetics [28, 29].

The placental transfer model

The co-implementation of placental transfer studies in the alternative *in vitro* assays could facilitate a prediction closer to the *in vivo* situation, which might be considered as a more realistic approach for studying developmental toxicities than *in vitro* alternatives alone [34, 35]. In **chapter 5**, the BeWo model has been combined with the rat WEC and the murine EST for re-calculating the effective concentrations of the tested azoles in these *in vitro* systems, as well as for estimating whether these compounds could be cytotoxic for the placenta itself. The correlations of the *in vitro* with the *in vivo* potency ranking were notably improved in **chapter 5** compared to the data in **chapter 3 and 4**, showing the importance of applying combined *in vitro* approaches in developmental toxicity testing. The pregnancy is a complex situation and the placenta is the coordinator of maternal and fetal communication [36-40]. Therefore, either the impact of chemicals on the placenta or the placental transfer of chemicals from the maternal to the embryonic side could lead to undesirable developmental outcomes [41, 42].

As illustrated in **chapter 5** and previous studies, the BeWo model is capable to predict the relative placental transfer rates of azoles and other compounds [43-45]. Despite the fact that azoles belong to the same chemical class, placental enzymatic and protein binding activity, physicochemical and molecular properties of compounds affect the ability of the compounds to cross the placenta [46-48]. The BeWo transfer model is a simplified method for mimicking the placental barrier [49-51], with which it is possible to roughly estimate the relative transport rates of chemicals [41]. The good agreement between the transport rates calculated with the BeWo assay and the placental perfusion system [45, 50], gives us confidence in interpreting the obtained results. On the other hand, it should be noted that the

origin of the cell line (malignant gestational choriocarcinoma of human male fetal placenta) and the stage of embryonic development (last stage of pregnancy) could be limitations of the system, which may lead to results irrelevant to the *in vivo* situation. Additionally, considering that the characteristics of the placenta are continuously altered during pregnancy and this may affect the overall capability of compounds to cause developmental effects, the development of a placental model that mimics the early and critical stages of pregnancy could increase the knowledge upon the effect of compounds in each relevant period of embryonic development. Therefore, the correction of effective concentrations calculated from the WEC and EST (**Chapter 5**) with more relevant kinetics of the early placenta could be more informative. Moreover, further assessment on how placental enzymatic activity and protein binding (albumin) could affect the transfer rates of compounds may give an additive value to this *in vitro* system [45]. BeWo cells do have enzymatic activity, represented by *Cyp1a1*, *Cyp3a4* and *Cyp2c9* enzymes, which may be capable of metabolizing the parent compound, when added in the apical compartment of the transwell plates [46, 48]. As presented in **Chapter 5**, taking MCZ as an example of a compound with difficult characteristics in the BeWo assay, we showed that the calculation of its transfer rate (Papp value) was not possible. This may be related to several potential confounding factors: MCZ may accumulate in the BeWo cells, such as in the case of B595, or be absorbed to the polycarbonate membrane or the transwell plate itself. Another scenario could be that because of MCZ's chemical instability and/or the increased enzymatic activity of the BeWo cells, MCZ might be converted to other products or metabolites that either cannot be detected or are unknown so far, but they might induce cytotoxicity in BeWo cells at concentrations higher that 10 µM. In clinical human studies, MCZ has been found to interact with many CYPs enzymes, among which CYP1A2, CYP3A4 and CYP2C9 [52], which are also present in the BeWo cell line [41]. Therefore, metabolism or transformation of MCZ to another potent product may explain its behaviour in the placental transfer assay in **Chapter 5**.

Molecular endpoints in developmental toxicity

The implementation of technologically advanced high throughput assays, varying from targeted single gene assays to whole genome screening, to detect gene expression changes as responses to embryotoxicants gives new dynamics in developmental toxicity testing [4, 19, 53, 54]. In **chapter 2** and in other studies, it has been shown that quantification and localization of FLU-mediated gene expression changes, related to toxic responses, are not only associated with morphological effects, but also precede them, revealing their value as a tool with increased sensitivity and predictability [55, 56]. Following the same rationale in

chapter 3, 4 and 5, the application of toxicogenomics in both rat WEC and murine EST aimed to better understand the mechanism by which azoles affect the biological systems inducing developmental toxicity. Therefore, toxicogenomics provides information for characterizing responses related to chemicals, for better classifying them based on the underlying mode of toxicological action and for linking toxic responses across different models and biological systems providing also insight in possible interspecies differences [54].

Characterization of transcriptional responses

The adverse outcome in embryonic development is dependent on the time and duration of exposure during pregnancy [57], as it has been also confirmed in **chapter 2 and 3**. Similarly, the homeostatic gene expression responses are highly dependent on the timing of exposure and the applied dose in both *in vivo* and *in vitro* systems [55, 58]. Effects of exposure of rat WEC to the same concentration of FLU among different developmental time windows were presented in **Chapter 2**, showing that the expression of genes during the earliest developmental window of 0-4 hours (GD 10) can predict effects on the same genes in subsequent developmental stages during the 2-day culture period, preceding the adverse developmental outcome. **Chapter 3** illustrates confirmatory information, indicating that the expression of developmental related genes fluctuates while the sterol related genes respond consistently more pronounced after longer exposure.

Studying the changes in expression of genes in both *in vivo* and *in vitro* (WEC) rat embryos during the time window of early embryogenesis (GD 10-12), it was shown that the absolute magnitude of expression of genes related to developmental processes was increased over the time [19]. The observed regulation of biological processes, including neural tube formation, early organogenesis of the central nervous system (CNS), ear, eye, limbs and heart, in *in vivo* and *in vitro* rat embryos was also correlated with the situation in human embryos, showing the same directionality and magnitude of gene expression changes corresponding to the developmental time window [19, 59]. Hartl *et al*. [60] and Mitiku and Baker [61] determined that murine transcripts and proteins associated with cell differentiation and early organ development are higher expressed in embryos during GD 8-8.5, which equals GD 10 in rat embryos. For example, murine embryonic exposure to methylmercury (MeHg) showed a time-dependent regulation of developmental related processes during early neurulation [62, 63]. Therefore, the transcriptional responses of biological systems demonstrate a time-dependent fingerprinting reflecting the dynamics of the progressive developmental processes. *In vivo* and *in vitro* exposure of rat embryos to RA (which is known as a common teratogen), illustrates the comparable time-dependent gene expression

changes during neurulation and early organogenesis, showing a peak in the number of regulated genes at the 4- and 6- hour exposure time point in *in vitro* and *in vivo* embryos, respectively [64]. Genes related to the anteroposterior patterning and RA metabolism were more significantly regulated in both systems in the same direction and with a comparable magnitude of the effect [64]

Additionally, in **chapter 3**, with increasing the concentrations of FLU, the number of regulated genes is also expanded. The dose of embryonic exposure to embryotoxicants is a factor that determines the number of regulated genes associated with the observed developmental effects. In developmental toxicology, taking advantage of the increased sensitivity of toxicogenomics, the assessment of transcriptome changes, at lower concentrations than those that cause developmental effects, is used to explore the characterization of chemicals in *in vivo* rodent systems or *in vitro* assays including the rat WEC [54], EST [65] and ZET [66]. For example, increasing the dose of cadmium in studies with *in vivo* exposed murine embryos, the number of regulated genes was monotonically increased. However, the pattern of gene expression was changed, showing different functional associations and revealing the most sensitively regulated pathways for the underlying mechanism of toxicological mode of action. Furthermore, we need to consider that the applied dose for studying the predictability of toxicogenomics influences the sensitivity of the method. Additionally, the excessive perturbations in the level of gene transcription may disturb the biological homeostasis and lead to adverse effects [67]. Overall, for concluding whether gene expression changes, occurring after embryonic exposure to xenobiotics, could lead or be linked to adverse effects, knowledge on the time- and dose- dependent functionality and sensitivity of gene expression is valuable.

Classification of transcriptional responses

Toxicogenomics has been reported to enhance the discrimination of chemical classes providing a better understanding of the molecular mode of action of embryotoxicants [53, 68]. Previous studies have indicated that compounds derived from different chemical classes could reveal the same adverse morphological effects in biological systems, but the different gene expression changes could nominate different underlying mechanisms of induced toxicity. Another study in the rat WEC showed that the morphological assessment of exposed embryos to four different chemicals (caffeine, MeHg, monobutyl phthalate and monoacetic acid) was not adequate for concluding upon the developmental toxicity of these compounds alone [69]. However, additional gene expression screening predicted the specificity of each compound in regulating the underlying mechanisms of toxicity [69]. Similar results were observed in a transcriptomic study with mouse embryos, which were exposed *in*

utero to arsenic and cadmium while undergoing neurulation [70]. Both these metals are neurotoxic and cause neural tube defects, while the unique embryotoxic responses on the transcriptome level to each of them were shown to be associated with specific functional groups of genes, as well as to the shared mechanisms of toxicity [70].

Furthermore, chemicals within the same class may share common chemical structural characteristics, physicochemical properties and similar modes of toxicological action [71]. Consequently, toxicogenomics approaches could classify compounds and distinguish toxicants and non-toxicants, as shown in **chapters 3, 4 and 5**. Identification of specific sets of genes, which are sensitive in predicting different transcriptional responses due to differences in the class or potency of chemicals, and their application as "differentiation track" improved the predictability of toxicogenomics studies [65, 72, 73]. The set of these genes, the so-called gene biomarkers, can reveal the regulation of pathways that are related to the induced developmental toxicity of chemicals (**Chapter 2 and 3**) and facilitate across-model comparisons (**Chapter 5**). Additionally, the regulation of biomarkers may be in agreement with the *in vivo* and *in vitro* potency ranking of chemicals, showing also similar effects on the regulation of the identified pathways of toxicity (mode of action) when evaluated at the same level of effective concentration (**Chapters 4 and 5**). For example, effects of triazole analogues on comparisons in the ZET, EST and rat WEC [74-76] demonstrated similar results regarding the sensitivity of the regulated gene-biomarkers related to the observed developmental toxic effects, which assisted a valuable cross-model comparison, while presenting results in high agreement with the *in vivo* situation [77].

In **chapter 3 and 4**, we showed that one of the tested azoles, MCZ, was in the same range of embryotoxic potency (ID_{10}) as the rest of the selected compounds in the WEC. Despite its potent *in vitro* and moderate *in vivo* profile, MCZ did not cause any statistically significant change on the level of WEC transcriptome. However, as analysed in **chapter 5**, MCZ caused an excessive effect on the developmental related gene (*Dhrs3*) under assessment in the EST, comparable to the effect of the most potent embryotoxicants; FLU and B599. Thus, the applicability domain of the selected *in vitro* test and the selected gene biomarker used is another factor that determines whether the embryotoxicity of a compound is correctly predicted and accurately classified [57, 78, 79]. Another example was given by Robinson *et al*. [69], who showed that four distinct teratogens induced equivalent levels of embryotoxicity in the rat WEC, with main effects on the closure of the neural tube and causing ear abnormalities. However, gene expression assessment revealed limited similarities in the identified functional groups of genes, showing the complexity of the underlying mechanism of embryotoxicity of each tested compound [69]. Similarly, Theunissen *et al*. [80] observed that neurodevelopmental toxicants did not reveal an overlap in regulation of gene responses

in the neural ETS (ESTn). Therefore, it is suggested that the category approach combined with the read-across concept can increase the accuracy of *in vitro* predictions, while enhancing at the same time the background knowledge on the underlying mechanisms of toxicity [81].

Interspecies comparisons of transcriptional responses

Transcriptomics can be a key approach for conducting interspecies comparison and extrapolate the observations from the *in vitro* to the *in vivo* situation on the level of their transcriptome dynamics, elucidating mechanisms of induced developmental toxicity, while understanding how the applicability domain of *in vitro* systems influences the accuracy of these predictions [79]. Interestingly, in **chapter 5**, we illustrated the good correlation of gene responses related to the developmental toxicity of the tested azoles in the rat WEC and murine EST. We also observed that the compounds with greatest manifestations of toxicity in the WEC and EST, also caused the greatest effect on regulating the sensitive genes related to embryotoxic responses (RA pathway), facts that were in agreement with the *in vivo* literature data. These observations may improve the extrapolation of *in vitro* observations to the human situation. The latter is also supported by Irie and Kuratabi [82], who have suggested that the “hourglass” model can better morphologically and genetically describe the developmental processes of vertebrates, in contrast to the “funnel-like” model. In the same study, a comparative transcriptome analysis of vertebrate embryos showed that the gene responses during the pharyngula stage were similar, while they were less conserved during the earlier and later stages of development [82]. Therefore, both the comparison and extrapolation of obtained results among different vertebrate models applied at comparatively similar developmental stages might be more accurate and relevant to the human situation. Confirmatory data were obtained from a comparative transcriptomics study between *in vivo* and *in vitro* (WEC) rat embryos, which showed similarities at the gene and functional level, without being exposed to any xenobiotic [19]. Moreover, the increased conservation of molecular pathways between the rat WEC, *in vivo* rat embryos [19] and human embryos [59, 83] suggests that gene biomarkers of developmental toxicity can facilitate a more accurate mechanistically-based approach for extrapolating an *in vitro* mechanism of toxicity to the human situation. Additionally similar functional MeHg-mediated transcriptional responses were observed between *in vitro* murine embryos, rat WEC and differentiated murine ESC in cardiomyocytes and neural cells, in contrast to the non-developing systems murine brain-, kidney- and embryonic fibroblast- derived tissues [84]. Overall, the correlation of transcriptome signatures in the *in vitro* developmental models with the *in vivo* developing systems supports the applicability and importance of the *in vitro*

developmental models in improving the genome-based predictions related also with the human situation.

Alternative approaches in risk assessment of embryotoxicants

Alternative approaches in developmental toxicity based on *in vitro* methodologies and gene expression analysis may improve the current strategies for conducting risk assessment on embryotoxicants [68, 81, 85-87].

The traditional risk assessment makes use of specific levels (NOAELs) and/or benchmark doses (BMDs, **chapter 3, 4 and 5**) or their lower confidence limits (BMDLs) for deciding on the safety of compounds and estimating acceptable levels of daily intake. Here, we selected the rat WEC (**chapter 2, 3 and 4**) and murine EST (**chapter 5**) as appropriate whole organism and cellular system, respectively, to study the pathway of toxicity of azoles. The calculated ID values of the tested compounds were further compared with the *in vivo* BMD values (calculated based on embryonic malformations) for concluding upon their reliability as alternative assays (**chapter 3, 4 and 5**). The addition of the kinetics of placental transport obtained from the BeWo placental model, as shown in **chapter 5**, confirmed the crucial role of biokinetics in enhancing the predictability of *in vitro* systems. Variances in the predictability of developmental toxicants may be observed due to differences in the exposure route, lack of maternal site and the developmental stages of the applied *in vivo* and *in vitro* biological systems. An additional important point of concern, which may increase the discrepancies between the *in vivo* and *in vitro* extrapolations, is the variety of observed malformations (skeletal, craniofacial, urogenital) and the limitations of the available *in vivo* data, which led us to conduct a qualitative analysis instead of a quantitative BMD-based approach, simplifying our observations (**chapter 4**).

Considering the increased sensitivity of transcriptomics, gene expression analysis may be applied as an extra endpoint for assessing possible changes in the genome level of biological systems after exposure to chemicals at the lowest part of their concentration-response curve, at which manifestations of developmental toxicity are absent [68, 88]. Studying the expression of single genes (**chapter 2 and 5**) or pathways (**chapter 3 and 4**) related with the molecular mechanism of teratogenicity in the WEC and EST exposed to the morphologically equally potent concentrations (ID_{10} and ID_{50}, respectively), we observed that the strong embryotoxic azoles *in vivo* caused also an increased upregulation of the biomarkers of RA-mediated pathway. However, it should be noted that the threshold for the effect on single genes or pathways might not be adequate for concluding on the threshold of relevant biological responses on the level of tissues, organs and whole organism (adaptation) and thus on the threshold of adversity [86]. Therefore, despite the fact that gene

expression regulation is considered more sensitive compared to morphological changes after exposure to chemicals, the question about the threshold of adversity of gene responses needs to be answered in a case by case manner.

In conclusion, for performing risk assessment on the developmental toxicity of azoles, dose-response curves derived from alternative to animal testing approaches, such as the WEC and EST combined with the BeWo assay, can accurately predict the morphological changes in high agreement with the *in vivo* studies. Additionally, defining/qualifying biomarkers of toxicity, at equally toxic concentrations (ID_{10} and ID_{50}), may give an insight in mechanistic aspects relevant for building an adverse outcome pathway (AOP) for azoles and assisting the quantitative *in vitro-in vivo* extrapolation (QIVIVE) [89-91], while possibly explaining the mechanism of retaining homeostasis in the level below the threshold of adversity [86, 92-94].

Retinoic acid and azoles: direct or indirect connection with teratogenic effects?

RA has been characterized as a morphogen, but also as a teratogen depending on its homeostasis in the biological systems under development [95]. Embryos exposed to RA and azoles suffered from similar morphological abnormalities, suggesting that they might share the same mechanism of developmental toxicity (**chapter 2, 3, 4, 5**) [64, 74]. Gene expression studies supported the idea of a shared toxicological mechanism of action between RA and azoles, revealing a common pattern in regulation of gene-sets that are related with RA metabolism (*Cyp26a1*, *Dhrs3*, *Rarb*) and general embryonic development (*Hox* family, *Gbx2*, *Cdx1*) (**chapter 2, 3, 4, 5**). In **chapters 3, 4 and 5**, we showed that the most potent embryotoxic azoles in both *in vitro* and *in vivo* assays, revealed a more pronounced effect on the upregulation of RA-related genes in the WEC and EST, when tested at equally toxic concentrations. The AOP of RA based on cellular *in vitro* systems (EST) and whole organisms (rat WEC and ZET) suggested that there is a molecular interaction of RA with enzymes that synthesize (*Dhrs3*) or metabolize (*Cyp26a1*) RA, affecting also genes that are responsible for anteroposterior patterning (*Hox* genes) and for caudal neural growth (*Gbx2* and *Rldh*) [96]. The dysregulation of the RA homeostasis triggers also the expression of *Fgf* and *Wnt* pathways, which have a crucial role in the general embryonic development, leading to skeletal and heart abnormalities [97-99]. Signals of RA-related coordinator-genes that belong to the *Crabp* family and suppression of transcriptional growth factors, such as the *Tgf-β* family, can also lead to the RA-related teratogenic outcome of azoles, such as cleft palate, craniofacial malformations and skeletal abnormalities (**chapter 3 and 4**) [22, 23, 100].

Alternatively, while trying to identify the exact mechanism of teratogenicity of azoles, it has also been suggested that the inhibition of the steroid related gene *Cyp19* could indirectly

lead to teratogenic outcomes after maternal exposure to azoles, causing in the first place placental hypertrophy, which further leads to increased transfer of azoles to the fetal side [22, 23]. Azoles have been designed to intervene with the fungal *Cyp51* gene, but they were also found to interact with mammalian *Cyp51* and other off-target *Cyp* genes [101]. Despite the fact that the potency is ten times lower in the mammalian systems compared to the fungal ones, dysregulation of mammalian *Cyp51* gene expression can lead to side-effects related to the sterol biosynthesis pathway and endocrine disruption, including steps of synthesis of bile acids, corticoids, glucocorticoids and sex steroids [22-24]. Therefore, *Cyp51* is considered as a classical gene biomarker for the fungicidal mode of action, related to the mammalian sterol biosynthesis pathway (**Chapter 2 and 3**). In **chapter 4 and 5**, we observed that the expression of *Msmo1*, which catalyses the same step of lanosterol synthesis in the sterol biosynthesis pathway as *Cyp51* and *Nsdhl*, shows a more extensive response in both WEC and EST when they were exposed to azoles, compared to *Cyp51*. Subsequently, *Msmo1* may be a more accurate gene biomarker for screening sterol-mediated responses among different *in vitro* testing systems. On the other hand, the inhibitory effect of azoles on *Cyp19*, which is the biomarker of the mammalian aromatase activity, might be of importance for detecting effects on steroidogenesis [23]. It is essential to mention here that it is not sure yet whether the detected magnitude of response of the sterol-related genes is actually causing the adverse effects, considering that this requires further consideration of the effective concentration, the duration of exposure and the extent of the effect itself. At present, the threshold of adversity is unknown and, therefore, more research is needed for setting the limits of adverse and adaptive responses using gene biomarkers.

❖ Future perspectives

Developmental toxicants could cause birth defects during any stage of pregnancy. The implemented *in vitro* assays and *in vivo* studies should be carefully designed for being able to detect embryotoxicity. Therefore, except for considering the complexity of the dynamic profile of a pregnancy, both the dynamics and kinetics of chemicals also need to be taken into account. Improving the alternative testing approaches to more intelligent testing strategies combining individual assays according to their different biological applicability domains, the selected read-outs will increase our knowledge leading to reliable extrapolation of data to the *in vivo* situation. However, further actions need to be taken for improving the alternatives for animal testing in order to provide future valuable information to expand our current knowledge on developmental toxicity and move the field one-step forward. The following points are some challenges that need more attention and are given here as suggestions for future advancements.

The role of alternative assays in the Risk Assessment

Risk assessment is still based on animal-derived dose-response data for evaluating possible risks for the human health and the environment [102]. The question here is whether we will ever be able to stop relying on animal data and start building risk assessment approaches based on *in vitro* data for predicting risks relevant to the human being. The present thesis provides information about the precision of *in vitro* assays for predicting the *in vivo* developmental toxicity of the selected azoles. Additional evaluation of more classes of chemicals could increase our knowledge and trust to alternative methods. Moreover, evaluation of the parent compounds and their metabolites could increase the correlation between *in vivo* and *in vitro* data [29]. The individual *in vitro* exposure of developmental toxicity assays to metabolites or the addition of systems with metabolic capacity may be valuable for building more precise QIVIVE [103]. Beside metabolism, other pharmacokinetic data concerning the absorption, distribution and excretion, will improve the prediction by accurately defining the dose and duration of exposure of the selected biological system to chemicals [103, 104]. In this thesis, an example on incorporating toxico- dynamic and kinetic information is given by combining both the WEC and EST with the BeWo assay (mimicking placental transfer), which resulted in more accurate predictions, better comparable to the *in vivo* developmental toxicity of the tested compounds. Future optimization of the BeWo assay may give more accurate results, especially focusing on to which extent the tested compounds could accumulate into the cells and/or bind to the polycarbonate membrane and the transwell plate itself, for exploring the underlying pharmacokinetic properties of azoles on

placental systems. Making use of *in vitro* concentration-response data in addition to *in vivo* observations, the implementation of physiologically based pharmacokinetic (PBPK) modelling is an emerging approach, which has been successfully incorporated for building *in vitro- in silico* predictions for *in vivo* developmental toxicity [105-107]. A retro-PBPK modelling has been also suggested, with which an association between the dose and the hazard will be created by calculating the dose needed to reach a tissue concentration as found in the *in vitro* test [91, 93].

Omics – A way to understanding biological responses

Omics approaches can boost the predictability of *in vitro* assays by identifying pathways and mechanisms of toxic responses of embryotoxicants for facilitating a better extrapolation of the obtained information to the human situation [54]. Defining molecular biomarkers of effects can enhance the knowledge about the dynamics of transcriptional responses during development and will support the interpretation of biological responses for understanding the underlying AOPs [87, 96]. The further combination of transcriptomics and metabolomics data would improve our knowledge on the role of transcriptome changes as toxic responses to alterations on the metabolome level [108], which may reflect the importance of these events in decoding the threshold of adversity of chemicals and the mechanism of restoring the homeostasis due to chemical stimuli [109, 110]. The present thesis provides information regarding transcriptional responses to equivalent toxic concentrations of azoles in the WEC and EST, showing the sensitivity of gene-biomarkers in detecting *in vivo* embryotoxic responses. However, for performing potency ranking based on the regulation of functional pathways related to teratogenic outcomes (for example, RA pathway), the applied concentration of the tested chemicals should be kept equal instead of testing equitoxic levels as needed when studying the modes of action.

Defining the threshold of adversity

Piersma *et al*. [57] have suggested that developmental toxicants have a threshold of adversity. Transcriptomics can detect toxic responses due to chemical exposure in a more sensitive way compared to evaluation of manifestations of toxicity. However, the level at which gene regulation leads to adversity requires a definition. Without clearly distinguishing adverse and adaptive gene responses, transcriptomics and other omics approaches cannot be implemented for conducting risk assessment of chemicals. Based on literature evidence, regulation of developmental toxic pathways may lead to adverse responses, while induction of transcriptional and metabolic pathways may be translated to adaptive responses [57, 67, 111, 112]. However, their exact definition is still a question, which could be answered only by further *in vitro* research for determining dose-response relationships of individual genes or

identified pathways of toxicity that are further associated with changes in the level of cells, tissues and organs [90, 94].

Innovative in silico models

The toxicokinetics and toxicodynamics knowledge derived from *in vivo* and *in vitro* assays of developmental toxicity testing, as well as the technological innovations could be further incorporated for building advanced *in silico* models for improving the prediction of the toxicity of chemicals and having an impact on future risk assessment strategies [89, 93, 113]. However, the understanding of physiological processes during development, which coordinate the cellular networks during normal morphogenesis (growth and differentiation processes) could give an insight to biological responses starting from the gene signalling until the organ formation. Knudsen *et al*. [88] have proposed computational systems that simulate the normal embryogenesis incorporating morphological data derived from *in vivo* and *in vitro* observations, as well as information obtained from developmental pathway analysis. The future vision is that advanced bioinformatics may be the key for building *in vitro* screening tools to integrate relevant complex biological information into statistical models, based on AOPs, omics approaches and *in vivo* observations [87, 114]. Therefore, the following aim of this "virtual embryo" system is to identify how chemical stimuli could affect the normal developmental processes and their association with perturbations of toxicity, such as neural tube defects, formation of cleft palate and other manifestations of embryotoxicity. The completion of this model would be a challenge and it may support the future mechanistic-based risk assessment for evaluating chemical and biological interactions, as well as for prioritizing further testing of chemicals [114].

❖ Conclusion

In this thesis, we have shown that the implementation of alternatives to *in vivo* assays could accurately predict the developmental toxicity of the selected azoles. The addition of even simple toxicokinetics, derived from the BeWo model, for evaluating the relative placental transfer rate of the tested chemicals, improved the correlation of *in vitro* with *in vivo* potency ranking, showing the importance of applying a battery of alternative assays in developmental toxicity testing approaches. Moreover, evaluating transcriptional responses in both the WEC and EST developmental toxicity tests, we identified sensitive gene biomarkers and pathways that preceded the manifestations of developmental toxicity, elucidating the mode of toxicological action of azoles and facilitating across-models comparisons.

References

1. van der Jagt K., M.S., Tørsløv J. & de Bruijn J. , *Alternative approaches can reduce the use of test animals under REACH* EUROPEAN COMMISSION DIRECTORATE GENERAL JRC JOINT RESEARCH CENTRE Institute for Health and Consumer Protection, http://home.kpn.nl/reach/downloads/reducingtheuseoftestanimalsunderreachihcprepor.pdf, 2004.
2. Adler, S., et al., *Alternative (non-animal) methods for cosmetics testing: current status and future prospects—2010.* Archives of Toxicology, 2011. **85**(5): p. 367-485.
3. Augustine-Rauch, K., C.X. Zhang, and J.M. Panzica-Kelly, *In vitro developmental toxicology assays: A review of the state of the science of rodent and zebrafish whole embryo culture and embryonic stem cell assays.* Birth Defects Res C Embryo Today, 2010. **90**(2): p. 87-98.
4. Piersma, A.H., *Alternative methods for developmental toxicity testing.* Basic Clin Pharmacol Toxicol, 2006. **98**(5): p. 427-31.
5. Scialli, A.R., *The challenge of reproductive and developmental toxicology under REACH.* Regul Toxicol Pharmacol, 2008. **51**(2): p. 244-50.
6. Sogorb, M.A., et al., *An integrated approach for detecting embryotoxicity and developmental toxicity of environmental contaminants using in vitro alternative methods.* Toxicol Lett, 2014. **230**(2): p. 356-67.
7. Marx-Stoelting, P., et al., *A review of the implementation of the embryonic stem cell test (EST). The report and recommendations of an ECVAM/ReProTect Workshop.* Altern Lab Anim, 2009. **37**(3): p. 313-28.
8. Spielmann, H., et al., *The embryonic stem cell test (EST), an in vitro embryotoxicity test using two permanent mouse cell lines: 3T3 fibroblasts and embryonic stem cells.* In Vitro Toxicology, 1997. **10**: p. 119-127.
9. Keller, G., *Embryonic stem cell differentiation: emergence of a new era in biology and medicine.* Genes Dev, 2005. **19**(10): p. 1129-55.
10. Lin, C., et al., *Dynamic transcriptional events in embryonic stem cells mediated by the super elongation complex (SEC).* Genes Dev, 2011. **25**(14): p. 1486-98.
11. Bruce, S.J., et al., *Dynamic transcription programs during ES cell differentiation towards mesoderm in serum versus serum-freeBMP4 culture.* BMC Genomics, 2007. **8**: p. 365.
12. Tsankov, A.M., et al., *Transcription factor binding dynamics during human ES cell differentiation.* Nature, 2015. **518**(7539): p. 344-349.
13. van Dartel, D.A., et al., *Disentangling cellular proliferation and differentiation in the embryonic stem cell test, and its impact on the experimental protocol.* Reprod Toxicol, 2009. **28**(2): p. 254-61.
14. Ellis-Hutchings, R.G. and E.W. Carney, *Whole embryo culture: a "New" technique that enabled decades of mechanistic discoveries.* Birth Defects Research Part B: Developmental and Reproductive Toxicology, 2010. **89**(4): p. 304-312.
15. Piersma, A.H., *Whole embryo culture and toxicity testing.* Toxicol In Vitro, 1993. **7**(6): p. 763-8.
16. New, D.A.T., *WHOLE-EMBRYO CULTURE AND THE STUDY OF MAMMALIAN EMBRYOS DURING ORGANOGENESIS.* Biological Reviews, 1978. **53**(1): p. 81-122.
17. Bechter, R. and H. Schon, *Use of the whole-embryo culture system in drug safety assessment?* Toxicol In Vitro, 1988. **2**(3): p. 195-203.
18. Brown, N.A. and S. Fabro, *Quantitation of rat embryonic development in vitro: a morphological scoring system.* Teratology, 1981. **24**(1): p. 65-78.
19. Robinson, J.F., A. Verhoef, and A.H. Piersma, *Transcriptomic analysis of neurulation and early organogenesis in rat embryos: an in vivo and ex vivo comparison.* Toxicol Sci, 2012. **126**(1): p. 255-66.
20. Webster, W.S., P.D. Brown-Woodman, and H.E. Ritchie, *A review of the contribution of whole embryo*

culture to the determination of hazard and risk in teratogenicity testing. Int J Dev Biol, 1997. **41**(2): p. 329-35.

21. Schmid, B.P., *Xenobiotic influences on embryonic differentiation, growth and morphology in vitro.* Xenobiotica, 1985. **15**(8-9): p. 719-26.
22. Chambers, J.E., et al., *Human and ecological risk assessment of a crop protection chemical: a case study with the azole fungicide epoxiconazole.* Crit Rev Toxicol, 2014. **44**(2): p. 176-210.
23. Marotta, F. and G.M. Tiboni, *Molecular aspects of azoles-induced teratogenesis.* Expert Opin Drug Metab Toxicol, 2010. **6**(4): p. 461-82.
24. Goetz, A.K. and D.J. Dix, *Toxicogenomic effects common to triazole antifungals and conserved between rats and humans.* Toxicol Appl Pharmacol, 2009. **238**(1): p. 80-9.
25. Brüggemann, R.J.M., et al., *Clinical Relevance of the Pharmacokinetic Interactions of Azole Antifungal Drugs with Other Coadministered Agents.* Clinical Infectious Diseases, 2009. **48**(10): p. 1441-1458.
26. Stahl, B., *JAU 6476 - Developmental toxicity study in rats after oral administration. Unpublished report No. M-012279-01-1 from Bayer AG, Wuppertal, Germany. Submitted to WHO by Bayer CropScience AG, Germany.* https://www.google.nl/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&ved=0ahUKEwjcjNCx5-HPAhWDrxoKHebSDzYQFgghMAE&url=http%3A%2F%2Fapps.who.int%2Fpesticide-residues-jmpr-database%2FDocument%2F110&usg=AFQjCNGpjQOE2xfb0QbWPWwA5-avbrC8kQ&sig2=ZFM7G3fgK4EdIA0e_zX0hQ&cad=rja. 1997.
27. Unger, T.M., Van Goethem, D. & Shellenberger, *A teratological evaluation of Bayleton in mated female rats.* Midwest Research Institute, Report No. 324 to Mobay Chemical Corporation Agricultural Chemical Division submitted to WHO by Bayer AG. (Unpublished), http://www.inchem.org/documents/jmpr/jmpmono/v83pr39.htm, 1982.
28. Flick, B., et al., *Embryotoxic potential of N-methyl-pyrrolidone (NMP) and three of its metabolites using the rat whole embryo culture system.* Toxicol Appl Pharmacol, 2009. **237**(2): p. 154-67.
29. Luijten, M., et al., *Application of a metabolizing system as an adjunct to the rat whole embryo culture.* Toxicol In Vitro, 2008. **22**(5): p. 1332-6.
30. Langsch, A. and H. Nau, *Metabolic Activation for In Vitro Systems.* ALTEX, Workshop 5.15 In vitro metabolism: Applications in pharmacology and toxicology, 2005. **22**(Special Issue 2): p. http://www.altex.ch/resources/354358_55.pdf.
31. Piersma, A.H., et al., *Biotransformation of Cyclophosphamide in Post-Implantation Rat Embryo Culture Using Maternal Hepatocytes in Co-Culture.* Pharmacology & Toxicology, 1991. **69**(1): p. 47-51.
32. Oglesby, L.A., et al., *Co-culture of rat embryos and hepatocytes: in vitro detection of a proteratogen.* Teratog Carcinog Mutagen, 1986. **6**(2): p. 129-38.
33. Rogiers, V., et al., *Hepatocyte-based in vitro models and their application in pharmacotoxicology Report of an EC DGXII meeting with representatives of the European pharmaceutical industry.* Toxicol In Vitro, 1995. **9**(5): p. 685-94.
34. Blaauboer, B.J., *Biokinetic modeling and in vitro-in vivo extrapolations.* J Toxicol Environ Health B Crit Rev, 2010. **13**(2-4): p. 242-52.
35. Strikwold, M., et al., *Combining in vitro embryotoxicity data with physiologically based kinetic (PBK) modelling to define in vivo dose-response curves for developmental toxicity of phenol in rat and human.* Arch Toxicol, 2013. **87**(9): p. 1709-23.
36. Hakkola, J., et al., *Xenobiotic-metabolizing cytochrome P450 enzymes in the human feto-placental unit: role in intrauterine toxicity.* Crit Rev Toxicol, 1998. **28**(1): p. 35-72.
37. Herman, N.L., et al., *Transfer of methohexital across the perfused human placenta.* J Clin Anesth, 2000. **12**(1): p. 25-30.
38. Miller, R.K., et al., *The role of the human placenta in embryonic nutrition. Impact of environmental and social*

factors. Ann N Y Acad Sci, 1993. **678**: p. 92-107.

39. Fabian, E., et al., *Activities of xenobiotic metabolizing enzymes in rat placenta and liver in vitro.* Toxicology in Vitro, 2016. **33**: p. 174-179.
40. Benirschke, K. and S.G. Driscoll, *The Pathology of the Human Placenta*, in *Placenta*, F. Strauss, K. Benirschke, and S.G. Driscoll, Editors. 1967, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 97-571.
41. Myllynen, P. and K. Vähäkangas, *Placental transfer and metabolism: An overview of the experimental models utilizing human placental tissue.* Toxicology in Vitro, 2013. **27**(1): p. 507-512.
42. Myllynen, P., M. Pasanen, and O. Pelkonen, *Human placenta: a human organ for developmental toxicology research and biomonitoring.* Placenta, 2005. **26**(5): p. 361-71.
43. Li, H., et al., *Extended evaluation on the ES-D3 cell differentiation assay combined with the BeWo transport model, to predict relative developmental toxicity of triazole compounds.* Arch Toxicol, 2016. **90**(5): p. 1225-37.
44. Li, H., et al., *Use of the ES-D3 cell differentiation assay, combined with the BeWo transport model, to predict relative in vivo developmental toxicity of antifungal compounds.* Toxicol In Vitro, 2015. **29**(2): p. 320-8.
45. Li, H., et al., *Assessment of an in vitro transport model using BeWo b30 cells to predict placental transfer of compounds.* Arch Toxicol, 2013. **87**(9): p. 1661-9.
46. Avery, M.L., C.E. Meek, and K.L. Audus, *The presence of inducible cytochrome P450 types 1A1 and 1A2 in the BeWo cell line.* Placenta, 2003. **24**(1): p. 45-52.
47. Mitra, P. and K.L. Audus, *In Vitro Models and Multidrug Resistance Mechanisms of the Placental Barrier*, in *Drug Absorption Studies: In Situ, In Vitro and In Silico Models*, C. Ehrhardt and K.-J. Kim, Editors. 2008, Springer US: Boston, MA. p. 368-396.
48. Pavek, P., et al., *Examination of Glucocorticoid receptor alpha-mediated transcriptional regulation of P-glycoprotein, CYP3A4, and CYP2C9 genes in placental trophoblast cell lines.* Placenta, 2007. **28**(10): p. 1004-11.
49. Pattillo, R.A., et al., *The hormone-synthesizing trophoblastic cell in vitro: a model for cancer research and placental hormone synthesis.* Ann N Y Acad Sci, 1971. **172**(10): p. 288-98.
50. Poulsen, M.S., et al., *Modeling placental transport: Correlation of in vitro BeWo cell permeability and ex vivo human placental perfusion.* Toxicology in Vitro, 2009. **23**(7): p. 1380-1386.
51. Bode, C.J., et al., *In Vitro Models for Studying Trophoblast Transcellular Transport.* Methods in molecular medicine, 2006. **122**: p. 225-239.
52. Kjaerstad, M.B., et al., *Systemic uptake of miconazole during vaginal suppository use and effect on CYP1A2 and CYP3A4 associated enzyme activities in women.* Eur J Clin Pharmacol, 2010. **66**(12): p. 1189-97.
53. Robinson, J.F., M.J. Gormley, and S.J. Fisher, *A genomics-based framework for identifying biomarkers of human neurodevelopmental toxicity.* Reprod Toxicol, 2016. **60**: p. 1-10.
54. Robinson, J.F. and A.H. Piersma, *Toxicogenomic approaches in developmental toxicology testing.* Methods Mol Biol, 2013. **947**: p. 451-73.
55. Balmer, N.V., et al., *From transient transcriptome responses to disturbed neurodevelopment: role of histone acetylation and methylation as epigenetic switch between reversible and irreversible drug effects.* Arch Toxicol, 2014. **88**(7): p. 1451-68.
56. Balmer, N.V. and M. Leist, *Epigenetics and transcriptomics to detect adverse drug effects in model systems of human development.* Basic Clin Pharmacol Toxicol, 2014. **115**(1): p. 59-68.
57. Piersma, A.H., et al., *Reproductive toxicants have a threshold of adversity.* Crit Rev Toxicol, 2011. **41**(6): p. 545-54.
58. Luijten, M., et al., *Transcriptomics analysis of retinoic acid embryotoxicity in rat postimplantation whole embryo culture.* Reprod Toxicol, 2010. **30**(2): p. 333-40.

59. Fang, H., et al., *Transcriptome analysis of early organogenesis in human embryos.* Dev Cell, 2010. **19**(1): p. 174-84.

60. Hartl, D., et al., *Transcriptome and proteome analysis of early embryonic mouse brain development.* PROTEOMICS, 2008. **8**(6): p. 1257-1265.

61. Mitiku, N. and J.C. Baker, *Genomic analysis of gastrulation and organogenesis in the mouse.* Dev Cell, 2007. **13**(6): p. 897-907.

62. Robinson, J.F., et al., *METHYLMERCURY INDUCED TOXICOGENOMIC RESPONSE IN C57 AND SWV MOUSE EMBRYOS UNDERGOING NEURAL TUBE CLOSURE.* Reproductive toxicology (Elmsford, N.Y.), 2010. **30**(2): p. 284-291.

63. Robinson, J.F., et al., *A Systems-Based Approach to Investigate Dose- and Time-Dependent Methylmercury-Induced Gene Expression Response in C57BL/6 Mouse Embryos Undergoing Neurulation.* Birth defects research. Part B, Developmental and reproductive toxicology, 2010. **89**(3): p. 188-200.

64. Robinson, J.F., et al., *A comparison of gene expression responses in rat whole embryo culture and in vivo: time-dependent retinoic acid-induced teratogenic response.* Toxicol Sci, 2012. **126**(1): p. 242-54.

65. van Dartel, D.A., et al., *Discriminating classes of developmental toxicants using gene expression profiling in the embryonic stem cell test.* Toxicol Lett, 2011. **201**(2): p. 143-51.

66. Hermsen, S.A., et al., *Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryotoxicity test and comparison with their in vivo potencies.* Toxicol In Vitro, 2011. **25**(3): p. 745-53.

67. Boekelheide, K. and M.E. Andersen, *A mechanistic redefinition of adverse effects - a key step in the toxicity testing paradigm shift.* Altex, 2010. **27**(4): p. 243-52.

68. Daston, G.P. and J.M. Naciff, *Predicting developmental toxicity through toxicogenomics.* Birth Defects Res C Embryo Today, 2010. **90**(2): p. 110-7.

69. Robinson, J.F., et al., *Embryotoxicant-specific transcriptomic responses in rat postimplantation whole-embryo culture.* Toxicol Sci, 2010. **118**(2): p. 675-85.

70. Robinson, J.F., et al., *Arsenic- and Cadmium-Induced Toxicogenomic Response in Mouse Embryos Undergoing Neurulation.* Toxicology and applied pharmacology, 2011. **250**(2): p. 117-129.

71. de Jong, E., et al., *Relative developmental toxicity of glycol ether alkoxy acid metabolites in the embryonic stem cell test as compared with the in vivo potency of their parent compounds.* Toxicol Sci, 2009. **110**(1): p. 117-24.

72. van Dartel, D.A., et al., *Monitoring developmental toxicity in the embryonic stem cell test using differential gene expression of differentiation-related genes.* Toxicol Sci, 2010. **116**(1): p. 130-9.

73. van Dartel, D.A.M., et al., *Transcriptomics-based identification of developmental toxicants through their interference with cardiomyocyte differentiation of embryonic stem cells.* Toxicology and Applied Pharmacology, 2010. **243**(3): p. 420-428.

74. Robinson, J.F., J.L. Pennings, and A.H. Piersma, *A review of toxicogenomic approaches in developmental toxicology.* Methods Mol Biol, 2012. **889**: p. 347-71.

75. Hermsen, S.A.B., et al., *Triazole-induced gene expression changes in the zebrafish embryo.* Reproductive Toxicology, 2012. **34**(2): p. 216-224.

76. Robinson, J.F., et al., *Triazole induced concentration-related gene signatures in rat whole embryo culture.* Reproductive Toxicology, 2012. **34**(2): p. 275-283.

77. de Jong, E., et al., *Comparison of the mouse Embryonic Stem cell Test, the rat Whole Embryo Culture and the Zebrafish Embryotoxicity Test as alternative methods for developmental toxicity testing of six 1,2,4-triazoles.* Toxicol Appl Pharmacol, 2011. **253**(2): p. 103-11.

78. Combes, R., M. Barratt, and M. Balls, *An overall strategy for the testing of chemicals for human hazard and risk*

assessment under the EU REACH system. Altern Lab Anim, 2006. **34 Suppl 1**: p. 15-27.

79. van der Burg, B., E. Kroese, and A.H. Piersma, *Towards a pragmatic alternative testing strategy for the detection of reproductive toxicants.* Reprod Toxicol, 2011. **31**(4): p. 558-61.
80. Theunissen, P.T., et al., *Compound-specific effects of diverse neurodevelopmental toxicants on global gene expression in the neural embryonic stem cell test (ESTn).* Toxicol Appl Pharmacol, 2012. **262**(3): p. 330-40.
81. Council, N.R., *Toxicity Testing in the 21st Century: A Vision and a Strategy*. 2007, Washington, DC: The National Academies Press. 216.
82. Irie, N. and S. Kuratani, *Comparative transcriptome analysis reveals vertebrate phylotypic period during organogenesis.* Nature Communications, 2011. **2**: p. 248.
83. Breschi, A., T.R. Gingeras, and R. Guigo, *Comparative transcriptomics in human and mouse.* Nat Rev Genet, 2017. **18**(7): p. 425-440.
84. Robinson, J.F., et al., *Comparison of MeHg-induced toxicogenomic responses across in vivo and in vitro models used in developmental toxicology.* Reproductive Toxicology, 2011. **32**(2): p. 180-188.
85. Thomas, R.S., et al., *Incorporating New Technologies Into Toxicity Testing and Risk Assessment: Moving From 21st Century Vision to a Data-Driven Framework.* Toxicological Sciences, 2013. **136**(1): p. 4-18.
86. Blaauboer, B.J., et al., *The use of biomarkers of toxicity for integrating in vitro hazard estimates into risk assessment for humans.* Altex, 2012. **29**(4): p. 411-25.
87. Adeleye, Y., et al., *Implementing Toxicity Testing in the 21st Century (TT21C): Making safety decisions using toxicity pathways, and progress in a prototype risk assessment.* Toxicology, 2015. **332**: p. 102-11.
88. Knudsen, T.B., et al., *Developmental toxicity testing for safety assessment: new approaches and technologies.* Birth Defects Res B Dev Reprod Toxicol, 2011. **92**(5): p. 413-20.
89. DeSesso, J.M., *Future of developmental toxicity testing.* Current Opinion in Toxicology, 2017. **3**: p. 1-5.
90. Bhattacharya, S., et al., *Toxicity Testing in the 21st Century: Defining New Risk Assessment Approaches Based on Perturbation of Intracellular Toxicity Pathways.* PLOS ONE, 2011. **6**(6): p. e20887.
91. Hartung, T., et al., *Integrated testing strategies for safety assessments.* Altex, 2013. **30**(1): p. 3-18.
92. Ankley, G.T., et al., *Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment.* Environ Toxicol Chem, 2010. **29**(3): p. 730-41.
93. Hartung, T., *From alternative methods to a new toxicology.* Eur J Pharm Biopharm, 2011. **77**(3): p. 338-49.
94. Loizou, G.D., *Animal-Free Chemical Safety Assessment.* Frontiers in Pharmacology, 2016. **7**(218).
95. Piersma, A.H., E.V. Hessel, and Y.C. Staal, *Retinoic acid in developmental toxicology: Teratogen, morphogen and biomarker.* Reprod Toxicol, 2017.
96. Tonk, E.C., J.L. Pennings, and A.H. Piersma, *An adverse outcome pathway framework for neural tube and axial defects mediated by modulation of retinoic acid homeostasis.* Reprod Toxicol, 2015. **55**: p. 104-13.
97. Huang, N., et al., *Diversity and function of mutations in p450 oxidoreductase in patients with Antley-Bixler syndrome and disordered steroidogenesis.* Am J Hum Genet, 2005. **76**(5): p. 729-49.
98. Jagtap, S., et al., *All-trans retinoic acid and basic fibroblast growth factor synergistically direct pluripotent human embryonic stem cells to extraembryonic lineages.* Stem Cell Res, 2013. **10**(2): p. 228-40.
99. Keber, R., et al., *Mouse knockout of the cholesterogenic cytochrome P450 lanosterol 14alpha-demethylase (Cyp51) resembles Antley-Bixler syndrome.* J Biol Chem, 2011. **286**(33): p. 29086-97.
100. Di Renzo, F., et al., *Molecular mechanism of teratogenic effects induced by the fungicide triadimefon: study of the expression of TGF-beta*

mRNA and TGF-beta and CRABPI proteins during rat in vitro development. Toxicol Appl Pharmacol, 2009. **234**(1): p. 107-16.

101. Georgopapadakou, N.H., *Antifungals: mechanism of action and resistance, established and novel drugs.* Curr Opin Microbiol, 1998. **1**(5): p. 547-57.

102. Krewski, D., et al., *Toxicity testing in the 21st century: a vision and a strategy.* J Toxicol Environ Health B Crit Rev, 2010. **13**(2-4): p. 51-138.

103. Wilk-Zasadna, I., et al., *Biotransformation in vitro: An essential consideration in the quantitative in vitro-to-in vivo extrapolation (QIVIVE) of toxicity data.* Toxicology, 2015. **332**: p. 8-19.

104. Blaauboer, B.J., et al., *Considering new methodologies in strategies for safety assessment of foods and food ingredients.* Food and Chemical Toxicology, 2016. **91**: p. 19-35.

105. Strikwold, M., et al., *Integrating in vitro data and physiologically based kinetic (PBK) modelling to assess the in vivo potential developmental toxicity of a series of phenols.* Archives of Toxicology, 2017. **91**(5): p. 2119-2133.

106. Fragki, S., et al., *In vitro to in vivo extrapolation of effective dosimetry in developmental toxicity testing: Application of a generic PBK modelling approach.* Toxicol Appl Pharmacol, 2017. **332**: p. 109-120.

107. Li, H., et al., *Use of physiologically based kinetic modeling-facilitated reverse dosimetry of in vitro toxicity data for prediction of in vivo developmental toxicity of tebuconazole in rats.* Toxicology Letters, 2017. **266**(Supplement C): p. 85-93.

108. Ramirez, T., et al., *Metabolomics in toxicology and preclinical research.* Altex, 2013. **30**(2): p. 209-25.

109. Bouhifd, M., et al., *Review: Toxicometabolomics.* Journal of applied toxicology : JAT, 2013. **33**(12): p. 10.1002/jat.2874.

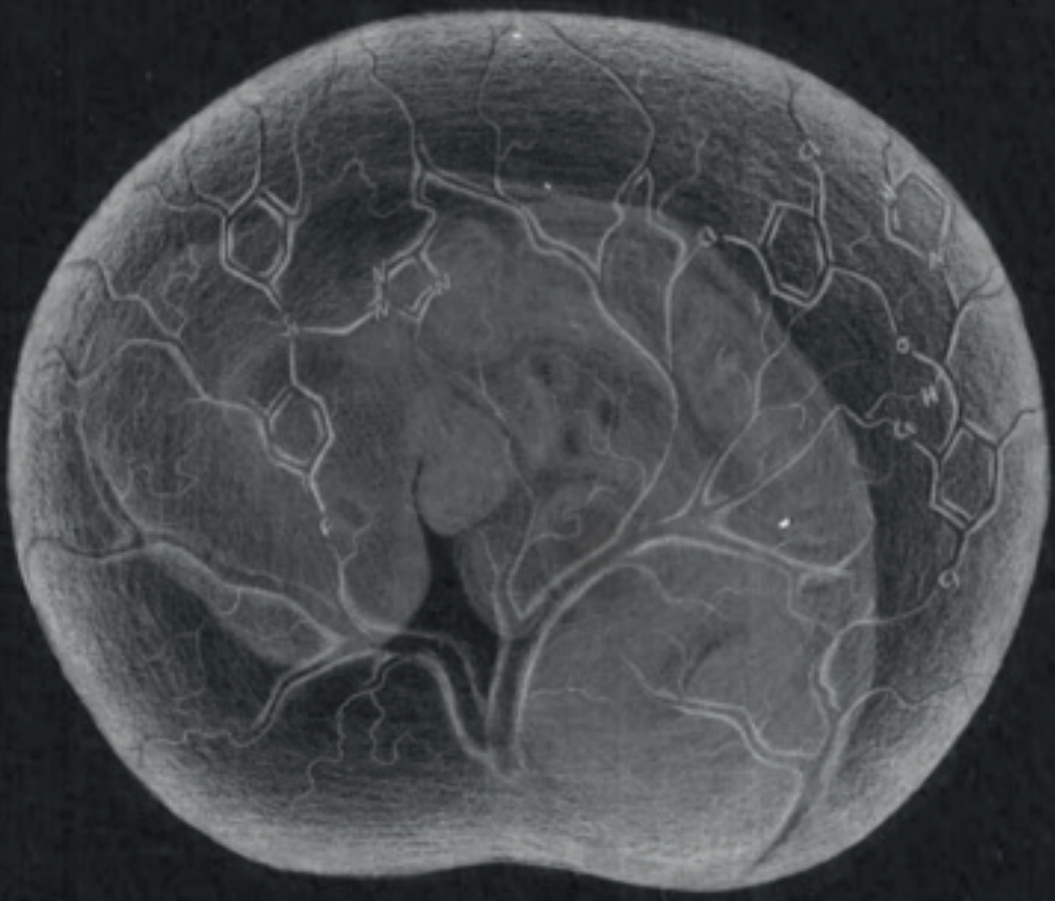
110. Ramirez, T., et al., *Metabolomics in Toxicology and Preclinical Research.* ALTEX, 2013. **30**(2): p. 209-225.

111. van Dartel, D.A., et al., *Concentration-dependent gene expression responses to flusilazole in embryonic stem cell differentiation cultures.* Toxicol Appl Pharmacol, 2011. **251**(2): p. 110-8.

112. Theunissen, P.T., et al., *Transcriptomic concentration-response evaluation of valproic acid, cyproconazole, and hexaconazole in the neural embryonic stem cell test (ESTn).* Toxicol Sci, 2012. **125**(2): p. 430-8.

113. Hartung, T., *Toxicology for the twenty-first century.* Nature, 2009. **460**(7252): p. 208-12.

114. Knudsen, T.B., et al., *FutureTox II: in vitro data and in silico models for predictive toxicology.* Toxicol Sci, 2015. **143**(2): p. 256-67.



Chapter 7

Summary

Summary of the thesis

The implementation of regulations for protecting both humans and the environment from potential chemical health hazards, as well as the increase of global pressure for reducing, refining and replacing animal experiments promote the development and application of alternatives to *in vivo* developmental toxicity studies. Due to the complexity of the reproductive cycle, combined *in vitro* approaches, focusing on morphological, molecular and toxicokinetic parameters, could better define the developmental toxicity of chemicals. In this thesis, azoles, which are a group of chemicals with antifungal activity, are under investigation. These compounds show marked differences in developmental toxicity potency and similarities with retinoic acid (RA)- related teratogenicity.

Chapter 1 of this thesis introduced information regarding the background of reproductive and developmental toxicology, including scientific concerns and the impact of past teratogenic outcomes on the society. For screening developmental teratogens, *in vitro* approaches have been proposed and successfully applied. Their combination may better mimic the *in vivo* embryo and, therefore, increase the accuracy in predicting possible developmental toxicants. Additional co-implementation of molecular approaches may give an insight in the mode of action underlying the observed effects. Azoles were selected in the present thesis due to evidence for possibly increasing developmental toxicity through dysregulating the balance of the RA pathways in the mammalian system. The chapter also described the objectives and outline of the research.

In **chapter 2**, we examined the time- dependent developmental effects in rat embryos exposed *in vitro* to flusilazole (FLU), and their link to RA mediated pathways. To this end, we assessed the effects of 4-hour exposure of whole embryo culture (WEC) embryos to 300μM FLU during four developmental time windows (0-4, 4-8, 24-28 and 44-48 h), evaluated morphological parameters, as well as expression and localization of five genes directly or indirectly linked with the RA pathway. A stage- specific gene expression response of cultured rat embryos exposed to FLU was detected, which preceded the development of morphologically observable malformations. During all the tested time windows, the most pronounced effect was observed in the regulation of RA-related genes. Therefore, it was concluded that such biomarkers can be employed as useful tools for early detection of possible teratogenic properties of compounds that belong to the triazole- group or of compounds with a similar teratogenic mode of action.

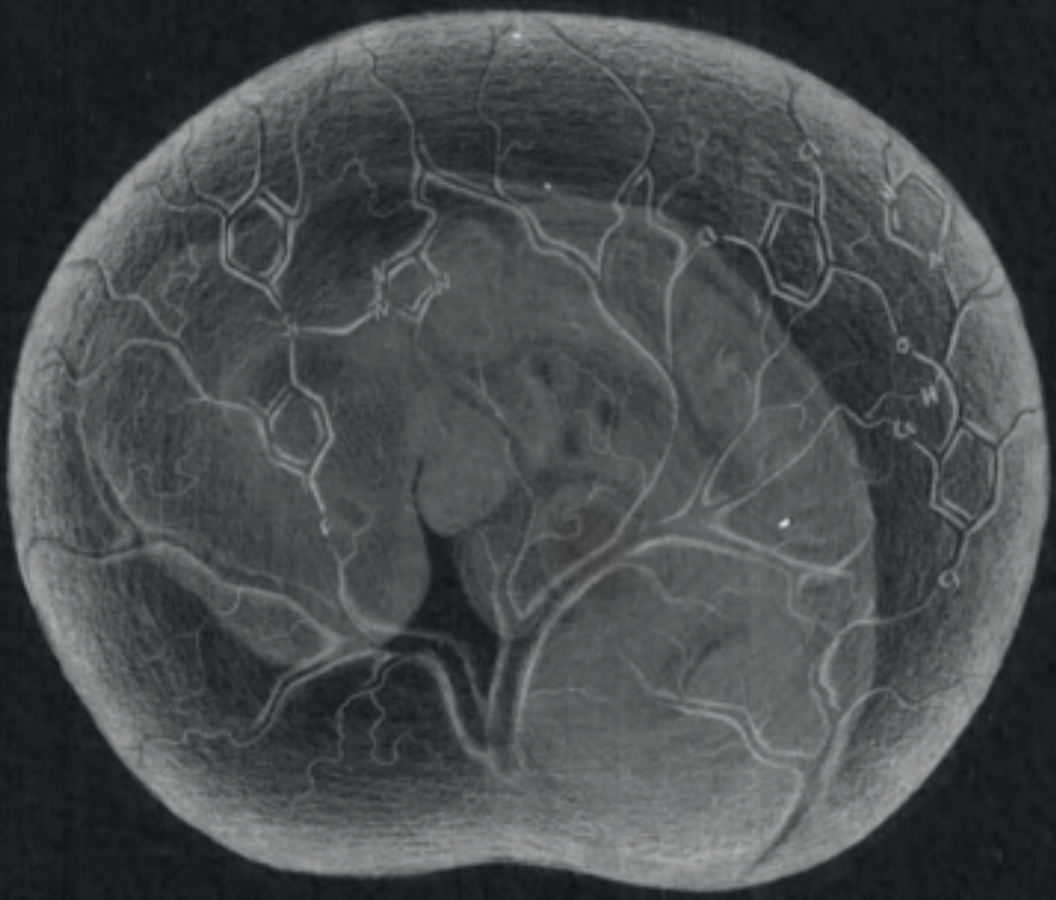
Chapter 3 provides mechanistic insight into the embryotoxicity of six azoles tested in the rat WEC. Here, we evaluated dose-dependent embryotoxicity of azoles in the rat WEC, calculating the concentration at which the total morphological score (TMS) is 10% decreased (ID_{10}). For the azoles tested we compared the *in vitro* ID_{10} for embryotoxicity to the *in vivo* effective doses, while we also performed a comparative analysis for understanding the toxicological and pharmacological mode of action of azoles in the rat WEC at the level of the transcriptome. Functional analysis of differential gene expression after 4 hours exposure at the ID_{10} revealed regulation of the sterol biosynthesis pathway and embryonic development genes, dominated by genes in the RA pathway, albeit in a differential way. FLU, ketoconazole and triadimefon were the most potent compounds affecting the RA pathway, while in terms of regulation of sterol function, difenoconazole and ketoconazole showed the most pronounced effects. A similar analysis at the 24-hour time point indicated an additional time-dependent difference in the aforementioned pathways regulated by FLU. Strong *in vivo* embryotoxic azoles showed also an increased regulation of the RA pathway when tested *in vitro*. On the other hand, weak or non- embryotoxic azoles showed a non-significant effect on genes that belong in the RA pathway. These observations led us to the conclusion that the toxicological mode of action of azoles was mediated through the RA pathway. In summary, the rat WEC assay in combination with transcriptomics could add mechanistic insight into the embryotoxic potency ranking and functional efficacy of the tested compounds, showing *Cyp26a1* and *Cyp51* as leader biomarkers of the off- and on- target effects, respectively.

Similarly to the previous chapter, in **chapter 4**, the potency ranking of the majority of the twelve tested azoles obtained based on the TMS in the WEC assay was in agreement with the *in vivo* potency ranking. Additionally, our expanded transcriptomics data, including gene specific responses of twelve azoles tested at their ID_{10} in the rat WEC for 4 hours, confirmed the observations of chapter 3 with another set of azoles. Potent embryotoxicants in both *in vivo* and *in vitro* assays caused more pronounced effects on the dysregulation of RA-mediated genes. Furthermore, azoles with more pronounced effects on the sterol biosynthesis mediated pathway were tested at a higher concentration, but with the same level of effect (ID_{10}). Due to the increased concentration needed for reaching the same level of morphological effects and the absence of RA-mediated pathway regulation, these azoles were considered as more favourable candidates for clinical and agricultural use. Focusing on monitoring the fungicidal activity of azoles, we also detected an increased sensitivity of the expression of *Msmo1*, which is an enzyme participating in converting lanosterol for synthesizing cholesterol in the mammalian sterol biosynthesis, together with *Cyp51*and *Nsdhl*. This observation led us to the conclusion that *Msmo1* could be a better biomarker of

effect on the sterol biosynthesis pathway compared to the classical biomarker of this pathway, *Cyp51*, and this may be of use for further improvement of the assessment of fungicidal activity of azoles or chemicals with similar mode of action.

Chapter 5 shows the value of combining toxico-dynamic and -kinetic *in vitro* approaches for embryotoxicity testing of azoles. We also report on the alterations in gene expression induced by azoles. Both the WEC assay and the embryonic stem cells test (EST) predicted the *in vivo* potency ranking of the twelve tested azoles with moderate accuracy. Combining these results with relative placental transfer rates (Papp values) as determined in the BeWo cell culture model, increased the predictability of both WEC and EST, with R^2 values increasing from 0.51 to 0.87 and from 0.35 to 0.60, respectively. The comparison of these *in vitro* systems correlated well (R^2 = 0.67), correctly identifying the strong and weak embryotoxicants. Evaluating specific gene responses related with the toxicological and fungicidal mode of action of the tested azoles in WEC and EST, we observed that the differential regulation of *Dhrs3* and *Msmo1* reached higher magnitudes in both systems compared to *Cyp26a1* and *Cyp51*. Establishing sensitive biomarkers across all the *in vitro* systems for studying the underlying mechanism of action of chemicals, such as azoles, is valuable for comparing alternative *in vitro* models and for improving insight in the mechanism of developmental toxicity of chemicals.

Chapter 6 of this thesis presented the general discussion and future perspectives on different topics raised based on the results obtained in the previously described experimental chapters. The results suggested that the combination of *in vitro* assays for screening the developmental toxicity of azoles may lead to predictions that are more accurate and in agreement with the *in vivo* observations. The addition of toxico-kinetics, which the BeWo placental transfer model offered, notably improved the correlations of *in vivo* and *in vitro* data. Furthermore, the co- implementation of transcriptomics and the identification of gene biomarkers revealed that despite the tested azoles were classified in the same chemical group, they might have a different mode of toxicological action. In conclusion, future combination of *in vitro* and *in silico* alternative approaches appear to be of advantage for screening and prioritizing chemical testing, in the process of assessing the consequences of chemical exposure for human health and the environment.



Appendix

Acknowledgments

First, I want to express my gratitude to my promotors Bennard van Ravenzwaay and Aldert Piersma, as well as to my co-promotor Ivonne Rietjens. I enjoyed our fruitful discussions during our meetings, which triggered me to grow up as a scientist.

Aldert, I really enjoyed our discussions and the freedom that you gave me to express myself. I learnt so many things from you that cannot be put into words. I will never forget your calm tips during my “storms” of stress. Thank you for everything.

Ivonne, thank you for giving me the opportunity of performing my PhD studies by proposing me to Ben as a possible candidate. I really appreciated your support and efficiency in reviewing my work with critical comments (especially during my writing months in the end of my PhD).

Ben, I remember our first meeting in Wageningen, when you and Ivonne interviewed me for the position. I was impressed with how calm and friendly you were. I enjoyed our collaboration and I appreciated your support and critical eyes during the years that we were working together. I also had the chance to meet you and have a great time outside of the working environment, in Seville, which I enjoyed a lot!

Aart, bedankt! I was planning to write this paragraph for you in Dutch, but since you left RIVM I forgot almost everything… I want to express to you my gratefulness for teaching me this amazing technique of whole embryo culture and for assisting me all these years. I really enjoyed our long Mondays and Wednesdays in the lab when we were performing the tests, while having relaxing talks - oefenen de Nederlandse taal - .

Jeroen, thank you for your “statistically significant” help! I have learnt so many things from you. I was amazed with your knowledge and, of course, the time, tolerance and patience that you showed to me when I was trying to learn the R programme for our omics analysis.

Marcia and Nacho (Ignacio), I am so so so happy that you are my paranymphs of this big event of my life! We started together exploring Wageningen back in 2011, when we were young and beautiful. Now, we are still together, with white hair and 10 kilos heavier, but w-i-s-e-r! Thank you for your valuable friendship, unlimited support and the soft tissues for my tears…

A very special “thank you” goes to Amalia, who is the young and very talented designer of my cover.

To Lidy, Irene, Gré and Letty thank you for helping me with all the administrative issues and bureaucracy. Special thanks go to Lidy for her great support during my last years of the PhD life!

Bert, Hans, Jochem, Laura and Sebas thank you for the great talks that we had from time to time. Nico, you are a great teacher and a cheerful person. Also, I always wanted to tell you how jealous I am with your aquarium in the office!

I would also like to thank my PhD colleagues in the Toxicology department, with whom I have shared moments of deep anxiety, but also big excitement. So, Abdul, Agata, Alexandros, Amer, Arif, Ashraf, Aziza, Barae, Diana, Diego, Felicia, Georgia, Hequn, Ixchel, Jia, Jing, Jonathan, Justine, Karsten, Kornphimol, Lenny, Lu, Marije, Marta, Mebra, Menno, Mengying, Miaoying, Myrthe, Qianrui, Reiko, Rozaini, Rung, Samantha, Shuo, Sunday, Suparmi and Wasma thank you! I would also like to thank my students, Ana, Anke, Arathi and Laura for their interest and contribution to my project.

Beside my TOX colleagues, I would like to express my gratitude to my colleagues at RIVM, with whom I have spent most of my time as a PhD. Many thanks go to my (bijna-) kamergenootjes: Astrid, Charlotte, Christina, Coen, Erna, Gina, Joantine, Hedwig, Kim, Kirsten, Linda, Peter, Sander, Sanne, Sjors, Tessel and Victoria. I would like to specially thank Conny, Edwin, Eric, Hennie and Liset for their technical support and interesting discussions during all these years! Finally yet importantly, I would like to express my appreciation to the VTS-members and the whole GZB department.

Many thanks go also to my friends from all over the places! You were so important and supportive for making me keep trying and “staying in shape”!

Most importantly, I would like to thank my parents and my brothers for their unlimited love and continuous support. Since I am Greek and we use to have big families with tight emotional connections, I want to extend my thankfulness to my uncle and aunt, my beloved cousins, who are my plus two brothers, Lina, Elena (the fresh generation) and the rest of my family, including my cute giagia who is not with us. Without these people, I would not be who I am and succeed in what I have done. Last but definitively not least, I want to truly thank my Angelos! You are my love and the source of motive and endless support to my – sometimes messy – life! Without you, I would have never achieved this and many other things.

Thank you all!

March 2018

Myrto

List of publications

Dimopoulou, M., A. Verhoef, B. van Ravenzwaay, I. M.C.M. Rietjens and A. H. Piersma (2016), "Flusilazole induces spatio-temporal expression patterns of retinoic acid-, differentiation- and sterol biosynthesis-related genes in the rat Whole Embryo Culture." Reproductive Toxicology 64: 77-85.

Dimopoulou, M., A. Verhoef, J. Pennings, B. van Ravenzwaay, I. M.C.M. Rietjens and A. H. Piersma (2017), “Embryotoxic and pharmacologic potency ranking of six azoles in the rat whole embryo culture by morphological and transcriptomic analysis”, Toxicology and Applied Pharmacology 322: 15-26.

Dimopoulou, M., A. Verhoef, J. L.A. Pennings, B. van Ravenzwaay, I. M.C.M. Rietjens and A. H. Piersma (2017), "A transcriptomic approach for evaluating the relative potency and mechanism of action of azoles in the rat Whole Embryo Culture", Toxicology 392: 96-105.

Dimopoulou, M., A. Verhoef, C. A. Gomes, C. W. van Dongen, I. M.C.M. Rietjens, A. H. Piersma and B. van Ravenzwaay. "A comparison of the embryonic stem cell test and whole embryo culture assay combined with the BeWo placental passage model for predicting the embryotoxicity of azoles." (accepted for publication, Toxicology Letters)

Curriculum Vitae

Myrto Dimopoulou was born in March 31st, 1988 in Patra, Greece. After receiving the Lyceum diploma in 2005 from Kastritsiou Lyceum in Patra, she started her bachelor studies in the Department of Chemistry at the University of Patra, from which she obtained her BSc degree in 2011. Myrto moved to the Netherlands in the same year to follow the two- year MSc programme in Food Safety at Wageningen University. During these studies, she performed her master thesis at the Department of Toxicology under the supervision of Prof. Ivonne M.C.M. Rietjens. Then, she conducted her internship at TNO Quality of life in Zeist for 6 months, on a Pediatric microdose study to evaluate drug metabolism, under the supervision of Dr. Wouter H.J. Vaes and Dr. Esther van Duijn. After graduating from her MSc studies in 2013, Myrto started her PhD research, entitled *Alternative developmental toxicity models for assessing the in vivo embryotoxicity of azoles* under the supervision of Prof. Aldert H. Piersma, Prof. Bennard van Ravenzwaay and Prof. Ivonne M.C.M. Rietjens. This project was funded by the chemical company BASF SE (Germany) and was a collaboration between the Department of Toxicology in Wageningen University and the Institute of Public Health and the Environment (RIVM), both located in the Netherlands. During her PhD, Myrto has been following the postgraduate education programme on Toxicology, which will enable her to register as a European Toxicologist after her graduation.

.

Overview of completed training activities

Discipline specific activities

Laboratory Animal Science, Postgraduate Education in Toxicology (PET), Utrecht, 2014

Molecular Toxicology, PET, Amsterdam, 2014

Organ Toxicology, PET, Nijmegen, 2015

Pathobiology, PET, Utrecht, 2016

Reproductive Toxicology, PET, Utrecht, 2014

Risk Assessment, PET, Wageningen, 2014

Toxicogenomics, PET, Maastricht, 2015

Conferences

Society of Toxicology, 56th Annual meeting, Baltimore, Maryland, USA, Poster presenter, 2017

European Teratology Society, 44th Annual meeting, Dublin, Ireland, Invited speaker & poster presenter, 2016

52nd European Congress of European Societies of Toxicology, Seville, Spain, Invited speaker & poster presenter, 2016

European Teratology Society, 43rd Annual meeting, Amsterdam, the Netherlands, Speaker, 2015

Nederlandse Vereniging voor Toxicologie (NVT), Speaker & poster presenter, 2015

General courses

Philosophy and Ethics of Food Science and Technology, VLAG, 2016

Scientific Publishing, Wageningen Graduate School, 2015

Scientific Writing, Wageningen Graduate School, 2014

VLAG PhD week, Venlo, 2014

Optional activities

Epidemiology, Wageningen, 2016

Environmental Toxicology, Wageningen, 2016

General Toxicology, Wageningen, 2014

Approved by Graduated School VLAG

The research described in this thesis was conducted within a collaborative project of Wageningen University, the Dutch National Institute of Public Health and Environment (RIVM) and BASF SE.

The research was financially supported by BASF SE (grant number 6153511466).

Financial support from Wageningen University and the Dutch National Institute of Public Health and the Environment (RIVM) for printing this thesis is gratefully acknowledged.

Cover design by Amalia Evangelidou
Printed by ProefschriftMaken - Digiforce, Vianen, the Netherlands

Propositions

1. Successful development of alternative testing strategies requires the combination of multiple assays.
   (this thesis)

2. Omics analysis at equally toxic concentrations can distinguish azoles according to their mode of toxicological action.
   (this thesis)

3. Instead of relying on human transplants, we can create tissues in the lab.

4. Cancer therapy requires personalized treatment.

5. Escaping from old ideas is harder than developing new ones.

6. Environmental consciousness builds the future of our children.

Propositions belonging to the thesis, entitled
"Alternative developmental toxicity models for assessing the *in vivo* embryotoxicity of azoles".

Myrto Dimopoulou
Wageningen, 05 March 2018.