Alternative testing strategies for predicting developmental toxicity of antifungal compounds

Hequn Li

THESIS COMMITTEE

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Wageningen University

Prof. Dr B. van Ravenzwaay
Professor of Reproduction and Developmental Toxicology
Wageningen University
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CHAPTER 1

General introduction

GENERAL INTRODUCTION AND AIM OF THE THESIS

Due to a high number of chemicals with only limited data on toxicity, there is a high demand for performing toxicological hazard identification and risk assessment of those chemicals. One toxic effect of great concern is developmental toxicity, i.e. adverse effects produced prior to conception, or during pregnancy and childhood. Exposure to agents affecting development can result in a wide range of adverse developmental effects, such as spontaneous abortions, stillbirths, malformations, early postnatal mortality, reduced birth weight, mental retardation, sensory loss, and other adverse functional or physical changes that are manifested postnatally [1]. Due to the high impact developmental toxicants exert on human society, the need for efficient methods to screen chemicals, drugs and environmental pollutants for their developmental toxicity is obvious.

In vivo developmental toxicity study

According to current international guidelines, developmental toxicity can be evaluated through exposure of pregnant animals, mostly rats and rabbits, and subsequent assessment of toxic effects in their fetuses [2-4]. A concurrent control group treated with the vehicle used for agent administration is a critical component of a well-designed study. At a minimum, a high dose, a low dose and one intermediate dose are included. Ideally, the high dose is selected to produce some minimal maternal or adult toxicity. The low dose is generally a no observed adverse effect level (NOAEL) for adult and offspring effects and if the low dose produces a biologically or statistically significant increase in response, it is considered the lowest observed adverse effect level (LOAEL). Normally, the test substance is administered to pregnant animals at least from implantation to one day prior to the day of scheduled sacrifice, which should be as close as possible to the normal day of delivery without risking loss of data resulting from early delivery [2, 3]. Evaluation of maternal responses is examined throughout pregnancy using clinical evaluations, as well as gross necropsy and histopathology. A number of endpoints that may be characterized as possible indicators of maternal toxicity include mortality, change in body weight, organ weights, food and water consumption. Shortly after caesarean section, the fetuses are evaluated. Endpoints of developmental toxicity include preimplantation loss, change in offspring body weight and the incidence of external, visceral, and skeletal malformations and variations that may indicate the organs or organ systems have been affected [2, 3].

The need for alternatives to animal studies

Recently, the European Commission has implemented the REACH (Registration, Evaluation, Authorization and restriction of CHemicals) legislation, aiming at greater protection of human health and the environment towards the adverse effects of

industrial chemicals. This REACH legislation aims at completing a comprehensive safety evaluation for existing and new commercial chemicals that are produced, imported and traded in Europe at amounts of more than one ton per year. However, implementation of the regulation may require 54 million vertebrate animals and testing costs of 9.5 billion euro [5]. Another estimation by the European Chemicals Bureau has shown that the developmental toxicity studies will require large amounts of animals and resources within REACH (23% of animals, 24% of resources) [6]. Because of the large number of animals needed, there is an urgent need for the development, validation and application of reliable alternative assays to in vivo developmental toxicity studies, which is in concordance with the replacement, reduction and refinement (3Rs) principle of animal use in toxicological research. The aim of the present thesis is to evaluate the applicability of an integrated in vitro-in silico approach for prediction of developmental toxicity using a series of antifungal compounds as the model compounds.

Antifungal compounds

The chemicals studied in the present thesis belong to the class of antifungal compounds. Antifungal compounds are widely used in agriculture and in human therapy. Their antifungal activity is based on their ability to inhibit cytochrome P450 (CYP) 51, a key enzyme in the formation of fungal wall components [7]. Human epidemiologic investigations have reported that antifungal compounds can pose a developmental toxicity hazard, with the evidence of inducing a lower birth weight and pregnancy loss, but without showing an increased risk of congenital malformations when administered at low therapeutic doses during pregnancy [7]. Several antifungal compounds tested in laboratory animals have been found to possess a common teratogenic potential to induce facial, axial skeleton, and limb defects. Although no evidence exists that shows that antifungal compounds induce malformations in human, some, but not all antifungal compounds have an effect on altering normal embryonic development leading to teratogenesis in animals indicating a possible teratogenic hazard in humans [7], which merits attention. Overall, assessment of the developmental toxic potential of antifungal compounds is of importance.

Two sets of antifungal compounds were studied in the present thesis. Our first set includes five reference antifungal compounds, i.e. ketoconazole, tebuconazole, propicoanzole, prothioconazole and fenarimol, with four of these being azoles (Fig.1). The azole family represents the largest family of antifungal compounds, which can be subdivided into the imidazole and triazole groups. We selected these five compounds, because of their widespread use as antifungal agents in medicine and crop protection and because they are known to cause developmental toxicity. For these chemicals an extensive toxicity data base is available, required for the evaluation of the predictions made in

the present thesis on their toxicity based on in vitro and in silico approaches [8-16]. The reference compounds used have demonstrated a varying degree of developmental toxicity in vivo (Table 1), allowing for a quantitative assessment of potency, which is the basis of the validation of the predictions of potency made in the present thesis based on the results from the in vitro assays.

The second set of compounds consists of six novel triazoles. For these novel triazoles, in vivo data are presented for the first time and are not available in the literature. In vivo studies were performed in addition to the framework of the present project at BASF SE, experimental toxicology and ecology, in the context of new fungicide development. All six novel triazoles showed to some degree a potential to cause prenatal developmental toxicity, including post-implantation loss, fetal weight decrease and skeletal malformation. Because of these findings development of these compounds was terminated and the compounds were made available as a second set of compounds for validation purposes of predictions made by in vitro developmental toxicity assays. After the in vitro assays have been validated in first instance using the five reference compounds, the evaluation of these assays is extended by testing the six novel triazoles and comparing the results obtained with in vivo findings.

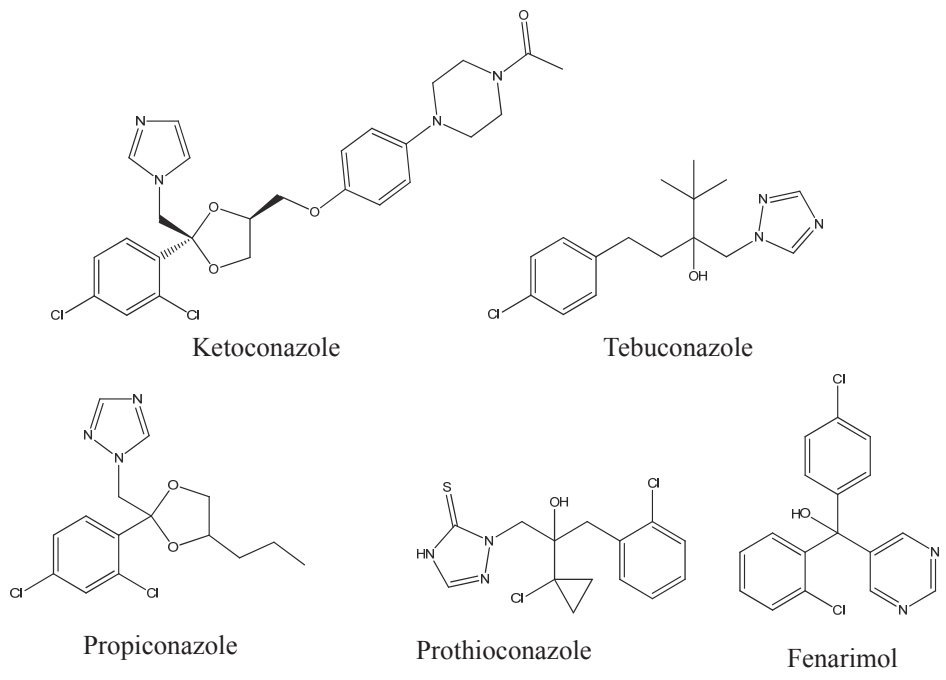

Fig. 1. Chemical structures of five reference antifungal compounds

Table 1. Developmental toxicity induced by antifungal compounds in rats upon oral administration.

Compound	Dose (mg/kg bw/day)	Exposure duration	Fetal effects	Ref.
Ketoconazole				
	25, 75	PI 6-17	Increased incidence of cleft palate	[8]
	80	GD 6-15	Increased embryolethality, decrease of fetal body weights, and fetal defects including cleft palate, frontal, parietal and interparietal incomplete ossification, limb anomalies, absent fibula and pubis, short femur, tibia and pubis	[9] [10]
Tebuconazole				
	30, 60, 120	GD 6-15	Increased incidences of skeletal variations.	[11]
	10, 30, 100	PM 6-15	Facial alteration dysplacia of scapula and long bones, exencephaly + spinia bifida, encephalomeningocele + macroglossia and other external alterations.	[12]
Propiconazole				
	30, 90, 300	GD 6–15	Increased incidence of rudimentary ribs, cleft palate, unossified sternebrae, as well as increased incidence of shortened and absent renal papillae	[13]
Prothioconazole				
	20, 80, 750	PC 6-19	Marginal increase in the incidence of fetal supernumerary rudimentary (comma-shaped) ribs	[14]
	80, 500, 1000	PC 6-19	Increased incidence of microphthalmia	[14]
Fenarimol				
	5, 13, 35	GD 6-15	Increased hydronephrosis without maternal toxicity. (This minor effect of delayed development is not of particular concern.)	[15]
	150, 300	GD 15–21	Increased neuromuscular and behavioral deficits in nursing pups	[16]

Note. PI = post-insemination; GD = gestational day; PC=post coitum; PM=post mating
- Data not available

The choice of the in vitro assay for developmental toxicity

The ES-D3 cell differentiation assay

Several in vitro methods have been developed to evaluate the developmental toxic potential of chemicals, such as the embryonic stem cell test (EST), the rodent whole embryo culture test and ex ovo assay of chicken embryos [17-19]. Among these tests the EST is the only validated in vitro assay that does not require the use of primary animal tissues. In the European Centre for the Validation of Alternative Methods'

validation study on 20 different chemicals with different in vivo embryotoxic potencies (classified as non, weak or strong embryotoxic), the EST provided a correct classification of the embryotoxic potential of 78% of the test chemicals, based on a prediction model that integrates effect data on ES-D3 cell viability, ES-D3 cell differentiation and 3T3 cell viability [20]. When only the strong embryotoxic chemicals were taken into account, a predictivity of 100% was obtained. However, in a second study, only 2 out of 13 test chemicals were correctly classified in the EST. Therefore, the applicability of this prediction model was questioned [20]. Several studies have used the ES-D3 cell differentiation assay as a stand-alone assay, using the chemical-induced inhibition of ES-D3 cell differentiation into functional (contracting) cardiomyocytes as a readout parameter, and have shown that the in vitro potency ranking of chemicals in this assay can be used to predict in vivo potency rankings within selected chemical classes for most of the investigated chemicals, including a set of antifungal compounds [21-23].

Despite the above-stated good predictive ability of the ES-D3 cell differentiation assay, the assay has also drawbacks. First of all it is relatively lengthy because it requires the exposure of cells to test chemicals for 10 days. Besides, a main weakness of the assay is the process of monitoring changes in the differentiation of the formed embryoid bodies (EBs) upon exposure to the test chemical, i.e. effects on the differentiation of the embryonic stem cells into contracting cardiomyocytes. Although an individual microscopic inspection of the 10-day differentiated EBs is performed to determine if they are functional (contractile), other relevant circumstances, such as the total beating area and beating intensity, are not taken into consideration for the toxicity assessment [20]. Another disadvantage is that in its present form, the ES-D3 cell differentiation assay focuses only on embryotoxicity and does not model any specific morphogenic events [24], so it is not able to detect the multitude of specific structural alteration events that may occur during pregnancy. However, since the assay is a cell-based assay, it holds promise for use in the high throughput testing of chemicals. Moreover, given the fact that ES-D3 cell differentiation assay as stand-alone has been shown to predict quite well the relative in vivo potencies of chemicals within chemical classes [21-23], we have used the ES-D3 cell differentiation assay in the present thesis to evaluate its applicability on predicting developmental toxicity of antifungal compounds.

The ex ovo assay of chicken embryos

Although the ES-D3 cell differentiation assay has been shown to be useful to predict the developmental toxicity potency of compounds, it is limited to the evaluation of the phenotypic endpoint, i.e. the differentiation of embryonic stem cells into contracting cardiomyocytes, and can therefore not be used to detect structural alterations induced by chemicals. Antifungal compounds have shown to possess a teratogenic potential, causing specific morphological alterations, such as craniofacial changes and inter alia cleft palate [25]. The mechanism of action of antifungal compounds on skeletal development has

been reported to result from alterations during hindbrain segmentation and branchial arches formation [26]. Since the teratogenic potential of chemicals is an important toxicological endpoint considered to be of high importance in risk assessment, it is important to employ an alternative assay that is capable of investigating structural alterations induced by antifungal compounds.

The ex ovo assay of chicken embryos can be used as such an assay as it assesses chemicals with regard to their developmental toxic potential based on morphological changes [27]. The ex ovo assay of chicken embryos was first established in 1976 by Jelinek et al. as a fast and cheap teratogenicity test [28]. Later on, their protocol has been modified by different researchers. In general, the culture duration (2 or 3 days) of the chicken embryos covers the major parts of organogenesis, offering the assessment of survival, growth and various morphological endpoints including specific development of different organ anlagen, i.e. head region with different brain -, eye -, ear anlagen and branchial arches, using microscopic inspection. The chicken embryos have been revealed to possess their own basic metabolic capacity, such as activities of CYP450 and glutathione S-transferases (GSTs), providing the possibility to screen for adverse effects of potential metabolites [29]. However, the ex ovo assay of chicken embryos has been criticized for the absence of mammalian maternal–fetal relations [30]. Moreover, although this assay has a short duration, i.e. 2 or 3 days, it is labor intensive and special equipment and highly educated and trained staff are needed.

Despite its disadvantages, in several inter-laboratory validation studies [31-33], the ex ovo assay of chicken embryos was found to be able to distinguish specific patterns of growth perturbations and anomalies induced by different chemicals in a concentration-dependent manner. At the present time, some laboratories in academia and industry are using the ex ovo assay of chicken embryos for routine embryotoxicity screening purposes and mechanistic studies. BASF SE has been using this assay for 10 years and obtained a good correlation to in vivo studies, within the limits of the applicability domain (compounds should not be toxic and/or irritant / corrosive) (Ben van Ravenzwaay, personal communication, October 1, 2015). Meanwhile, it has been reported that teratogenic potentials of tested compounds detected by the ex ovo assay of chicken embryos were similar to those determined in vivo [29, 34]. Therefore, the ex ovo assay of chicken embryos seems to be a suitable tool to study the potential to cause structural alterations of the antifungal compounds to gain additional information about their embryotoxic potential.

The importance of toxicokinetic information for predicting in vivo toxicity based on in vitro toxicity data

Although the ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos have exhibited their usefulness in predicting developmental toxicity of chemicals [21-23] [31-33], these in vitro systems have the generic limitations of most in vitro systems

with regard to the lack of in vivo kinetic processes, phenomena that are of influence in the in vivo situation. Toxicokinetics (TK), which is typically defined as absorption, distribution, metabolism and excretion (ADME) processes of a chemical, describes the fate of a toxic substance within the body. For the risk assessment using in vivo animal testing, TK data are key information for various extrapolations needed in risk assessment, such as interspecies extrapolation, route-to-route extrapolation and for mechanistic considerations [35]. Therefore, TK information is important in the in vivo animal testing based risk assessment.

With the implantation of the REACH legislation, in vitro tests are being developed to reduce the use of animals for REACH chemicals. There is a general consensus that the TK information is necessary to convert the in vitro results, generated at tissue/cell or sub-cellular level, into dose response curves or potency levels for the entire target organism [36]. More and more attention has been paid in recent years to the (further) development and implementation of in vitro or/and in silico models to measure the various ADME [37-39]. In the present thesis, we have developed and validated an in vitro cell-based model to obtain kinetic information on placental transfer, and combined that with data from in vitro developmental toxicity tests, in order to assess whether the prediction of in vivo developmental toxicity using in vitro models improves by adding kinetic data on placental transfer.

Placental transfer

The placenta is an important organ required for the development of the embryo or fetus. During normal pregnancy, the placenta attaches to the uterine wall and serves as a structure separating the maternal and fetal blood circulations. The functions of the placenta include signaling, production and release of hormones and enzymes, transfer of nutrients and waste products, implantation, cellular growth and maturation [40]. Placental transfer plays a crucial role in developmental toxicity. Only a few decades ago, it was still commonly believed that the placenta protects the conceptus from harmful agents [41]. However, the thalidomide catastrophe in the 1960s made people start to consider that chemicals can cross the placenta and give rise to serious developmental toxicity [41]. Ever since this catastrophe, several studies have been conducted providing evidence that toxicants, such as alcohol, nicotine or harmful drugs, can pass the placental barrier, reach the conceptus and induce toxicity [42-47]. It is important to note that different chemicals may transfer through the placental barrier to a different extent [42, 44, 46-51]. Several in vitro developmental toxicity studies using the ES-D3 cell differentiation assays have suggested that discrepancies of toxicity potencies between in vitro and in vivo could be due to the lack of a measure of placental transfer in the in vitro system [21-23]. The inclusion of data on placental transfer may therefore provide additional information, leading to the improvement of the predictive values of in vitro developmental toxicity tests.

Chemicals cross the placenta mainly by simple passive diffusion. Other possible mechanisms of placental transport are facilitated diffusion, active transport, pinocytosis and filtration. Important influencing factors on the rate and extent of placental transfer by passive diffusion include molecular weight, ionization (pKa), lipid solubility and protein binding. The passage through placental membranes can be also influenced by transporter proteins as many chemicals have been shown to be substrates of transporters in the placenta [51].

Placental transfer of compounds can be studied in the human ex vivo placental perfusion model, which is one of the main techniques for investigating transport of compounds across the maternal-fetal barrier. This model uses human term placenta, and therefore resembles the in vivo situation in human best. However, since the placentas used are donated by mothers and collected when they give birth, the number of available placentas is limited and the planning of experiments is difficult. An in vitro model using BeWo cells may provide an easier and faster method for predicting placental transfer of compounds [52]. BeWo cells are derived from a human choriocarcinoma that has retained cell properties and hormonal profiles of mononucleated cytotrophoblasts [53]. The BeWo b30 clone has a cell layer forming ability and has been used to study placental distribution of drugs and nutrients [54]. When grown on transwell inserts, the cell layer separates an apical maternal compartment from a basolateral fetal compartment and the cells get polarized with respect to the expression of functional transporters specific to the apical and basal membranes, such as P-glycoprotein [55], although P-glycoprotein expression levels in BeWo b30 cells have been reported to be lower than those in primary trophoblasts [56]. In the present study, the BeWo cell transport model is evaluated by comparing relative transport rates in this model to relative transport rates in the ex vivo human placental model, using a series of compounds that show low, middle and high transfer rates in the human ex vivo placental model [57]. Further, in the present thesis the BeWo model is used to study placental transfer of chemicals and to evaluate whether inclusion of placental transfer data may improve predictions of in vivo developmental toxicity based on in vitro developmental toxicity data.

The use of physiologically based kinetic modeling facilitated in vitro-in vivo extrapolations using a reverse dosimetry approach to apply in vitro toxicity data for risk assessment

With the help of the in vitro kinetic assays, such as the BeWo cell transport model, the predictive value of in vitro developmental toxicity tests may be improved. However, the challenge of predicting developmental toxicity using in vitro assays still remains that the effect concentrations obtained from those assays cannot be used for risk assessment, because risk assessment requires dose response curves from which points of departure to define safe levels of exposure for humans can be derived. The quantitative conversion of in vitro effect concentrations of compounds into in vivo effect doses, using in silico

approaches such as physiologically based kinetic (PBK) modeling, is a promising strategy in developing alternatives for toxicity testing for risk assessment [35, 58].

PBK modeling

With PBK modeling the fate of compounds in the body can be simulated and can be used to relate external (toxic) doses to internal (toxic) concentrations [58]. Briefly, in a PBK model the animal or human is described as a set of tissue compartments and these basic compartments can each be depicted as a single box. Then this conceptual model is translated into a mathematical model, describing the ADME kinetic processes in each compartment by differential equations. In the next step model parameter values need to be determined, including values for 1) physiological and anatomical parameters (e.g. cardiac output, tissue volumes and tissue blood flows), 2) physico-chemical parameters (e.g. tissue/blood partition coefficients) and 3) other kinetic parameters (e.g. kinetic constants for transport as well as for biotransformation reactions of the chemical and/or its metabolites). The values for physiological and anatomical parameters of a species can be obtained from the literature. Physico-chemical and kinetic parameter values may be obtained using in vivo data or using in silico or in vitro techniques. After the mathematical model has been defined and the parameter values have been obtained, simulations can be made using computational software, such as Berkeley Madonna (Macey and Oster, UC Berkeley, CA, USA), to calculate the concentrations of the chemical and its metabolites in the different compartments over time as a function of the dose. In order to examine the validity of the model and the reliability of subsequent predictions, the performance of the model is evaluated by comparing the predictions made to experimental in vivo kinetic data. This evaluation process helps discover discrepancies between the model predictions and the in vivo experimental data, which may point out if a certain important kinetic process has been overlooked or its model parameter value imprecisely incorporated in the model, requiring a new hypothesis and improvements of the model [58].

In vitro to in vivo extrapolations with a reverse dosimetry approach

After the PBK models have been developed, the application of so-called PBK modeling-facilitated reverse dosimetry enables the translation of in vitro concentration–response curves into in vivo dose–response curves, providing a platform to use in vitro toxicity data for risk assessment [37, 39, 59]. In reverse dosimetry approaches aiming to predict in vivo toxic dose levels, in vitro toxic effect concentrations are considered as surrogate tissue or blood concentrations that could cause an adverse effect in the in vivo situation. The corresponding in vivo toxic dose levels can be predicted using a PBK model to calculate the doses that are needed to reach these internal effect concentrations, indicating that PBK modeling-facilitated reverse dosimetry is an adequate method for the quantitative translation of in vitro data to the in vivo situation. Therefore, there is increased interest

in using the reverse dosimetry approach to define dose–response curves that enable the definition of reference values for risk assessment, such as Benchmark Dose (BMD) values, based on the translation of in vitro concentration–response data obtained from in vitro toxicity assays [35, 60]. Our group has shown that reverse dosimetry of developmental toxicity data obtained in the ES-D3 cell differentiation assay resulted in the correct prediction of in vivo dose–response curves of glycol ethers, phenol and retinoic acid [37, 39, 59]. No proof-of-principles of chemicals from other chemical categories are available so far. In the present thesis, the use of the PBK modeling-facilitated reverse dosimetry approach with the antifungal compound tebuconazole is applied to extrapolate in vitro concentration-response curves obtained in the ES-D3 cell differentiation assays and the ex ovo assay of chicken embryos into in vivo dose-response curves, in order to assess whether the in vitro developmental toxicity data of antifungal compounds can be applied in toxicological risk assessment.

OUTLINE OF THESIS

In **Chapter 1** of the thesis, background information on the topic is given and the aim of the thesis is presented. As outlined above the aim of the present thesis is to evaluate the applicability of integrated in vitro and in silico approach for prediction of developmental toxicity using a series of antifungal compounds as the model compounds.

Chapter 2 presents the assessment of the usefulness of an in vitro BeWo cell transport model to predict placental transfer. To this end, BeWo cells, derived from a human choriocarcinoma, were grown on transwell inserts to form a confluent cell layer, separating an apical maternal compartment from a basolateral fetal compartment. For a set of nine selected model compounds, including the reference compound antipyrine, the transport velocity from the apical to the basolateral compartment is determined. Relative transport rates obtained were compared with the relative transport rates of these compounds derived from data of ex vivo placental perfusion studies reported in the literature.

Chapters 3 investigated the applicability of the ES-D3 cell differentiation assay combined with the in vitro BeWo transport model to predict relative in vivo developmental toxicity potencies. To this purpose, the in vitro developmental toxicity of five antifungal compounds is investigated by characterizing their inhibitory effect on the differentiation of ES-D3 cells into cardiomyocytes. The BeWo transport model is used to determine the relative placental transport velocity. The ES-D3 cell differentiation data, combined with the relative transport rates obtained from the BeWo model, were compared to in vivo developmental toxicity data of the compounds studied, as reported in the literature.

Chapter 4 extended the evaluation on this combined approach by testing another set of six antifungal compounds. To this end, we combined ES-D3 cell differentiation data of the six compounds with relative transport rates obtained from the BeWo model and compared the obtained ranking to the developmental toxicity ranking as derived from in vivo data.

In **Chapter 5** we investigated the applicability of the ex ovo assay of chicken embryos to predict the potential to cause alterations in development of five antifungal compounds also studied in Chapter 3 and investigated if the combination of the ex ovo assay with the kinetic information on placental transfer would improve its predictive capacity. To this end, the toxicity data inducing the increase in the incidence of the number of structurally altered embryos, combined with the relative transport rates obtained for the various model compounds in the BeWo model, were compared with in vivo data on developmental toxicity of the compounds studied as reported in the literature.

In **Chapter 6** the in vitro concentration-response curves of the antifungal compound tebuconazole, obtained in the ES-D3 cell differentiation assay (Chapter 3) and the ex ovo assay of chicken embryos (Chaper 5), were translated into in vivo dose-response curves for developmental toxicity of tebuconazole in rat, using the reverse dosimetry approach.

The PBK model for tebuconazole developed and used for this purpose is solely based on kinetic parameter values derived using in vitro and in silico techniques. To assess whether in vivo developmental toxicity could be predicted for this chemical, using the reverse dosimetry approach, predicted dose-response curves for in vivo developmental toxicity in rats were compared with literature data on tebuconazole-induced in vivo developmental toxicity in rats.

Finally, **Chapter 7** summarizes and discusses the results of the present thesis and provides future perspectives. It discusses possible implications of the use of the integrated in vitro-in silico approach for toxicological risk assessment and gives directions for future research in the field.

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CHAPTER 2

Assessment of an in vitro transport model using BeWo b30 cells to predict placental transfer of compounds

Hequn Li, Bennard van Ravenzwaay, Ivonne M.C.M. Rietjens, Jochem Louisse

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ABSTRACT

The human ex vivo placental perfusion model has regularly been used to study the transplacental transport of compounds. However, this method is laborious and dependent on the presence of fresh human placenta, hampering its use for the assessment of large numbers of compounds. An in vitro model for the placental barrier using BeWo b30 cells may provide an alternative to the ex vivo system. The present study aims to assess whether such an in vitro model could be used to reliably predict placental transfer. To this end, BeWo b30 cells, derived from a human choriocarcinoma, were grown on transwell insert to form a cell layer, separating an apical maternal compartment from a basolateral fetal compartment. For a set of nine selected model compounds, including the reference compound antipyrine, the transport velocity from the apical to the basolateral compartment was determined. Relative transport rates obtained were compared with the transfer indices (a measure for the transport relative to antipyrine) of these compounds obtained in ex vivo placental perfusion studies as reported in the literature. The relative transport rates in the in vitro BeWo model were in good correlation (R^2=0.95) with the transfer indices reported for the ex vivo model. This indicates that the BeWo model could be a valuable in vitro model for prediction of placental transfer of compounds.

INTRODUCTION

The implementation of the REACH legislation results in the increased use of animals for toxicity testing, especially for reproductive and developmental toxicity testing [1]. Therefore, the development and validation of in vitro and in silico alternatives for these in vivo studies are urgently needed [2]. A number of in vitro methods have been developed to screen compounds for possible developmental toxicity, using primary cultures of embryonic cells or embryonic stem cell lines, such as the Embryonic Stem cell Test (EST), as well as test methods using whole embryos, such as the rodent Whole Embryo Culture (WEC) test and the Chicken Embryotoxicity Screening Test (CHEST) [3-5]. Although these assays have been proven to be useful in ranking compounds in relative potency, they are at present not used in risk assessment. An important reason is that in vitro assays only provide in vitro concentration-response curves, whereas in vivo dose-response curves are required for setting safe exposure levels in risk assessment. The application of so-called reverse dosimetry enables the translation of in vitro concentration-response curves into in vivo dose-response curves, providing a platform to use in vitro toxicity data for risk assessment [6]. In the reverse dosimetry approach, in vitro toxic concentrations are set as internal concentrations in a physiologically based kinetic (PBK) model, which is used to calculate which doses result in these internal concentrations [6, 7]. In a simple reverse dosimetry approach, it can be assumed that concentrations in the maternal circulation equal the concentrations in the conceptus, implying that placental transfer is not taken into account in the PBK modeling [6]. However, this is not true for all compounds, due to restricted or enhanced transport across the placenta, which is for example the case for B vitamins, vitamin E, vaccenic acid and cotinine [8-11]. It is therefore important to obtain information on the transport of compounds across the placenta, in order to enable incorporation of placental transfer in PBK models used for reverse dosimetry when predicting in vivo developmental toxicity dose levels based on in vitro toxicity assays.

Placental transfer of compounds can be studied in the human ex vivo placental perfusion model, which is one of the main techniques for investigating transport of compounds across the maternal-fetal barrier. This model resembles the in vivo situation in humans best. Furthermore, the size of the human placenta enables a great number of different parameters to be studied, e.g. placental metabolism, hormones and enzyme production and release [12, 13]. However, since the placentas used are donated by mothers and collected when they give birth, the number of available placentas is limited and the planning of experiments is difficult.

An in vitro model using BeWo cells may provide an easier and faster method for predicting placental transfer of compounds [14]. BeWo cells are derived from a human choriocarcinoma that has retained cell properties and hormonal profiles of mononucleated cytotrophoblasts [15]. The b30 BeWo clone has a monolayer forming ability and has been used to study placental distribution of drugs and nutrients [16]. Furthermore, these

cells express placental differentiation markers, such as human chorionic gonadotrophin (HCG) [17] and the major cytochrome P450 isoforms (CYP1A1 and 1A2) present in placenta [18]. When grown on transwell inserts, the cells get polarized with respect to the expression of functional transporters specific to the apical and basal membranes, such as P-glycoprotein [19], although P-glycoprotein expression levels in BeWo b30 cells have been reported to be lower than those in primary trophoblasts [20]. Although the BeWo model may possess these characteristics of a functional placenta, it represents a simplification of the in vivo situation. It is therefore important to obtain information on the comparison of the in vitro BeWo model with the ex vivo placental perfusion model, in order to assess the usefulness of the BeWo model.

The objective of the present study was to evaluate the ability of the in vitro BeWo model in predicting placental transfer of compounds. To this end, the transport rates of a set of nine selected model compounds (amoxicillin, antipyrine, digoxin, indomethacin, ketoprofen, levofloxacin, lidocaine, ofloxacin and spiramycin), which exhibit a wide range of transport indices (TIs) in the ex vivo placental perfusion model, were determined in the BeWo model and compared with data reported for the ex vivo model, as reported in the literature. In addition, the influence of protein binding on the transfer of the test compounds was studied.

MATERIALS AND METHODS

Reagents and chemicals

Digoxin, spiramycin, antipyrine, amoxicillin, ofloxacin, levofloxacin, ketoprofen, indomethacin, lidocaine, human placental collagen, bovine serum albumin (BSA), and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Fetal calf serum (FCS) was obtained from HyClone-Perbio (Etten-Leur, the Netherlands) and dimethyl sulfoxide (DMSO) was purchased from Acros Organics (Geel, Belgium). Penicillin, streptomycin, L-glutamine, trypsin-EDTA, phosphate-buffered saline (PBS), and Hank's balanced salt solution (HBSS) were obtained from Invitrogen (Breda, the Netherlands). Trifluoroacetic acid was purchased from J.T. Baker (Deventer, the Netherlands) and acetonitrile was obtained from Biosolve (Valkenswaard, the Netherlands).

Cell culture

The BeWo cell line (clone b30) was kindly provided by the Institute of Public Health of the Faculty of Health Sciences of the University of Copenhagen (Denmark) with permission from Dr. Alan Schwartz (Washington University, St. Louis, MO), and confirmed to be mycoplasma negative. BeWo b30 cells (passages 27 to 45) were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 10,000 U/ml penicillin, 10 mg/ml streptomycin, and 2 mM L-glutamine. The cells were maintained

in polystyrene cell-culture flasks (Corning, USA), under a humidified atmosphere of 5% CO_2 at 37 °C. The cells were harvested by exposure to a 0.05% trypsin-EDTA solution and transferred to transwell® polycarbonate membranes (12 mm diameter, 0.4 μm pore size) (VWR International BV, Amsterdam, the Netherlands) coated with human placental collagen. Cells were seeded at a density of 100,000 cells/cm^2 and the medium (0.5 ml apical compartment, 1.5 ml basolateral compartment) was replaced daily. At day 6 post-seeding the BeWo b30 cell layers were used for transport experiments. According to the findings of Liu et al., a confluent monolayer has been formed on day 6 post-seeding [21].

Determination of transepithelial electrical resistance values

The barrier forming capacity of the BeWo b30 cell layers was evaluated by measuring the transepithelial electrical resistance (TEER) of the cell layer using a Millicell ERS-2 Volt-Ohm Meter (Millipore, USA) at day 2 to 7 post-seeding. The TEER value was determined after replacing culture medium with HBSS and was corrected for the TEER of the collagen-coated filters in the absence of BeWo b30 cells.

Transport experiments

At day 6 post-seeding, following the examination of cell layer integrity using TEER measurements, the cells were equilibrated in transport buffer (HBSS) in both the apical and basolateral compartment for 30 min at 37 °C. Only the cell layers showing TEER values between 80 and 100 Ω·cm^2 were used for transport experiments. Transport experiments were initiated by adding 0.5 ml of transport buffer containing the test compound, added from a 200 times concentrated stock solution in DMSO, to the apical compartment and 1.5 ml transport buffer to the basolateral compartment. Subsequently, the plate was incubated in a humidified atmosphere with 5% CO_2 at 37 °C. After 15, 30, 60, and 120 min a sample of 0.2 ml was taken from the basolateral compartment and replaced by an equal volume of transport buffer. At the end of each experiment, a 0.2 ml sample was also taken from the apical compartment. Subsequently, the filters with the BeWo b30 cell layers were washed 3 times with PBS, cut out of the insert, dissolved in 0.25 ml 65% (v/v) methanol and sonificated for 15 min in a Bandelin Sonorex RK100 (Berlin, Germany) in order to determine the amount of compound accumulated in the cells. After each experiment mass-balance calculations were performed. The transport of levofloxacin, indomethacin and antipyrine was investigated using apical concentrations of 50, 100 and 200 μM. The transport of digoxin, amoxicillin, ofloxacin, ketoprofen and lidocaine was studied at a concentration of 100 μM. In each transport study, amoxicillin was included allowing data comparison of different studies.

In the ex vivo placental perfusion studies performed with indomethacin, levofloxacin, ofloxacin, ketoprofen and lidocaine described in literature, albumin was added to maternal and fetal perfusates. Therefore, the effect of protein addition

to the transport buffer on the transfer rates of these compounds was investigated in the BeWo model in the present study. To this end, BSA was added to the transport buffer, present in the same concentrations as the albumin concentration reported to be added in the perfusates of the respective human ex vivo placental perfusion studies. The albumin concentrations used in these experiments are presented in Table 1.

Table 1. Albumin concentrations (g/l) in transport buffer in the BeWo model and albumin concentrations in the perfusates in human ex vivo placental perfusion studies.

	Transport buffer BeWo model		Human placental perfusion perfusate		
Test compound	**Apical**	**Basolateral**	**Maternal**	**Fetal**	**ref**
Indomethacin	2	2	2	2	[22]
Levofloxacin	3	3	3	3	[23]
Ofloxacin	3	3	3	3	[23]
Ketoprofen	8.7	10.2	8.7	10.2	[24]
Lidocaine	35	40	35 [a]	40	[25]

[a] 100% fresh frozen plasma was used as maternal perfusate [25]. The physiological serum albumin concentration in late pregnancy is 35 g/l [26].

High Performance Liquid Chromatography analysis

Samples were analyzed using High Performance Liquid Chromatography (HPLC) to quantify the amount of test compound present in the collected samples in order to determine the transport rate. The HPLC system used consisted of a Waters (Milford, MA) 600 controller and a 600 pump, equipped with a photodiode array detector set to record absorption of wavelengths between 200 and 400 nm. A Waters 717 plus autosampler was used for sample injection. The temperature of the autosampler was kept at 7 ˚C. Sample (50 µl) was applied to a C18 5µm reverse phase column (150mm×4.6mm I.D.) with a guard column (7.5mm×4.6 mm I.D.) (Alltech, Bergen op Zoom, the Netherlands). Prior to analysis, collected samples containing BSA were mixed with 0.4 ml methanol. After centrifugation at 16,000 g for 5 min, the supernatant was injected for HPLC analysis. After sonification, the samples containing cells and filters were centrifuged at 16,000 g for 5 min, after which the supernatant was used for HPLC injection.

The mobile phase used for analysis of all the test compounds consisted of (A) 0.1% trifluoroacetic acid in nanopure water and (B) HPLC-grade acetonitrile. For analysis of amoxicillin, ofloxacin, spiramycin, levofloxacin, indomethacin, ketoprofen and antipyrine, elution was applied at a flow rate of 1 ml/min, starting at 10% B with a

linear increase to 100% B in 20 min. Subsequently, the gradient returned linearly to the initial condition in 2 min and remained 10 min at this condition prior to the next injection. For analysis of digoxin and lidocaine, the solvent gradient started at 22% B at a flow rate of 0.8 ml/min for 2 min, followed by a linear increase to 100% B in 8 min. Subsequently, the gradient returned to the initial conditions by a linear gradient over 2 min, followed by an equilibration of 10 min. In each experiment, calibration curves were included for quantification.

Data analysis

For each compound, the linear appearance rate in the basolateral compartment was determined. These linear appearance rates were used to calculate apparent permeability (P_{app}) coefficients (P_{app} coefficient (cm/s) = $(\Delta Q/\Delta t) / (A \cdot C_0)$), where ΔQ is the amount of test compound (µmol) transported to the receiver chamber in a certain time span (Δt (s)), A is the cell surface area (cm^2) and C_0 is the initial concentration of the test compound (mM). To calculate ΔQ for the second, the third and the fourth time point, a correction was made to compensate for the removal of compound at the earlier time points (ΔQ at t_{x+1} = amount measured at t_{x+1} (nmol) (basolateral concentration at t_{x+1} (µM) * 1.5 (mL)) supplemented with the amount removed at t_x (nmol) (basolateral concentration sample at t_x (µM) * 0.2 (mL))). Subsequently, relative P_{app} ratios were determined by expressing the P_{app} coefficient as a fraction of the P_{app} coefficient obtained for antipyrine. Relative P_{app} ratios can be written as $(A_{f,\ compound\ X}(t) * A_{m,\ antipyrine}(t_0)) / (A_{f,\ antipyrine}(t) * A_{m,\ compound\ X}(t_0))$, in which $A_{f,\ compound\ X}(t)$ = amount of compound X in the fetal (basolateral) compartment at time point t, $A_{m,\ antipyrine}(t_0)$ = amount of antipyrine added to the maternal (apical) compartment at time point t_0, $A_{f,\ antipyrine}(t)$ = amount of antipyrine in the fetal compartment at time point t, and $A_{m,\ compound\ X}(t_0)$ = amount of compound X added to the maternal compartment at time point t_0. The relative P_{app} ratios were used for the comparison with TIs reported for the ex vivo placental perfusion model. A TI value represents the transfer of the compound of interest as a fraction of the transfer of the reference compound antipyrine in ex vivo human placental perfusion studies [22-25, 27-30]. In the original studies, different formulas for TI values are reported, of which most can be rewritten as $(A_{f,\ compound\ X}(t) * A_{m,\ antipyrine}(t)) / (A_{f,\ antipyrine}(t) * A_{m,\ compound\ X}(t))$, in which $A_{f,\ compound\ X}(t)$ = amount of compound X in the fetal compartment at time point t, $A_{m,\ antipyrine}(t)$ = amount of antipyrine in the maternal compartment at time point t, $A_{f,\ antipyrine}(t)$ = amount of antipyrine in the fetal compartment at time point t, and $A_{m,\ compound\ X}(t)$ = amount of compound X in the maternal compartment at time point t. In the ex vivo study of lidocaine, however, the TI value was expressed slightly different, and can be rewritten as $(A_{f,\ compound\ X}(t) * A_{m,\ antipyrine}(t_0)) / (A_{f,\ antipyrine}(t) * A_{m,\ compound\ X}(t_0))$, being equal to the in vitro relative P_{app} values.

RESULTS

TEER values of BeWo b30 cell layers

BeWo b30 cells grown on collagen-coated polycarbonate membranes demonstrated a gradual increase in TEER value in time, with the maximal TEER value of 95 Ω·cm^2 being reached at day 6 post-seeding (Figure 1), indicating an increasing integrity of the cell layer until day 6 post-seeding. The TEER value decreased to 64 Ω·cm^2 at day 7 post-seeding indicating that the integrity of the barrier decreased. This finding was similar to the results reported by Poulsen *et al.* [29], showing that day 6 post-seeding is the optimal time point to perform transport experiments.

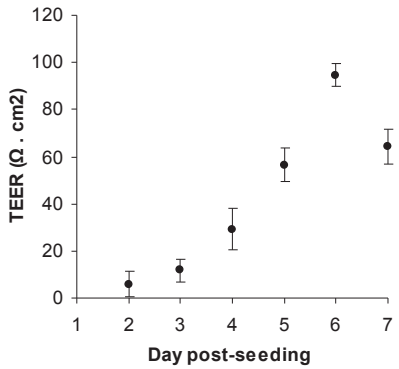

Fig. 1. Transepithelial electrical resistance (TEER) of BeWo b30 cell layers at different days post-seeding. Data are presented as mean ± standard deviation (n=6)

Transfer of test compounds in the in vitro BeWo model

For all transport experiments, the mass balances were between 91 and 99%. Figure 2a shows the amount of levofloxacin, indomethacin and antipyrine in the basolateral compartment of the BeWo model with increasing time, after adding 50 nmol to the apical compartment (0.5 ml of a 100 μM solution). Up to 60 min, the transport of all compounds to the basolateral compartment was linear in time. Therefore, the linear appearance rate of mass in the basolateral compartment could be determined using data at 60 min for the calculation of P_{app} coefficients (Table 2). Furthermore, the amount transported to the basolateral compartment was linearly related to the amount added to the apical compartment (Figure 2b).

The effect of albumin on the transport of levofloxacin, ofloxacin, ketoprofen, indomethacin and lidocaine was studied by adding albumin in both compartments at the same concentrations as in the perfusates used in the ex vivo perfusion studies

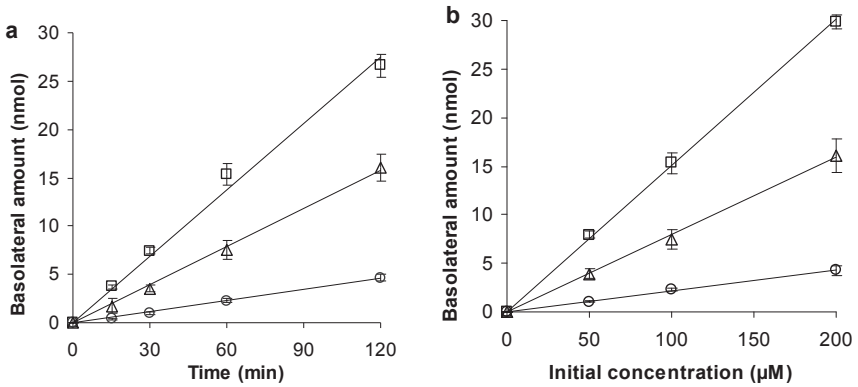

Fig. 2. Amount of levofloxacin (circle), indomethacin (triangle) and antipyrine (square) in the basolateral compartment in the in vitro BeWo model (a) with increasing time using initial concentrations of 100 μM in the apical compartment and (b) at 60 min with increasing initial concentrations of 50, 100, 200 μM in the apical compartment. Data are presented as mean ± standard deviation (n=6)

(Table 1). The obtained P_{app} coefficients for these studies are shown in Table 2 and were between 2.0- and 8.4-fold lower than the values obtained in the absence of BSA. Figure 3 shows the transport of antipyrine and ketoprofen in the BeWo model in the absence or presence of BSA, using BSA concentrations as used in the ex vivo placental perfusion study on ketoprofen (and antipyrine as reference compound). The results presented show that the presence of 8.7 g/l BSA in the apical and 10.2 g/l BSA in the basolateral compartment reduced the transfer of antipyrine by 31% (Figure 3a), while the transfer of ketoprofen was reduced by 88 % under these conditions (Figure 3b), indicating that the addition of BSA can affect the transfer of different compounds to different extents.

Table 2. Papp coefficients (10^{-6} cm/s) of 9 test compounds in BeWo model in the presence and absence of BSA.

	No BSA	With BSA
Digoxin	4.6	
Spiramycin	5.7	
Amoxicillin	7.2	
Levofloxacin	8.7	3.1
Ofloxacin	10.3	4.4
Indomethacin	23.2	9.9
Ketoprofen	23.6	2.8
Lidocaine	36.1	17.4
Antipyrine	38.0	

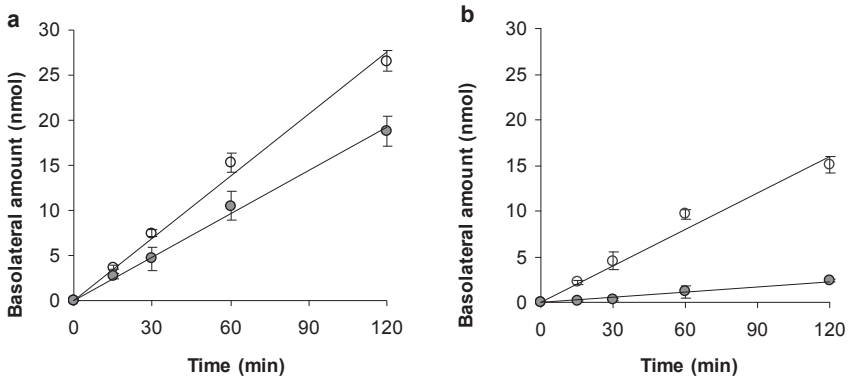

Fig. 3 Amount of antipyrine (a) and ketoprofen (b) in the basolateral compartment of the BeWo model with increasing time using initial concentrations of 100 µM in the apical compartment in the absence (open circle) or presence (closed circle) of BSA in the apical and basolateral compartment. The concentrations of BSA used were 8.7 g/l in the apical compartment and 10.2 g/l in the basolateral compartment, similar to what was applied in the corresponding ex vivo placental perfusion study [24]. Data are shown as mean ± standard deviation (n=6)

Comparison of the placental transfer in the in vitro BeWo model and the transport in the ex vivo human placental perfusion model

The selection of the compounds tested in the BeWo model was based on the broad range of TI values as reported for these compounds in the ex vivo human placental perfusion studies (ranging from 0.13 to 0.83) (Table 3). Table 3 presents the TI values of the selected model compounds reported for the ex vivo placental perfusion model as well as the relative Papp values obtained in the in vitro BeWo model in the absence and presence of BSA. In order to extend the dataset for comparison between the ex vivo and in vitro models, transport data of caffeine, benzoic acid and glyphosate obtained in both placental transport models reported by [29] were included in the comparison. The relative transport rates in the in vitro BeWo placental transport model showed a high correlation (R^2=0.95) with the ex vivo TI values (Figure 4a) when in vitro transport studies were carried out in the absence of BSA. In the presence of BSA, the correlation to ex vivo placental perfusion was slightly lower (Figure 4b; R^2=0.92).

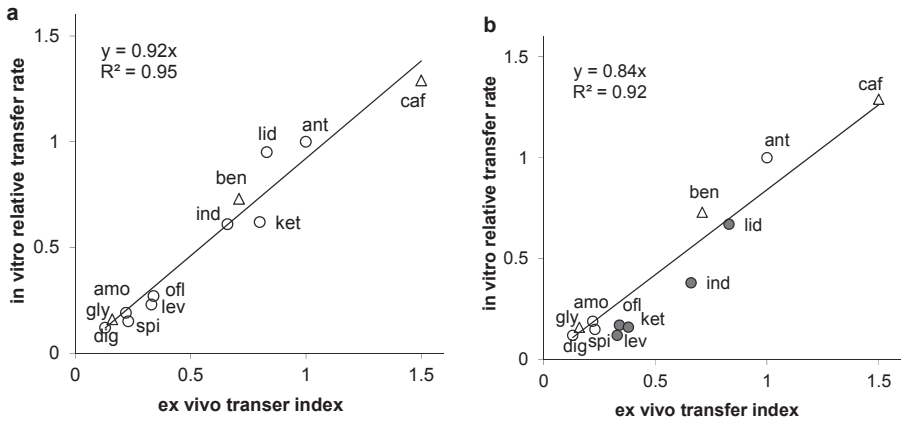

Fig. 4. Correlation between TIs from the ex vivo placental perfusion model and relative transport rates from the in vitro BeWo model for selected model compounds. A: all in vitro studies were carried out in the absence of albumin in the transport medium. B: in vitro studies were carried out in the presence of albumin in the transport medium, when the respective ex vivo studies were performed in the presence of albumin in the perfusates (closed symbols). Ex vivo data were taken from literature. In vitro data were obtained in present study for 9 test compounds (circles) and taken from [29] for 3 compounds (triangles). amo=amoxicillin, ant=antipyrine, ben=benzoic acid, caf=caffeine, dig=digoxin, gly=glyphosate, ind=indomethacin, ket=ketoprofen, lev=levofloxacin, lid=lidocaine, ofl=ofloxacin, spi=spiramycin

DISCUSSION

The objective of the present study was to evaluate the ability of the in vitro BeWo model to predict placental transfer of compounds. To this end, the relative transport rates of a set of nine model compounds obtained in the in vitro BeWo model, supplemented with the transport data of three compounds in this model reported by Poulsen et al. (2009), were compared with TIs of these compounds in the ex vivo human placental perfusion model as reported in the literature. The study shows that the relative transfer rates of the selected compounds in the in vitro BeWo model correlated well with the TIs reported for the ex vivo human placental perfusion model (R^2=0.95), indicating that the in vitro BeWo model is a useful tool to predict placental transfer of compounds.

The relative transport rates in the in vitro placental transport model correlated strongly with the ex vivo TIs in absence of BSA in the in vitro studies. A good correlation (R^2=0.92) was also found for the in vitro transport rates obtained under experimental conditions in which BSA was present. When albumin was present in the transport buffer, the transport rates of the six test compounds (Table 2) were lower than when albumin was absent, showing that the placental transfer of these compounds was influenced by the presence of proteins. It may be expected that in general only the free fraction of

the compound will be able to cross the placenta [31]. Binding to proteins decreases the free drug concentrations in maternal and fetal compartments. It has been shown in ex vivo studies that changes in protein concentrations in the maternal and fetal perfusates affect placental transfer [32, 33]. In the ex vivo perfusion study of ketoprofen, which has a high binding affinity for albumin [24], the TI decreased 2.1-fold, from 0.80 in the absence of albumin to a TI of 0.38 in the presence of albumin [24]. Using the same conditions regarding albumin concentrations in the BeWo model, the in vitro transport rate of ketoprofen relative to antipyrine decreased 4.9-fold, from 0.54 in the absence of albumin to 0.11 in the presence of albumin (Table 3). This indicates that the effect of protein binding on the transport of ketoprofen relative to the transport of antipyrine appears to be larger in the in vitro BeWo model than in the ex vivo placental perfusion model. This may be related to the fact that the in vitro BeWo model is a static system, without flows of perfusates, whereas the ex vivo model is a dynamic system consisting of flows of maternal and fetal perfusates controlled by peristaltic pumps [34].

Although we found a good correlation between the relative P_{app} ratios in the in vitro BeWo model and the TIs obtained in the ex vivo placental perfusion model, a difference between relative P_{app} ratios and TI values is that with TI values, the change in the amount of compound in the maternal circulation is included in its calculation. This implicates that a TI value does not exactly represent a relative transport rate, whereas the relative P_{app} ratio does. Therefore, a better correlation may be found between relative P_{app} ratios and relative transfer rates of the compounds in the ex vivo model. Unfortunately, the data reported for the ex vivo studies did not allow the calculation of relative transfer rates of the compounds in this model. However, if the decrease in the amount of compound in the maternal compartment is relatively low compared to the amount added at t_0, the TI value will be close to the actual relative transfer rate.

Although the data obtained in the in vitro BeWo model was in good correlation with data obtained in the ex vivo placental perfusion model, it must be noted that it is not clear how well both models predict placental transfer at early stages of pregnancy. The ex vivo human placental perfusion method uses term placenta [34] and the obtained data may therefore be especially relevant for the last period of pregnancy. Since many structural abnormalities occur at early stages of pregnancy [35], it is important to predict placental transfer at these early stages. Therefore, knowledge is needed on whether placental transfer in early stages of pregnancy differs from placental transfer in the last period of pregnancy, in order to determine how well the BeWo model predicts placental transfer in these critical stages of pregnancy.

Since we found a good correlation between the relative transport rates in the in vitro BeWo model and the TIs in the ex vivo placental perfusion model, the BeWo model may be a useful tool to predict whether compounds are likely to reach the fetus when being present in the maternal circulation. Together with data on in vitro developmental

toxicity of these compounds, for example obtained in the EST, the BeWo transport data may give indications which compounds would be of highest concern for the developing conceptus. This information could be used for prioritization for eventual further testing. Furthermore, the transport data generated from the BeWo model may be used in future studies to make quantitative predictions of fetal exposure, if the data would be integrated in PBK models that describe placental transfer from the maternal circulation to a fetal compartment. This addition of placental transfer in PBK modeling may improve the prediction of in vivo toxic dose-response curves for developmental toxicity when translating in vitro concentration-response curves to the in vivo situation using reverse dosimetry, enabling a non-animal based developmental toxicity testing strategy.

CONCLUSION

The present study shows that the in vitro BeWo model is capable of predicting similar relative placental transfer of compounds compared to the ex vivo placental perfusion model. The in vitro BeWo model may therefore provide an alternative to the ex vivo placental perfusion model, as an easier and faster method to predict placental transfer, enabling the assessment of a larger number of compounds. The data obtained in the BeWo model may be useful for the prioritization for eventual further testing of compounds. Furthermore, the parameters for placental transfer generated from this in vitro model may be used in future studies to establish parameter values for placental transfer in PBK models that can be used for reverse dosimetry, enabling the translation of in vitro concentration-response curves into in vivo dose-response curves that can be used for risk assessment, thereby contributing to the replacement, reduction and refinement of animal use in toxicity testing.

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NOTES

The authors declare that they have no conflict of interest.

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CHAPTER 3

Use of the ES-D3 cell differentiation assay, combined with the BeWo transport model, to predict relative in vivo developmental toxicity of antifungal compounds

Hequn Li, Ivonne M. C. M. Rietjens, Jochem Louisse, Martine Blok, Xinyi Wang, Linda Snijders, Bennard van Ravenzwaay

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ABSTRACT

We investigated the applicability of the ES-D3 cell differentiation assay combined with the in vitro BeWo transport model to predict the relative in vivo developmental toxicity potencies. To this purpose, the in vitro developmental toxicity of five antifungal compounds was investigated by characterizing their inhibitory effect on the differentiation of ES-D3 cells into cardiomyocytes. The BeWo transport model, consisting of BeWo b30 cells grown on transwell inserts and mimicking the placental barrier, was used to determine the relative placental transport velocity. The ES-D3 cell differentiation data were first compared to benchmark doses (BMDs) for in vivo developmental toxicity as derived from data reported in the literature. Correlation between the benchmark concentration for 50% effect (BMC_d50) values, obtained in the ES-D3 cell differentiation assay, with in vivo BMD10 values showed a reasonable correlation ($R^2 = 0.57$). When the ES-D3 cell differentiation data were combined with the relative transport rates obtained from the BeWo model, the correlation with the in vivo data increased ($R^2 = 0.95$). In conclusion, we show that the ES-D3 cell differentiation assay is able to better predict the in vivo developmental toxicity ranking of antifungal compounds when combined with the BeWo transport model, than as a stand-alone assay.

INTRODUCTION

The implementation of the REACH legislation results in the increased use of animals for (eco) toxicity testing, especially for developmental toxicity testing [1, 2]. Therefore, it is urgently needed to develop and validate in vitro and in silico alternative methods for in vivo developmental toxicity studies [3]. Several in vitro methods have been developed to evaluate the developmental toxic potential of chemical substances, such as the embryonic stem cell test (EST), the rodent whole embryo culture test and the chicken embryotoxicity screening test [4-6]. Among these tests only the EST does not require the use of primary animal tissues. The EST uses the blastocyst-derived mouse embryonic stem cell line D3 (ES-D3) that spontaneously differentiates into contracting cardiomyocytes when cultured as embryoid bodies. The inhibition of the ES-D3 cell differentiation into contracting cardiomyocytes by test compounds is the in vitro endpoint for developmental toxicity [7]. In the European Centre for the Validation of Alternative Methods' validation study on 20 different chemicals with different in vivo embryotoxic potencies (classified as non, weak or strong embryotoxic), the EST provided a correct classification of the embryotoxic potential of 78% of the test chemicals, based on a prediction model that integrates effect data on ES-D3 cell viability, ES-D3 cell differentiation and 3T3 cell viability. When only the strong embryotoxic chemicals were taken into account, a predictivity of 100% was obtained. However, in a second study, only 2 out of 13 test chemicals were correctly classified in the EST. Therefore, the applicability of this prediction model was questioned [8]. Several studies that have used the ES-D3 cell differentiation assay as a stand-alone assay, have shown that in vitro potency ranking of chemicals in this assay can be used to predict in vivo potency rankings within selected chemical classes for most of the investigated chemicals [9-11]. Any incorrect ranking was pointed out to be possibly due to the lack of in vivo kinetic processes in this in vitro assay, phenomena that are of influence in the in vivo situation. A further point is that some chemicals may be tested at higher concentrations in the EST than could be achieved in vivo. Therefore, it is of importance to combine the in vitro model for developmental toxicity with data on in vivo kinetics, to better predict in vivo developmental toxicity potencies.

One of the main in vivo kinetic processes involved in developmental toxicity is placental transfer, as the transport of compounds through the placental barrier may differ, thereby influencing the final concentrations of compounds that could reach the fetus. Placental transfer of compounds can be studied in the human ex vivo placental perfusion model, which is a useful model for investigating transport of compounds across the maternal-fetal barrier [12-15]. However, this method is laborious and dependent on the presence of fresh human placenta, hampering the use for the assessment of large numbers of compounds. Another method to study placental transfer is the in vitro BeWo transport model, which is easy, fast and cheap [16]. In this model, BeWo b30 cells, derived from a human choriocarcinoma, are used. When grown on a transwell insert, the cells

get polarized and form a cell layer, separating an apical compartment from a basolateral compartment, representing maternal and fetal compartment in vivo, respectively [17]. Besides transmembrane diffusion, compounds can be transported across the BeWo cell membrane via active transport or paracellular diffusion. These different transport mechanisms have been elucidated in inhibition and specific substrate studies which demonstrated the expression of specific transporters such as the P-glycoprotein[18], and the multidrug resistance-associated proteins, as well as the breast cancer resistance protein (Mitra and Audus, 2010). We have previously shown that this in vitro model is capable of predicting relative placental transfer of a set of 9 model compounds with a good correlation to the transport observed in the ex vivo placental perfusion model (R^2=0.95), indicating the in vitro BeWo transport model is useful to study placental transfer of compounds [19].

The aim of the present study was to determine the usefulness of combining the ES-D3 cell differentiation assay of the EST with the in vitro BeWo transport model to predict the relative in vivo developmental toxicity potencies of a series of five antifungal developmental toxicants, being ketoconazole, tebuconazole, propiconazole, prothioconazole and fenarimol. The five compounds have shown to be developmental toxicants in animal studies as they cause, e.g., increased embryolethality, cleft palate, reduced fetal weight, and skeletal malformations [20, 21]. The in vitro developmental toxicity data of the five test compounds obtained in this study were compared to the historical in vivo data.

MATERIALS AND METHODS

Chemicals

Ketoconazole, tebuconazole, propiconazole, prothioconazole and fenarimol (with purity for the five compounds all > 98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Dimethyl sulfoxide (DMSO) was purchased from Acros Organics (Geel, Belgium). Hank's balanced salt solution (HBSS) was obtained from Invitrogen (Breda, the Netherlands).

ES-D3 cell culture

The murine ES-D3 cell line was purchased from ATCC (Wesel, Germany). The cells were maintained in polystyrene cell culture flasks (Corning, the Netherlands) in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Breda, The Netherlands), supplemented with 20% heat-inactivated fetal calf serum (Lonza, BioWhittaker, Verviers, Belgium), 50 U/ml penicillin with 50 µg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich) and 1% (v/v) non-essential amino acids (Invitrogen), at 37°C and 5% CO_2 in a humidified atmosphere. Cells were kept undifferentiated with 1000 U/ml murine leukemia inhibiting factor (LIF)

(Sigma-Aldrich) and subcultured every 2 to 3 days using non-enzymatic cell dissociation solution (Sigma-Aldrich) to detach the cells.

Cytotoxicity assay with ES-D3 cells

To determine cytotoxicity of the compounds, a WST-1 assay was performed. This assay measures the influence of test compounds on mitochondrial activity, by using WST-1 reacting with the mitochondrial succinate-tetrazolium reductase forming the water-soluble formazan reaction product. The cytotoxicity of a test compound inversely correlates to the absorbance of the produced formazan quantified spectrophotometrically as described previously [22]. ES-D3 cells were exposed to test compounds for the duration of one day or five days. Briefly, cells were seeded in 96-well plates (Greiner bio-one) at concentrations of 20 x 10^4 cells/ml (one-day exposure) or 1 x 10^4 cells/ml (five-day exposure) in 100 µL culture medium in the absence of LIF and incubated for one day to allow cell adherence. Then the cells were exposed to the test compounds at concentrations up to 200 µM (final solvent concentration: 0.2% DMSO) and subsequently cultured for one or five days at 37°C and 5% CO_2 in a humidified atmosphere. Solvent DMSO (0.2%) was used as a negative control and Triton X-100 served as a positive control in all cytotoxicity assays. After incubation of one or five days, 20 µL WST-1 reagent (Roche, Woerden, the Netherlands) was added to each well and the plates were incubated for another 3 hours. Then absorbance was measured at 450 nm using SpectraMax M2 (Molecular Devices, Sunnyvale, USA). Three wells were used per treatment in each independent experiment. Three independent experiments were done for each compound. The cell viability was expressed as % of control, with the solvent control set at 100% viability. Reproducible results were obtained from the treatments of negative and positive controls in all of the cytotoxicity assays.

Differentiation assay with ES-D3 cells

Differentiation assays were carried out to detect the effects of test compounds on the differentiation of ES-D3 cells into contracting cardiomyocytes using culture medium in the absence of LIF. On day 1, droplets of 20 µl cell suspension (3.75 x 10^4 cells/ml) were placed as hanging drops, to which the test compounds were added at concentrations up to 100 µM (final solvent concentration: 0.2% DMSO), on the inner side of the lid of a 96-well plate. Sterile lids of eppendorf tubes were placed on the corner wells of the plates to prevent contact of the drops with the plate. The wells of the 96-well plate were filled with 250 µl phosphate buffered saline (PBS) (Invitrogen) and the plate was sealed with Micropore tape (3M, Neuss, Germany) to prevent evaporation of the hanging drops. Plates were incubated for three days at 37°C and 5% CO_2 in a humidified atmosphere. In the drops, cells formed embryonic bodies (EBs), which were transferred to non-tissue culture treated Petri dishes (diameter 6 cm, Greiner) with 5 ml of medium with test compound. On day 5, the EBs were transferred to a 24-well plate (Corning) with 1 ml

of medium with test compound, with one EB per well. On day 10, the number of wells containing contracting EBs was determined by visual inspection using a light microscope. Solvent control (DMSO) was included in each experiment. The solvent control was also used to assess the quality of the batch of ES-D3 cells used in each individual test (cells being randomly distributed over test groups and solvent control). Tests were accepted for further analysis if at least 21 of the 24 wells of the solvent control contained contracting cardiomyocytes. For each test compound, three independent assays were performed, at concentrations ranging from 0.1 to 100 µM. The results were expressed as "fraction of total", with 1.0 implying all EBs in one 24-well plate differentiated into contracting cardiomyocytes.

BeWo transport experiments

To study placental transfer of test compounds, BeWo transport experiments were performed as described previously [19]. Briefly, BeWo b30 cells (passages 27–45) were cultured in DMEM (Zwijndrecht, the Netherlands), supplemented with 10 % (v/v) heat-inactivated FCS (HyClone-Perbio, Etten-Leur, the Netherlands), 10,000 U/ml penicillin, 10 mg/ml streptomycin and 2 mM l-glutamine. The cells were seeded at a density of 1 x 10^5 cells/cm^2 on transwell® polycarbonate membranes (12 mm diameter, 0.4 µm pore size) (VWR International BV, Amsterdam, the Netherlands) coated with human placental collagen. The medium (0.5-ml apical compartment, 1.5-ml basolateral compartment) was replaced daily. At day 6 post-seeding, the BeWo b30 cell layers were used for transport experiments. The barrier forming capacity of the BeWo cell layers was evaluated by measuring the transepithelial electrical resistance (TEER) of the cell monolayer using a Millicell ERS-2 Volt-Ohm Meter (Millipore, USA) at day 6 post-seeding as described previously [19]. Only the cell layers showing TEER values between 80 and 100 Ω·cm^2 were used for transport experiments.

Transport experiments were initiated by adding 0.5 ml of transport buffer HBSS containing the test compound, added from a 200 times concentrated stock solution in DMSO, to the apical compartment and 1.5 ml transport buffer to the basolateral compartment (final solvent concentration: 0.5% DMSO). Subsequently, the plate was incubated in a humidified atmosphere with 5 % CO_2 at 37 °C. After 15, 30, 60 and 90 min, a sample of 0.2 ml was taken from the basolateral compartment and replaced by an equal volume of transport buffer. At the end of each experiment, a 0.2 ml sample was also taken from the apical compartment. The amount of compound accumulated in the cells was determined and mass-balance calculations were performed as described previously [19]. The transport of each test compound was investigated at an apical amount of 50 nmol (0.5 ml of a 100 µM solution) to allow detection of compounds transported to the basolateral compartment. In each transport study, amoxicillin was included as a control for monolayer integrity since it is a compound known to be transported only to a limited extent and antipyrine was included as a control for optimal transport and

a reference compound to enable calculation of relative transport rates. The transport of the five test compounds, as well as the standards amoxicillin and antipyrine, through the permeable membrane of the transwell filter in the absence of BeWo cells was determined as well. It was shown to be equally fast among these compounds (data not shown), ensuring that any differences observed in the BeWo model were related to the BeWo cell system and not to the filter.

As described in the analysis certificate sheets from Sigma-Alltech, ketoconazole and propiconaozle have two isomers. For tebuconazole, prothioconazole and fenarimol, only one dominant form is present in the product with >99% purity. To determine transport rates of isomers of ketoconazole and propiconazole across BeWo cell layers, additional transport experiments were carried out and samples were taken at 60 min (during the linear phase of transport, as determined in the earlier assays) from both apical and basolateral compartment. At this time we also determined the intracellular accumulation in the BeWo cells as described previously [19].

High-performance liquid chromatography analysis

Samples were analyzed using high-performance liquid chromatography (HPLC) to quantify the amount of test compound in order to determine the transport rate and to perform mass-balance calculations. The HPLC system used consisted of a Waters (Milford, MA) 600 controller and a 600 pump, equipped with a photodiode array detector set to record absorption of wavelengths between 200 and 400 nm. A Waters 717 plus autosampler was used for sample injection. The temperature of the autosampler was kept at 7 °C.

For analysis of all compounds, 50 µl sample was applied to a C18 5 µm reverse-phase column (150 mm × 4.6 mm I.D.) with a guard column (7.5 mm × 4.6 mm I.D.) (Alltech, Bergen op Zoom, the Netherlands). The mobile phase used for analysis of all the test compounds consisted of (A) 0.1 % trifluoroacetic acid in nanopure water and (B) HPLC-grade acetonitrile. Elution was at a flow rate of 0.8 ml/min, starting at 22 % B with a linear increase to 100 % B in 8 min. Subsequently, the gradient returned linearly to the initial condition in 10 min and remained 2 min at this condition prior to the next injection. In each experiment, calibration curves were included for quantification. Using this HPLC method the two isomers of ketoconazole appeared as one peak, whereas the two isomers of propiconazole were separated as two peaks. Isomers of ketoconazole were identified and quantified as described in the analysis certificate sheets from Sigma-Alltech with small modifications. To this end, 50 µl sample was applied to a CYCLOBOND I 2000 HP-RSP 5 µm column (250 mm x 4.6 mm I.D.) (Alltech, Bergen op Zoom, the Netherlands). The mobile phase used for analysis consisted of (A) 0.1 % formic acid in nanopure water and (B) HPLC-grade acetonitrile. Two isomers were separated at a flow rate of 1 ml/min by using an isocratic elution system with 70% A and 30% B.

Data analysis

BeWo transport data

For each compound, the linear appearance rate in the basolateral compartment was determined. These linear appearance rates were used to calculate apparent permeability (Papp) coefficients (Papp coefficient (cm/s) = $(\Delta Q/\Delta t) / (A \cdot C_0)$), where ΔQ is the amount of test compound (nmol) transported to the receiver chamber in a certain time span (Δt (s)), A is the cell surface area (cm^2) and C_0 is the initial concentration of the test compound (μM). To calculate ΔQ, a correction was made to compensate for the removal of compound when taking samples (ΔQ at t_{x+1} = amount measured at t_{x+1} (nmol) (basolateral concentration at t_{x+1} (μM) × 1.5 (ml)) supplemented with the amount removed at t_x (nmol) (basolateral concentration sample at t_x (μM) × 0.2 (ml))). Subsequently, relative Papp values were determined by expressing the Papp coefficient as a fraction of the Papp coefficient obtained for antipyrine. Amounts (nmol) of two isomers in the basolateral compartment, apical compartment and BeWo cell layer were analyzed at 60 min. Pairwise comparison of the isomers was performed using a two-sided student's T-test considering differences statistically significant when $p < 0.05$.

In vitro data

Different dichotomous concentration-response models were fitted to the developmental toxicity data obtained from the ES-D3 cell differentiation assay of the EST to calculate benchmark concentrations (BMC) using Environmental Protection Agency benchmark dose software (BMD) version 2.4. For each test compound, the BMC_d50, representing the concentration for a 50% reduction in the number of differentiated EBs, was derived. Models included in the evaluation were the gamma, logistic, loglogistic, probit, logprobit, multistage, weibull and the quantal-linear model. Goodness-of-fit of the models was evaluated to accept a model, based on the P-values, the scaled residuals and the graphical displays obtained. The lowest BMC_d50 value was chosen from the accepted models.

To combine in vitro developmental toxicity data obtained from the ES-D3 cell differentiation assay of the EST with placental transfer data obtained from the BeWo transport model, a corrected BMC_d50 value was calculated by dividing the BMC_d50 values by the relative Papp values.

In vivo data

A literature study was performed to determine the in vivo developmental toxicity potencies of each test compound. Search terms included the name of test compound together with combinations of the search terms teratogenicity, developmental toxicity, malformation, embryotoxicity and cleft palate. For the purpose of this paper, the results of studies performed in rats were used, mostly published in reports

of the Food and Agriculture Organization (FAO), World Health Organization (WHO), Environmental Protection Agency (EPA), European Commission (EC) and the European Food Safety Authority (EFSA) or in published papers on developmental toxicity. A study was selected if it included at least one control group and two dose groups, which enables analysis by the BMD approach to derive a BMD value. The incidence of cleft palate or skeletal malformations was selected as the in vivo endpoint for developmental toxicity, as they have shown to be the profound developmental toxic effects caused by antifungal compounds. For fenarimol, hydronephrosis was used as the in vivo endpoint since data were not available on cleft palate or skeletal malformations. A benchmark response (BMR) for the BMD for each compound was defined as a 10% extra incidence of malformations. The BMD10 was calculated using dichotomous concentration-response models as described for the in vitro data.

RESULTS

Cytotoxicity assay with ES-D3 cells

WST-1 assays for both one-day and five-day exposure were performed to evaluate the cytotoxic effects of the compounds on the ES-D3 cells (Figure 1). For all compounds, the concentrations tested (up to 100 μM) were non-cytotoxic as determined in the one-day cytotoxicity assay. Among the five compounds, fenarimol was the most potent one in the five-day cytotoxicity assay, reducing the cell viability to 50% at 100 μM. Exposure of 100 μM ketoconazole, prothioconazole and tebuconazole to the ES-D3 cells resulted in 40%, 25%, and 20% decline in cell viability, respectively, reflecting lower cytotoxic properties of these compounds than of fenarimol. Propiconazole did not cause any reduction in cell viability up to 100 μM.

Differentiation assay with ES-D3 cells

To study the in vitro developmental toxicity of the antifungal compounds, the effects of the compounds on the differentiation of ES-D3 cells into contracting cardiomyocytes were evaluated. All test compounds induced a concentration-dependent inhibition of the differentiation of the ES-D3 cells into contracting cardiomyocytes (Figure 1). The BMC_d50 values occurred at concentrations that did not cause cytotoxicity, indicating that inhibitory effects on the differentiation of EBs are not due to cytotoxicity of test compounds. BMC_d50 values are summarized in Table 1, showing that ketoconazole was the most potent in inhibiting the differentiation of EBs, followed by tebuconazole, fenarimol, prothioconazole and propiconazole.

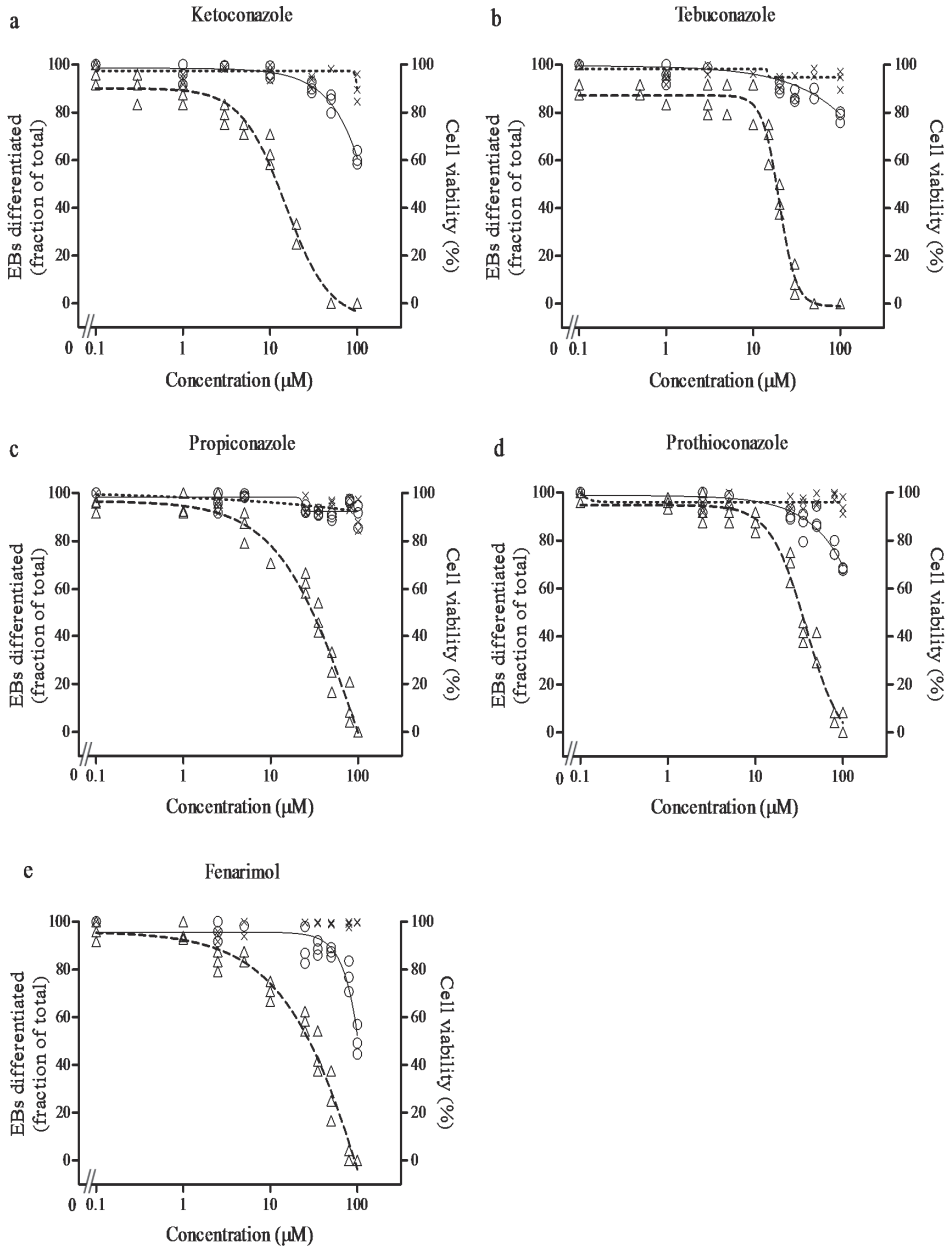

Fig. 1. Concentration-dependent effects of test compounds ketoconazole (a), tebuconazole (b), propiconazole (c), prothioconazole (d) and fenarimol (e) on cell viability for one-day (×) and five-day (○) exposure and on inhibition of ES-D3 cell differentiation (Δ). Results represent at least three independent experiments and are presented as mean ± standard deviation (n = 3).

Table 1. BMC_d50 and corrected BMC_d50 values for in vitro developmental toxicity of test compounds in the ES-D3 cell differentiation assay and BMD10 values for in vivo developmental toxicity of test compounds in rats.

	BMC_d50 (µM)	**Corrected BMC_d50 (µM)**	**BMD10 (cleft palate or skeletal malformation) (µmol/kg bw/day)**
Ketoconazole	10.63	13.46	20.14
Tebuconazole	18.85	21.92	27.55
Fenarimol	24.27	44.13	68.86
Propiconazole	38.64	55.19	90.99
Prothioconazole	35.42	88.55	212.4

In vitro BeWo transport

Ketoconazole, tebuconazole, propiconazole, prothioconazole and fenarimol were tested in the BeWo transport model to assess their transport across the placental barrier. For all transport experiments, the mass balances were between 91 and 99 %. Figure 2 shows the amount of test compounds in the basolateral compartment of the BeWo model with increasing time, after adding 50 nmol to the apical compartment. Antipyrine was included as a reference compound known to be efficiently transported across the BeWo cell layer [19] and amoxicillin was used as a control compound to check the integrity of the cell layer. The slow transfer of amoxicillin indicated an integrate BeWo cell

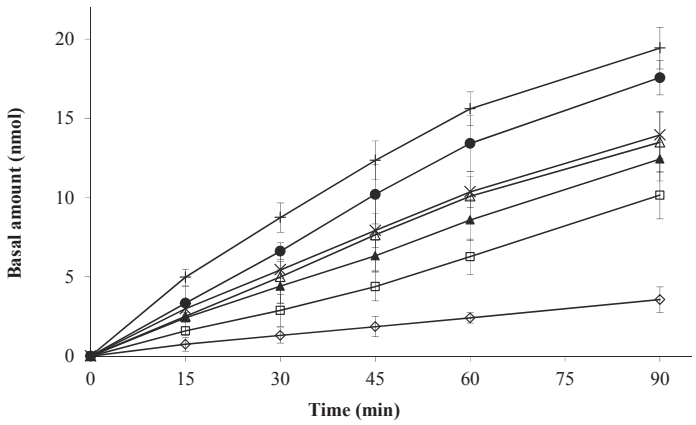

Fig. 2. Amount of amoxicillin (◊), prothioconazole (□), fenarimol (▲), ketoconazole (△), propiconazole (×), tebuconazole (●) and antipyrine (+) in the basolateral compartment in the in vitro BeWo model with increasing time using initial concentrations of 100 µM (50 nmol) in the apical compartment. Data are presented as mean ± standard deviation (n = 6).

layer. Up to 60 min, the transport of all compounds to the basolateral compartment was linear in time. Therefore, the linear appearance rate of mass in the basolateral compartment could be determined using data at 60 min for the calculation of Papp coefficients (Table 2). The data in Table 2 show a wide range of Papp coefficients for the five test compounds, illustrating different placental transfer rates among them, with tebuconazole being transported at the highest rate and prothioconazole at the lowest rate.

Table 2. Papp coefficients and relative Papp values of five test compounds and control compounds amoxicillin and antipyrine in the BeWo model.

	Papp coefficient (10^{-6} cm/s)	**Relative Papp value**
Amoxicillin	6 ± 0.8[a]	0.15
Prothioconazole	16 ± 2.7	0.40
Fenarimol	21 ± 3.2	0.55
Propiconazole	27 ± 3.8	0.70
Ketoconazole	31 ± 2.4	0.79
Tebuconazole	33 ± 4.3	0.86
Antipyrine	39 ± 2.6	1.00

[a] Mean ± SD

Extra transport experiments were performed to study placental transfer of different isomers across BeWo cell layers. Table 3 shows the amount of isomers of ketoconazole and propiconazole in the basolateral and apical compartment, as well as the amount accumulated in the BeWo cell layer, at 60 min after adding 50 nmol to the apical compartment in the BeWo transport model. The results of the t-test show that the difference between the amounts (basolateral, apical and intracellular) of the two isomers for both ketoconazole and propiconazole is not significant ($p>0.05$), indicating the same degree of transport across the BeWo cell layer for the isomers of both test compounds.

Table 3. Amount (nmol, mean ± SD) of isomers of ketoconazole and propiconazole in the basolateral compartment, apical compartment and BeWo cell layer at 60 min using initial concentrations of 100 μM (50 nmol) in the apical compartment.

		Isomer 1 (nmol)	Isomer 2 (nmol)
Ketoconazole	Basolateral	5.1 ± 0.4	4.8 ± 0.6
	Apical	17.4 ± 1.1	17.9±1.3
	Intracellular	1.3± 0.1	1.1 ± 0.1
Propiconazole	Basolateral	5.4 ± 0.8	5.2 ± 0.9
	Apical	17.3 ± 0.9	17.7± 1.5
	Intracellular	2.2± 0.2	2.1± 0.3

Combination of in vitro developmental toxicity data with BeWo transport data

To combine in vitro developmental toxicity data obtained from the ES-D3 cell differentiation assay of the EST with placental transfer data obtained from the BeWo transport model, a corrected BMC_d50 value was calculated by dividing the BMC_d50 values by the relative Papp values (Table 1). After the correction, the potency ranking was altered, with ketoconazole being the most toxic compound and prothioconazole the least potent one.

BMD derivation from in vivo data

A literature study on in vivo developmental toxicity was performed to obtain a potency ranking of the test compounds in vivo. For this ranking, studies performed in rats with oral administration were used. Table 4 provides an overview of the details of the animal studies used to derive BMD values. BMD10 values that correspond with a 10% extra incidence of cleft palate or skeletal malformations on litters or on fetuses were derived. When the best fits of BMD10 values derived on both litters and fetuses for a test compound were determined, the lower value (relating to a more sensitive endpoint) of the two was selected as the final BMD10 value for the compound. Table 1 shows the results of BMD analysis of these in vivo data (for details see Supplementary file).

Comparison of in vivo and in vitro developmental toxicity

Both BMC_d50 and corrected BMC_d50 values were compared with in vivo BMD10 values to assess the usefulness of taking into account BeWo transport data (Table 1). Based on the BMD10 values, ketoconazole was the most potent compound in vivo and prothioconazole was the least potent one. The data of these compounds in the ES-D3 cell differentiation assay of the EST correlate to some extent with the BMD10

values derived from the in vivo data with R^2 being 0.57 (Figure 3). When the ES-D3 cell differentiation data are combined with the results obtained in the BeWo transport model to obtain corrected BMC_d50 values, a better correlation with the in vivo BMD10 values was obtained indicated by an increase of the R^2 to 0.95 (Figure 3). These data thus demonstrate the power of including a component of kinetics when predicting relative in vivo toxicity potencies based on in vitro toxicity data.

Table 4. Developmental data on the incidence of cleft palate or skeletal malformation in rat

Compound	Days	Dose (mg/kg bw/day)	No. of fetuses (litters) examined	Incidence of cleft palate or skeletal malformation on fetuses (litters)
Ketoconazole[a]	PI	0	- (22)	- (0)
	6-17	25	- (22)	- (3)
		75	- (3)	- (3)
Tebuconazole[b]	GD	0	144(24)	29(14)
	6-15	30	137(24)	40(17)
		60	127(22)	38(17)
		120	116(24)	48(20)
Propiconazole[c]	GD	0	141(22)	0(0)
	6–15	30	148(21)	1(1)
		90	156(22)	4(4)
		300	148(22)	53(16)
Prothioconazole[d]	PC	0	-	23.5%(95.2%)
	6-19	20	-	18.2%(77.8%)
		80	-	27.6%(88.9%)
		750	-	33.6%(95.7%)
Fenarimol[e]	GD	0	-	9%(25%)
	6-15	5	-	0(0)
		13	-	0(0)
		35	-	30%(62%)

Note. PI = post-insemination; GD = gestational day; PC=post coitum

[a] data from [31] based on increased incidences of cleft palate.

[b] data from [32] based on increased incidences of skeletal variations.

[c] data from [33] based on increased incidence of skeletal malformations.

[d] data from [34] based on increased incidence of skeletal variations.

[e] data from [35] based on increased hydronephrosis, as no cleft palate or skeletal malformations were reported.

- data not available.

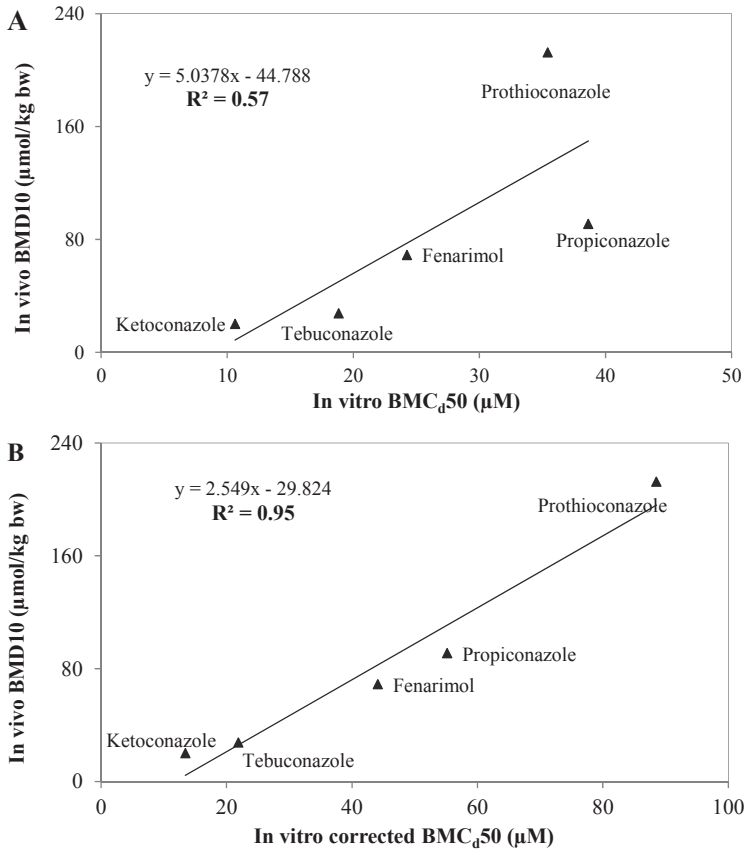

Fig. 3. Correlation between in vivo BMD10 values of five test compounds ketoconazole, tebuconazole, propiconazole, prothioconazole and fenarimol, as derived based on data reported in the literature, with (A) in vitro BMC_d50 values, obtained from the ES-D3 cell differentiation assay of the EST, and with (B) in vitro corrected BMC_d50 values, obtained from combining in vitro BMC_d50 values from the ES-D3 differentiation assay with relative P_{app} values from the BeWo transport model.

DISCUSSION

Several in vitro studies have been performed with the ES-D3 cell differentiation assay of the EST to predict in vivo developmental toxicity potency ranking of series of structurally related compounds, and it has been suggested that the capacity of this assay to predict in vivo potency ranking could be improved by combining kinetic information with the in vitro ES-D3 cell differentiation data [9-11]. Therefore, in the present study, one of the important in vivo kinetic processes for developmental toxicity, i.e. placental transfer, was taken into account when in vivo developmental toxicity ranking of five antifungal

compounds was predicted based on in vitro toxicity data obtained in the ES-D3 cell differentiation assay of the EST.

The in vitro BeWo transport model was used in the present study, as it was previously shown to be suitable to adequately characterize relative placental transfer rates of compounds [19, 23, 24]. Our intention was to determine the Papp values and to use relative Papp values to adjust the BMC_d50 values, obtained from the differentiation assay, for placental transfer. We did not aim to test the transport mechanisms of the compounds in this study. The five test compounds showed different transfer rates through the placental barrier in the BeWo model, indicating that transport velocities to the fetus in vivo may differ as well. However, how this exactly translates to differences in fetal exposure is not known, due to the fact that in vivo experimentation of placental transport in humans is not feasible on a large scale for obvious ethical reasons and little has been published on the fetal bioavailability of antifungal compounds in human. Therefore, we hypothesize that the extent of fetal exposure would be positively correlated to the relative transport velocity in the BeWo model. This means that a compound with a relatively low Papp coefficient may cause a relatively low fetal exposure, whereas a compound with a relatively high Papp coefficient may cause a relatively high fetal exposure. Therefore, we investigated whether the correlation between in vitro BMC_d50 values and in vivo developmental toxicity dose levels improved when we corrected the in vitro BMC_d50 values for differences in placental transfer, by dividing these values for the five model compounds in the ES-D3 cell differentiation assay of the EST by relative Papp values obtained in the BeWo model.

The results obtained reveal that the corrected BMC_d50 values correlated better than the uncorrected ones with the in vivo BMD10 values. This indicates that combining ES-D3 cell differentiation assay results with placental transfer kinetics improved the ability of this in vitro assay to predict relative in vivo developmental toxicity potencies of antifungal compounds. It should be noted that the BeWo cell model is not suitable to determine the absolute transport rates of compounds, given that the BeWo cell model is a simplification of the in vivo placental transfer system. This is related to several parameters that are different from the in vivo situation, e.g. circulation, flow rate and the volumes of maternal and fetal compartments. In those cases where it is important to use exact fetal bioavailability values, physiologically based kinetic (PBK) models which describe the in vivo absorption, distribution, metabolism, and excretion processes of a compound should be used. Other important in vivo kinetic processes than placental transfer, such as intestinal absorption, maternal metabolism and placental metabolism, were not taken into account in the present study. Therefore, incorporating more kinetic information is likely to further improve the predictive value of the ES-D3 cell differentiation assay of the EST. Therefore, a PBK model, should be the ultimate goal for the translation of in vitro toxicity data to the in vivo situation [25, 26]. The present study demonstrates that placental transfer should be one of the components in these PBK models.

In the ES-D3 cell differentiation assay of the EST, the developmental toxicity potency of test compounds is investigated by evaluating their inhibitory effects on the differentiation of mouse ES-D3 cells into contracting cardiomyocytes. The ES-D3 cell differentiation assay of the EST is considered to represent the fetal component of developmental toxicity. As the (azole) antifungal compounds are also known for their abilities to inhibit cytochrome enzymes (Marotta and Tiboni 2010), and in particular may inhibit estrogen biosynthesis through CYP 19 aromatase inhibition, reducing the conversion of androgens to estrogens [27], this aspect, which takes place in the maternal organism, merits attention. The phenotypic analysis of the inhibition of cardiac differentiation in the ES-D3 cell differentiation assay of the EST cannot take this part of developmental toxicity into account. Therefore, other relevant in vitro assays, such as steroidogenesis assay, may be needed. In addition, although the ES-D3 cell differentiation assay of the EST combined with the BeWo transport assay shows to be an appropriate in vitro toxicological system to achieve an enhanced predictivity of the relative developmental toxicity for antifungal compounds, combined testing strategies based on in vitro assays should be used to provide relevant mechanistic information for human risk assessment. For example, the ES-D3 cell differentiation assay could be combined with transcriptomics to be able to get information on gene expression changes after exposure and the underlying mechanisms [28].

It is well known that the placental transfer rates of the compounds can be influenced by their physicochemical and molecular properties [29]. In fact, small molecules (molecular weight < 500) were reported to diffuse more readily than larger ones and low lipophilicity may exhibit restricted transport across the placental barrier [29]. It has also been found that the number of hydrogen bonding acceptor or donor sites was a very important descriptor that correlate negatively with the permeability values across the placental barrier. Moreover, a positive dependence of the placental transfer on log D has been reported [30]. Each of these physicochemical and molecular properties may provide useful information on evaluating permeability characteristic of compounds, but cannot be used independently to determine transport rate, as placental transfer is a complex process and the influence on the transfer of compounds is a conglomeration of different physicochemical and molecular properties. Therefore, a quantitative structure-activity relationship (QSAR) methodology for modeling transport across the human placental barrier was established using a multivariate data analysis approach on 84 compounds by Giaginis et al. [30]. The QSAR model used 16 descriptors and the predicted permeability values, relative to antipyrine, were compared with the relative transport values to antipyrine obtained from ex vivo human placental perfusion experiments compiled from numerous sources. A good correlation was found, with r^2 being 0.73, showing that this model is an adequate and robust tool, providing an informative illustration of the contributing physicochemical, molecular and structural properties of the compounds

in placental transfer process. However, in their study, 4 compounds were defined as outliers and excluded from the model, rendering the prediction model less reliable due to its incapability to analyze the permeability of the compounds sharing the similar physicochemical and molecular properties with those 4 compounds [30]. In addition, this in silico model exhibits limited reflection on the interactions between compounds and biological systems, for example the interaction with the cell membrane transporters, which are essential information for compound's permeation through a placental barrier. In contrast, the relative Papp values obtained our BeWo cell model were proven to correlate very well with the transport in the ex vivo placental perfusion model based on 9 model compounds, with an even higher r^2 being 0.95 [19], and it allows the direct examination on the interaction of compounds with a biological system, indicating it outperforms a simple in silico membrane model. Although, as compared to an in silico model, the BeWo cell model is relatively more time-consuming and laborious, it can still be used in a high-throughput manner.

Some of the compounds investigated are present in the form of different isomers (stereoisomerism), and each of these isomers may have different physical properties and biological effects. We therefore considered that it was important to know if different isomers could transfer through the placental barrier at different rates. Both ketoconazole and propiconazole have two isomers and each compound was tested in vivo and in the in vitro systems as a mixture of isomers. The results obtained with the BeWo model in the present study revealed that the isomers of both compounds transfer through BeWo cell layers at a comparable rate. Therefore, an equal exposure of the fetus to the isomers may be expected, which indicates that the exposure to the combination of the two isomers in the ES-D3 cell differentiation assay is expected to be relevant for the in vivo situation.

In conclusion, in the present study we showed, for the first time, that the ES-D3 cell differentiation assay of the EST, combined with the in vitro BeWo transport model, is able to better predict the in vivo developmental toxicity ranking of the antifungal compounds, than as a stand-alone assay, which could be a valuable alternative for developmental toxicity screening and prioritization. More compounds with different modes of action should be tested to further evaluate the capacity of this approach in predicting the developmental toxicity ranking of chemicals. At this stage the applicability domain of this combined in vitro toxico-dynamics and –kinetics approach is not known. From a conceptual point of view, it would seem that there is no reason why the value of this approach should be limited to the class of (azole) antifungal compounds. Since the method uses only in vitro assays to predict in vivo developmental toxicity ranking of chemicals, it can contribute to the 3Rs of animal testing. The predictive ability of the ES-D3 cell differentiation assay of the EST can most likely be further enhanced if it is combined with more kinetic data.

FUNDING

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NOTES

The authors declare that they have no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table 1. Overview of BMD analysis performed using BMDS software version 2.4, a BMD of 10% extra risk and default settings. BMD10 values are derived from the incidence of cleft palate on litters in rats exposed to ketoconazole. The data used as input for the BMD analysis are presented in Table 3.

Model Name	No. of parameters	Log Likelihood	P-value	Accepted[a]	BMD10 (µmol/kg bw/day)
Null	1	-14.548	-	-	-
Full	3	-8.762	-	-	-
Gamma	1	-8.763	1	Yes	44.82
Logistic	2	-8.763	0.99	Yes	45.96
LogLogistic	2	-8.762	0.99	Yes	46.12
LogProbit	2	-8.763	0.99	Yes	45.59
Multistage	1	-9.465	0.64	Yes	34.21
Probit	2	-8.763	0.99	Yes	45.01
Weibull	2	-8.763	0.99	Yes	43.51
Quantal-Linear	1	-11.193	0.16	Yes	20.14

[a] Fitted model not significantly different (worse) than the full model at $p<0.05$.

Table 2. Overview of BMD analysis performed using BMDS software version 2.4, a BMD of 10% extra risk and default settings. BMD10 values are derived from the incidence of skeletal variation on litters in rats exposed to tebuconazole. The data used as input for the BMD analysis are presented in Table 3.

Model Name	No. of parameters	Log Likelihood	P-value	Accepted[a]	BMD10 (µmol/kg bw/day)
Null	1	-55.432	-	-	-
Full	4	-53.392	-	-	-
Gamma	2	-53.475	0.92	Yes	42.59
Logistic	2	-53.523	0.88	Yes	50.84
LogLogistic	3	-53.399	0.99	Yes	27.55
LogProbit	3	-53.597	0.81	Yes	77.63
Multistage	2	-53.475	0.92	Yes	42.59
Probit	2	-53.542	0.86	Yes	53.65
Weibull	2	-53.475	0.92	Yes	42.59
Quantal-Linear	2	-53.475	0.92	Yes	42.59

[a] Fitted model not significantly different (worse) than the full model at $p<0.05$.

Table 3. Overview of BMD analysis performed using BMDS software version 2.4, a BMD of 10% extra risk and default settings. BMD10 values are derived from the incidence of skeletal variation on fetuses in rats exposed to tebuconazole. The data used as input for the BMD analysis are presented in Table 3.

Model Name	No. of parameters	Log Likelihood	P-value	Accepted[a]	BMD10 (µmol/kg bw/day)
Null	1	-318.207	-	-	-
Full	4	-311.237	-	-	-
Gamma	2	-311.607	0.92	Yes	139.33
Logistic	2	-311.721	0.88	Yes	170.50
LogLogistic	3	-311.573	0.97	Yes	127.71
LogProbit	3	-311.613	0.98	Yes	214.94
Multistage	2	-311.607	0.92	Yes	139.33
Probit	2	-311.703	0.86	Yes	166.72
Weibull	2	-311.607	0.92	Yes	139.33
Quantal-Linear	2	-311.607	0.92	Yes	139.35

[a] Fitted model not significantly different (worse) than the full model at $p<0.05$.

Table 4. Overview of BMD analysis performed using BMDS software version 2.4, a BMD of 10% extra risk and default settings. BMD10 values are derived from the incidence of skeletal malformation on litters in rats exposed to propiconazole. The data used as input for the BMD analysis are presented in Table 3.

Model Name	No. of parameters	Log Likelihood	P-value	Accepted[a]	BMD10 (µmol/kg bw/day)
Null	1	-48.081	-	-	-
Full	4	-27.342	-	-	-
Gamma	2	-27.397	0.95	Yes	161.95
Logistic	2	-28.511	0.44	Yes	282.92
LogLogistic	2	-27.466	0.88	Yes	165.46
LogProbit	2	-27.638	0.73	Yes	157.67
Multistage	2	-27.342	1	Yes	258.96
Probit	2	-28.252	0.53	Yes	163.14
Weibull	2	-27.363	0.98	Yes	90.99
Quantal-Linear	1	-28.490	0.56	Yes	161.95

[a] Fitted model not significantly different (worse) than the full model at $p<0.05$.

Table 5. Overview of BMD analysis performed using BMDS software version 2.4, a BMD of 10% extra risk and default settings. BMD10 values are derived from the incidence of skeletal malformation on fetuses in rats exposed to propiconazole. The data used as input for the BMD analysis are presented in Table 3.

Model Name	No. of parameters	Log Likelihood	P-value	Accepted[a]	BMD10 (µmol/kg bw/day)
Null	1	-185.075	-	-	-
Full	4	-115.666	-	-	-
Gamma	3	-116.191	0.35	Yes	283.20
Logistic	2	-116.449	0.62	Yes	367.70
LogLogistic	3	-116.123	0.36	Yes	283.06
LogProbit	3	-116.305	0.33	Yes	277.05
Multistage	1	-116.203	0.8	Yes	266.55
Probit	2	-116.143	0.76	Yes	337.06
Weibull	3	-116.051	0.36	Yes	284.61
Quantal-Linear	1	-126.274	0.0007	No	169.92

[a] Fitted model not significantly different (worse) than the full model at $p<0.05$.

Table 6. Overview of BMD analysis performed using BMDS software version 2.4, a BMD of 10% extra risk and default settings. BMD10 values are derived from the incidence of skeletal variations on litters in rats exposed to prothioconazole. The data used as input for the BMD analysis are presented in Table 3.

Model Name	No. of parameters	Log Likelihood	P-value	Accepted[a]	BMD10 (µmol/kg bw/day)
Null	1	-33.807	-	-	-
Full	4	-31.199	-	-	-
Gamma	3	-32.976	0.06	Yes	486.86
Logistic	2	-33.020	0.17	Yes	222.31
LogLogistic	3	-32.976	0.06	Yes	431.27
LogProbit	3	-32.966	0.07	Yes	302.93
Multistage	2	-36.274	0.15	No	972.94
Probit	2	-33.016	0.17	Yes	238.49
Weibull	3	-32.982	0.06	Yes	1853.11
Quantal-Linear	2	-33.023	0.17	Yes	212.40

[a] Fitted model not significantly different (worse) than the full model at $p<0.05$.

Table 7. Overview of BMD analysis performed using BMDS software version 2.4, a BMD of 10% extra risk and default settings. BMD10 values are derived from the incidence of skeletal malformation on fetuses in rats exposed to prothioconazole. The data used as input for the BMD analysis are presented in Table 3.

Model Name	No. of parameters	Log Likelihood	P-value	Accepted[a]	BMD10 (µmol/kg bw/day)
Null	1	-57.016	-	-	-
Full	4	-56.179	-	-	-
Gamma	2	-56.442	0.77	Yes	1416.99
Logistic	2	-56.451	0.77	Yes	1541.92
LogLogistic	3	-56.421	0.49	Yes	1361.97
LogProbit	3	-56.408	0.50	Yes	1803.279
Multistage	2	-52.922	0.62	Yes	1321.42
Probit	2	-56.450	0.77	Yes	1527.41
Weibull	2	-56.442	0.77	Yes	1417.00
Quantal-Linear	2	-56.442	0.77	Yes	1417.00

[a] Fitted model not significantly different (worse) than the full model at $p<0.05$.

Table 8. Overview of BMD analysis performed using BMDS software version 2.4, a BMD of 10% extra risk and default settings. BMD10 values are derived from the incidence of hydronephrosis on litters in rats exposed to fenarimol. The data used as input for the BMD analysis are presented in Table 3.

Model Name	No. of parameters	Log Likelihood	P-value	Accepted[a]	BMD10 (µmol/kg bw/day)
Null	1	-52.372	-	-	-
Full	4	-30.66	-	-	-
Gamma	2	-38.122	0.001	no	72.91
Logistic	2	-42.276	0	no	37.05
LogLogistic	2	-38.114	0.001	no	91.76
LogProbit	3	-38.114	0.0002	no	81.73
Multistage	2	-40.622	0.0002	no	42.83
Probit	2	-42.851	0	no	33.91
Weibull	3	-38.114	0.0002	no	93.74
Quantal-Linear	2	-45.896	0	no	26.47

[a] Fitted model not significantly different (worse) than the full model at $p<0.05$.

Table 9. Overview of BMD analysis performed using BMDS software version 2.4, a BMD of 10% extra risk and default settings. BMD10 values are derived from the incidence of hydronephrosis on fetuses in rats exposed to fenarimol. The data used as input for the BMD analysis are presented in Table 3.

Model Name	No. of parameters	Log Likelihood	P-value	Accepted[a]	BMD10 (µmol/kg bw/day)
Null	1	-31.956	-	-	-
Full	4	-22.835	-	-	-
Gamma	2	-25.378	0.098	Yes	88.48
Logistic	2	-26.787	0.02	no	67.42
LogLogistic	3	-25.377	0.03	no	98.37
LogProbit	3	-25.377	0.03	no	92.30
Multistage	2	-25.128	0.04	Yes	68.86
Probit	2	-27.051	0.02	no	64.042
Weibull	3	-25.377	0.03	no	99.11
Quantal-Linear	2	-28.520	0.02	no	60.54

[a] Fitted model not significantly different (worse) than the full model at $p<0.05$.

CHAPTER 4

Extended evaluation on the ES-D3 cell differentiation assay combined with the BeWo transport model, to predict relative developmental toxicity of triazole compounds

Hequn Li, Burkhard Flick, Ivonne M. C. M. Rietjens, Jochem Louisse, Steffen Schneider, Bennard van Ravenzwaay

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ABSTRACT

The mouse embryonic stem D3 (ES-D3) cell differentiation assay is based on the morphometric measurement of cardiomyocyte differentiation and is a promising tool to detect developmental toxicity of compounds. The BeWo transport model, consisting of BeWo b30 cells grown on transwell inserts and mimicking the placental barrier, is useful to determine relative placental transport velocities of compounds. We have previously demonstrated the usefulness of the ES-D3 cell differentiation assay in combination with the in vitro BeWo transport model to predict the relative in vivo developmental toxicity potencies of a set of reference azole compounds. To further evaluate this combined in vitro toxicokinetic and toxicodynamic approach, we combined ES-D3 cell differentiation data of 6 novel triazoles with relative transport rates obtained from the BeWo model and compared the obtained ranking to the developmental toxicity ranking as derived from in vivo data. The data show that the combined in vitro approach provided a correct prediction for in vivo developmental toxicity, whereas the ES-D3 cell differentiation assay as stand-alone did not. In conclusion, we have validated the combined in vitro approach for developmental toxicity, which we have previously developed with a set of reference azoles, for a set of six novel triazoles. We suggest that this combined model, which takes both toxicodynamic and toxicokinetic aspects into account should be further validated for other chemical classes of developmental toxicants.

INTRODUCTION

The EU REACH legislation requires the safety assessment for new and existing chemicals. At present, regulatory safety assessment is predominantly performed using animal models, with large numbers of animals needed particularly for developmental toxicity testing. Therefore, REACH stimulates the use of animal-free approaches wherever possible [1, 2]. Alternative test methods for in vivo developmental toxicity testing, accepted for use in regulatory toxicity testing, are urgently needed [3]. The mouse embryonic stem D3 (ES-D3) cell differentiation assay is a valuable tool that can be used to predict in vivo developmental toxicity potency rankings within selected chemical classes for most of the chemicals investigated so far [4-6]. However, as this assay does not take kinetic processes into account, it was suggested to combine the ES-D3 cell differentiation assay with data on kinetics, e.g. placental transfer of compounds, in order to better predict in vivo potency of the developmental toxicants [4-6]. We have previously shown that an in vitro BeWo transport model is capable of predicting relative placental transfer rates of a set of 9 model compounds with a good correlation to the relative transport rates observed in the ex vivo placental perfusion model (R^2=0.95), indicating that the in vitro BeWo transport model is useful to obtain data with respect to placental transfer of compounds [7]. When combining the BeWo model with the ES-D3 cell differentiation assay, for a set of reference azoles, the coefficient of determination (R^2) for correlation of relative in vitro potency with relative in vivo potency increased from 0.57 to 0.95 [8], showing that the combined approach is able to better predict the in vivo developmental toxicity of azole antifungal compounds than the stand-alone ES-D3 cell differentiation assay.

Our first study included data on five reference antifungal compounds, with four of them being azoles. The azole family represents the largest family of antifungal compounds, which can be subdivided into the imidazole and triazole groups. We selected the class of azoles, because of their widespread use as antifungal agents in medicine and crop protection [9] and because they are known to cause developmental toxicity, for which an extensive toxicity data base is available, required for evaluation of predictions made. The reference azoles used demonstrated a varying degree of developmental toxicity [10, 11], allowing for a quantitative assessment of potency, which was the basis of the first validation of the combined ES-D3 and BeWo assay strategy.

The aim of the present study is to further validate the combined ES-D3 cell differentiation assay and BeWo transport model for predicting in vivo potency of developmental toxicants. To this end, six novel triazoles (Table 1) were investigated in the combined in vitro approach and the obtained predictions for these compounds were compared with the in vivo data that are presented in this paper for the first time, not taken from the literature.

Table 1. Chemical information of six triazoles tested in the present study.

Code	Name	MW (g/mol)
0594	[5-(4-Chloro-2-fluoro-phenyl)-3-(2,4-difluoro-phenyl)-isoxazol-4-yl]-pyridin-3-yl-methanol	416.8
0595	[2,4-Bis-(2,4-difluoro-phenyl)-thiophen-3-yl]-pyridin-3-yl-methanol	415.4
0596	4-{2-[2-(4-Fluoro-phenyl)-2-hydroxy-1-methyl-3-[1,2,4] triazol-1-yl-propyl]-thiazol-4-yl}-benzonitrile	419.5
0599	4-[2-[2-(4-fluorophenyl)-2-hydroxy-3-imidazol-1-yl-1-methyl-propyl] thiazol-4-yl]benzonitrile	418.5
0600	1-[[2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4,6-dimethyl-1,3-dioxan-2-yl]methyl]-1,2,4-triazole	434.3
0618	4-methyl-1,3-dioxolan-2-yl-methyl-1,2,4-triazole derivate	440.0

MATERIALS AND METHODS

Chemicals

BASF triazoles 0594, 0595, 0596, 0599, 0600 and 0618 were kindly provided by BASF SE (Ludwigshafen, Germany). Dimethyl sulfoxide (DMSO) was purchased from Acros Organics (Geel, Belgium).

BeWo transport experiments

To study placental transfer of test compounds, BeWo transport experiments were performed as described previously [7]. Briefly, BeWo b30 cells (passages 27–45) were cultured in DMEM (Zwijndrecht, the Netherlands), supplemented with 10 % (v/v) heat-inactivated FCS (HyClone-Perbio, Etten-Leur, the Netherlands), 10,000 U/ml penicillin, 10 mg/ml streptomycin and 2 mM L-glutamine. The cells were seeded at a density of 1 x 10^5 cells/cm^2 on transwell® polycarbonate membranes (12 mm diameter, 0.4 µm pore size) (VWR International BV, Amsterdam, the Netherlands) coated with human placental collagen. The medium (0.5-ml apical compartment, 1.5-ml basolateral compartment) was replaced daily. At day 6 post-seeding, the BeWo b30 cell layers were used for transport experiments. The barrier forming capacity of the BeWo cell layers was evaluated by measuring the transepithelial electrical resistance (TEER) of the cell monolayer using a Millicell ERS-2 Volt-Ohm Meter (Millipore, USA) at day 6 post-seeding as described previously [7]. Only the cell layers showing TEER values between 80 and 100 Ω·cm^2 were used for transport experiments.

Transport experiments were initiated by adding 0.5 ml of transport buffer Hank's balanced salt solution (HBSS, Invitrogen, Breda, the Netherlands) containing the test compound, being 0594, 0595, 0596, 0599, 0600 and 0618, dissolved in DMSO (final solvent concentration 0.5% DMSO), at concentration of 50 µM, to the apical

compartment and 1.5 ml transport buffer to the basolateral compartment. Subsequently, the plate was incubated in a humidified atmosphere with 5 % CO_2 at 37 °C. After 15, 30, 60 and 90 min, a sample of 0.2 ml was taken from the basolateral compartment and replaced by an equal volume of transport buffer. At the end of each experiment, a 0.2 ml sample was also taken from the apical compartment. Subsequently, the filters with the BeWo b30 cell layers were washed 3 times with PBS, cut out of the insert, dissolved in 0.25 ml 65 % (v/v) methanol and sonificated for 15 min in a Bandelin Sonorex RK100 (Berlin, Germany) in order to determine the amount of compound accumulated in the cells. After each experiment, mass-balance calculations were performed. In each transport study, amoxicillin and antipyrine were included as control and reference compounds. Amoxicillin transport was included to assess for monolayer integrity since it is a compound known to be transported only to a limited extent across the placenta, whereas antipyrine was included as a control for optimal transport and a reference compound to enable calculation of relative transport rates [7]. The transport of the six test compounds, as well as the reference compounds amoxicillin and antipyrine, through the permeable membrane of the transwell filter in the absence of BeWo cells was determined as well. The transport to the basolateral compartment was shown to be equally fast for all compounds (data not shown), ensuring that any differences observed in the transport studies were related to the BeWo cell layer and not to the filter. For each test compound, three independent experiments were performed, with three technical replicates per experiment.

High-performance liquid chromatography analysis

Samples were analyzed using high-performance liquid chromatography (HPLC) to quantify the amount of test compound in order to determine the transport rate and to perform mass-balance calculations. The HPLC system used consisted of a Waters (Milford, MA) 600 controller and a 600 pump, equipped with a photodiode array detector set to record absorption of wavelengths between 200 and 400 nm. A Waters 717 plus autosampler was used for sample injection. The temperature of the autosampler was kept at 7 °C.

For analysis of all compounds, 50 µl sample was injected to a C18 5 µm reverse-phase column (150 mm × 4.6 mm I.D.) with a guard column (7.5 mm × 4.6 mm I.D.) (Alltech, Bergen op Zoom, the Netherlands). The mobile phase used for analysis of all the test compounds consisted of (A) 0.1 % trifluoroacetic acid in nanopure water and (B) HPLC-grade acetonitrile. Elution was at a flow rate of 0.8 ml/min, starting at 22 % B with a linear increase to 100 % B in 8 min. Subsequently, the gradient returned linearly to the initial condition in 10 min and remained 2 min at this condition prior to the next injection. In each analysis, calibration curves of all compounds were included for quantification.

ES-D3 cell culture

The murine ES-D3 cell line was purchased from ATCC (Wesel, Germany). The cells were maintained in polystyrene cell culture flasks (Corning, the Netherlands) in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Breda, The Netherlands), supplemented with 20% heat-inactivated fetal calf serum (Lonza, BioWhittaker, Verviers, Belgium), 50 U/ml penicillin with 50 µg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich) and 1% (v/v) non-essential amino acids (Invitrogen), at 37°C and 5% CO_2 in a humidified atmosphere. Cells were kept undifferentiated with 1000 U/ml murine leukemia inhibiting factor (LIF) (Sigma-Aldrich) and subcultured every 2 to 3 days using non-enzymatic cell dissociation solution (Sigma-Aldrich) to detach the cells.

Cytotoxicity assay with ES-D3 cells

To determine cytotoxicity of the compounds, a WST-1 assay was performed. This assay measures the influence of test compounds on the formation of the water-soluble formazan reaction product from WST-1 by mitochondrial succinate-tetrazolium reductase enzymes. The cytotoxicity of a test compound inversely correlates to the absorbance of the produced formazan quantified spectrophotometrically as described previously [12]. ES-D3 cells were exposed to test compounds for the duration of one day or five days as described before [8]. Briefly, cells were seeded in 96-well plates (Greiner bio-one) at concentrations of 20 x 10^4 cells/ml (one-day exposure) or 1 x 10^4 cells/ml (five-day exposure) in 100 µL culture medium in the absence of LIF and incubated for one day to allow cell adherence. Then the cells were exposed to the test compounds at concentrations up to 60 µM (final solvent concentration: 0.2% DMSO) and subsequently cultured for one or five days at 37°C and 5% CO_2 in a humidified atmosphere. Solvent DMSO (0.2%) was used as a negative control and 1% Triton X-100 served as a positive control in all cytotoxicity assays. After incubation of one or five days, 20 µL WST-1 reagent (Roche, Woerden, the Netherlands) was added to each well and the plates were incubated for another 3 hours. Then absorbance was measured at 450 nm using a SpectraMax M2 (Molecular Devices, Sunnyvale, USA). Three wells were used per treatment in each independent experiment. Three independent experiments were done for each compound. The cell viability was expressed as % of the solvent control, with the solvent control set at 100% viability. Reproducible results were obtained from the treatments of negative and positive controls in all of the cytotoxicity assays.

Differentiation assay with ES-D3 cells

Differentiation assays were carried out to detect the effect of test compounds on the differentiation of ES-D3 cells into contracting cardiomyocytes using culture medium in the absence of LIF. On day 1, droplets of 20 µl cell suspension (3.75 x 10^4 cells/ml) were placed as hanging drops, to which the test compounds 0594, 0595, 0596, 0599,

0600 and 0618 were added at concentrations ranging from 0.2 to 60 µM (final solvent concentration: 0.2% DMSO), on the inner side of the lid of a 96-well plate. Sterile lids of eppendorf tubes were placed on the corner wells of the plates to prevent contact of the drops with the plate. The wells of the 96-well plate were filled with 250 µl phosphate buffered saline (PBS) (Invitrogen) and the plate was sealed with Micropore tape (3M, Neuss, Germany) to prevent evaporation of the hanging drops. Plates were incubated for three days at 37°C and 5% CO_2 in a humidified atmosphere. In the drops, cells formed embryonic bodies (EBs), which were transferred to non-tissue culture treated Petri dishes (diameter 6 cm, Greiner) with 5 ml of medium with test compound. On day 5, the EBs were transferred to a 24-well plate (Corning) with 1 ml of medium with test compound, with one EB per well. On day 10, the number of wells containing contracting EBs was determined by visual inspection using a light microscope. Solvent control (0.2% DMSO in culture medium) was included in each experiment. The solvent control was also used to assess the quality of the batch of ES-D3 cells used in each individual test (cells being randomly distributed over test groups and solvent control). Tests were accepted for further analysis if at least 21 of the 24 wells of the solvent control contained contracting cardiomyocytes. For each test compound, three independent assays were performed. The results were expressed as "fraction of total", with 1.0 implying all EBs in one 24-well plate differentiated into contracting cardiomyocytes.

In vivo experiments

The in vivo investigations of maternal and prenatal developmental toxicity were performed as a screening approach following the general principles of OECD 414 and OPPTS 870.3700 test guidelines, and the OECD and United States Environmental Protection Agency Good Laboratory Practice Standards [40 CFR Part 160 (FIFRA) and Part 792 (TSCA)]. The major differences to a full guideline study were the smaller sample size, using 9-10 instead of at least 16 pregnant rats, per test substance. Furthermore, the fetal evaluation was performed using 4 to 10 litters, the lower number being used if sufficient (i.e. positive) results were obtained to assess the prenatal developmental toxicological potential of the test compounds. The in vivo studies were performed according to the German Animal Welfare Act, the European Council Directive 2010/63/EU and in an AAALAC-accredited facility. This screening study of developmental compounds was approved by the local authorizing agency for animal experiments (Landesuntersuchungsamt Koblenz, Germany) as referenced by the approval number 23 177-07/G08-3-008).

Test Animals

The animals were paired by the breeder (time-mated Wistar rats, Crl: WI [Han], Charles River Laboratories, Sulzfeld, Germany), between 10 and 12 weeks of age, and supplied on the day of evidence of mating; this day is referred to as gestational day

(GD) 0 and following day as GD 1. All animals showed no clinical signs of disease. This strain has been extensively used, both in our laboratory and elsewhere, and is sensitive to reproductive toxicants. All rats were housed individually in Makrolon cages with Lignocel PS 14 fibres dust-free bedding and wooden enrichment blocks. The cages were kept in climate controlled rooms at 20–24°C with a relative humidity of 30–70%, an air exchange rate of 15 times per hour, and a 12-hr light/dark cycle. Diet (Ground Kliba SA, Switzerland) and tap water were provided ad libitum. In-life data (mortality, clinical signs, body weights, and food consumption) were recorded throughout the study, but presented only if relevant for the interpretation of prenatal developmental effects.

Experimental Procedure

Studies were conducted with daily oral administration of test substances by gavage (test compounds 0594, 0595, 0596, 0599, and 0600) or via diet (test compound 0618) from GD 6 to GD 19 (Table 4). The individual dose levels were selected based on the results of a repeated exposure to non-pregnant female rats over 14 days (data not shown). According to the OECD 414 test guideline it was aimed to cause signs of maternal toxicity as recommended at the high dose level. The test compounds administered by gavage were administrated between 100 and 600 mg/kg bw/day using the vehicle 1% carboxymethylcellulose in deionized water. The standard dose volume was 10 mL/kg bw. The test compound 0618 was administered via the diet at dose levels of 300 ppm (dose was reduced from 2500 ppm on GD 6 and 7 causing severe clinical signs) and 1000 ppm in the diet. The exposures were corresponding to a substance intake of 28 mg/kg bw/day (GD 8-19) and 69 mg/kg bw/day (GD 6-19), respectively.

Necropsy and Fetus Preparation

On GD 20, the surviving dams were anesthetized with isoflurane, sacrificed by decapitation, and examined macroscopically. For each dam, the uterus was opened and the number, distribution, and classification of implantation sites (live fetus, early and late fetal resorptions, and dead fetus) were determined. The fetuses were removed, sexed and fetal body weight determined. Gross-pathological examination of the fetuses, including assessment of abnormalities of the fetal membranes, placentas, amniotic fluid and umbilical cord, was performed. Subsequently, all fetuses were sacrificed by injection of pentobarbital. About half of the fetuses of each dam were fixed in ethyl alcohol and after fixation, stained according to a modified method of Dawson (1926) to show the skeleton. The other half of the fetuses of each dam was fixed in Harrison´s fluid. After fixation, the soft tissue of these fetuses was examined according to a modified microdissection method [13]. The glossary of [14] and its updated version of [15] was essentially used to describe findings in fetal morphology. Classification of these findings

was based on the terms and definitions proposed by [16] and [17, 18]. A permanent structural change that is likely to affect adversely the survival or health was assessed as malformation. A change that also occurs in the fetuses of control animals and/or is unlikely to affect adversely the survival or health was assessed as variation. This includes delays in growth or morphogenesis that have otherwise followed a normal pattern of development.

Data analysis

BeWo transport data

For each compound, the linear appearance rate in the basolateral compartment was determined. These linear appearance rates were used to calculate apparent permeability (Papp) coefficients (Papp coefficient (cm/s) = $(\Delta Q/\Delta t) / (A \cdot C_0)$), where ΔQ is the amount of test compound (nmol) transported to the receiver chamber in a certain time span (Δt (s)), A is the cell surface area (cm^2) and C_0 is the initial concentration of the test compound (μM). To calculate ΔQ, a correction was made to compensate for the removal of compound when taking samples (ΔQ at t_{x+1} = amount measured at t_{x+1} (nmol) (basolateral concentration at t_{x+1} (μM) × 1.5 (ml)) supplemented with the amount removed at t_x (nmol) (basolateral concentration sample at t_x (μM) × 0.2 (ml))). Subsequently, relative Papp values were determined by expressing the Papp coefficient as a fraction of the Papp coefficient obtained for antipyrine.

In vitro ES-D3 differentiation data

Different dichotomous concentration-response models were fitted to the in vitro developmental toxicity data obtained from the ES-D3 cell differentiation assay to calculate benchmark concentrations (BMC) using Environmental Protection Agency benchmark dose (BMD) software version 2.4. For each test compound, the BMC_d50, representing the concentration for a 50% reduction in the number of differentiated EBs, was derived. Models included in the evaluation were the gamma, logistic, loglogistic, probit, logprobit, multistage, weibull and the quantal-linear model. Goodness-of-fit of the models was evaluated to accept a model, based on the P-values, the scaled residuals and the graphical displays obtained. The lowest BMC_d50 value was chosen from the accepted models.

Figures of concentration-response curves for both differentiation and cytotoxicity were made using Graphpad Prism 5 using a 4-parameter logistic model. These curves were not used for the derivation of the BMD_d50 values since BMD_d50 values were derived as described above using BMD modeling.

To combine in vitro developmental toxicity data obtained from the ES-D3 cell differentiation assay with placental transfer data obtained from the BeWo transport model, a corrected BMC_d50 value was calculated by dividing the BMC_d50 values by the relative Papp values, as described in [8].

In vivo data

Data obtained for food consumption, body weight, carcass weight, weight of unopened uterus, weight of placentas and fetuses, the number of implantations, number of late fetal resorptions, and percentage of postimplantation loss were analyzed by a simultaneous comparison of all dose groups with the control group using Dunnett´s test [19, 20]. The number of pregnant animals at the end of the study, mortality rate of the dams, and number of litters with fetal findings was analysed by Fisher´s exact test [21] and the proportion of fetuses with findings per litter by Wilcoxon signed-rank test [21, 22]. Maternal toxicity was classified as slight if body weight and/or carcass weight reduction is not above 10%, moderate if body weight and/or carcass weight reduction is between 10% and 20% and severe if body weight and/or carcass weight reduction is above 20%. All the in vivo data are expressed as the affected fetuses/litter.

RESULTS

In vitro BeWo transport

For all transport experiments, the mass balances were between 91 and 99 %. Figure 1 shows the increasing amount of test compounds in the basolateral compartment of the BeWo model over time, after adding 25 nmol to the apical compartment. Antipyrine was included as a reference compound known to be efficiently transported across the BeWo cell layer and amoxicillin was used as a control compound to check the integrity of the cell layer [7]. The slow transfer of amoxicillin indicated an intact BeWo cell layer. For up to 60 min, the transport of all compounds to the basolateral compartment was linear in time. Therefore, the linear appearance rate of compound in the basolateral compartment could be determined using data at 30 min for the calculation of Papp coefficients. The data in Table 2 show a wide (up to 8-fold difference) range of Papp coefficients for the six test compounds, illustrating different placental transfer rates among them, with 0596 being transported at the highest rate and 0595 at the lowest rate. Besides the transport rate, intracellular accumulation of six test compounds and the control compounds at 90 min was quantified and the results obtained are shown in Table 2. The data show that all triazoles tended to accumulate in the BeWo cells and that amoxicillin and antipyrine did not. Higher amounts of 0595, 0600 and 0618 were detected in the cells than of the other three triazoles.

Cytotoxicity assay with ES-D3 cells

WST-1 assays for both one-day and five-day exposure were performed to evaluate the cytotoxic effects of the compounds on the ES-D3 cells (Figure 2). For all compounds, the concentrations tested (up to 60 μM) were non-cytotoxic as determined in the one-day cytotoxicity assay. Among the six compounds, 0618 was the most potent one in the five-day cytotoxicity assay, reducing the cell viability to 10% at 60 μM. Exposure of 60 μM 0599, 0596, and 0594 to the ES-D3 cells resulted in 80%, 70% and 30% decline in cell

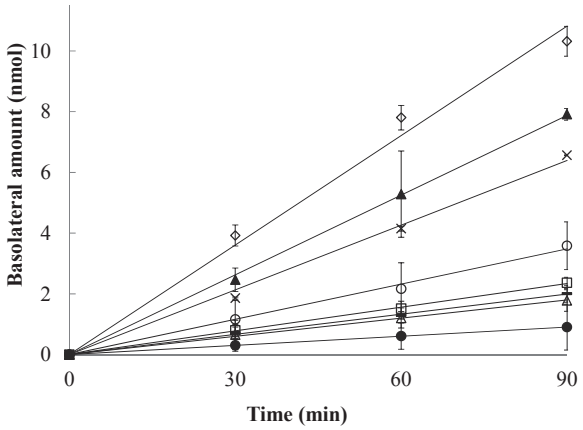

Fig. 1. Amount of 0594 (○), 0595 (●), 0596 (▲), 0599 (×), 0600 (□), 0618 (-), amoxicillin (△) and antipyrine (◊) in the basolateral compartment in the in vitro BeWo model with increasing time using initial concentrations of 50 μM (25 nmol) in the apical compartment. Data are presented as mean ± standard deviation (n = 3).

Table 2. Papp coefficients at 30 min and relative Papp values of six test compounds and the reference compounds amoxicillin and antipyrine in the BeWo model.

Compounds	Intracellular accumulation (% of added amount)	Papp coefficient (10^{-6} cm/s)	Relative Papp value
Amoxicillin	0%	6.5 ± 0.2[a]	0.16
0594	24%	11.5 ± 2.6	0.30
0595	79%	3.0 ± 0.4	0.08
0596	12%	24.4 ± 2.7	0.63
0599	22%	18.4 ± 1.1	0.47
0600	66%	7.9 ± 1.0	0.20
0618	56%	7.1 ± 0.2	0.18
Antipyrine	0%	38.9 ± 3.0	1.00

[a] Mean ± SD

viability, respectively, reflecting lower cytotoxic properties. The least cytotoxic triazoles were 0595 and 0600, which caused slight reduction in cell viability (20%) up to 60 μM.

Differentiation assay with ES-D3 cells

To study the in vitro developmental toxicity of the antifungal triazoles, the effect of the compounds on the differentiation of ES-D3 cells into contracting cardiomyocytes was evaluated. All test compounds induced a concentration-dependent inhibition of

the differentiation of the ES-D3 cells into contracting cardiomyocytes (Figure 2). The calculated BMC_d50 values were at concentrations that did not cause cytotoxicity (after one and five days), indicating that inhibitory effects on the differentiation of EBs are not due to cytotoxicity of the test compounds. BMC_d50 values are summarized in Table 3, showing that 0599 was the most potent in inhibiting the differentiation of EBs, followed by 0600 > 0618 > 0594 > 0595 > 0596.

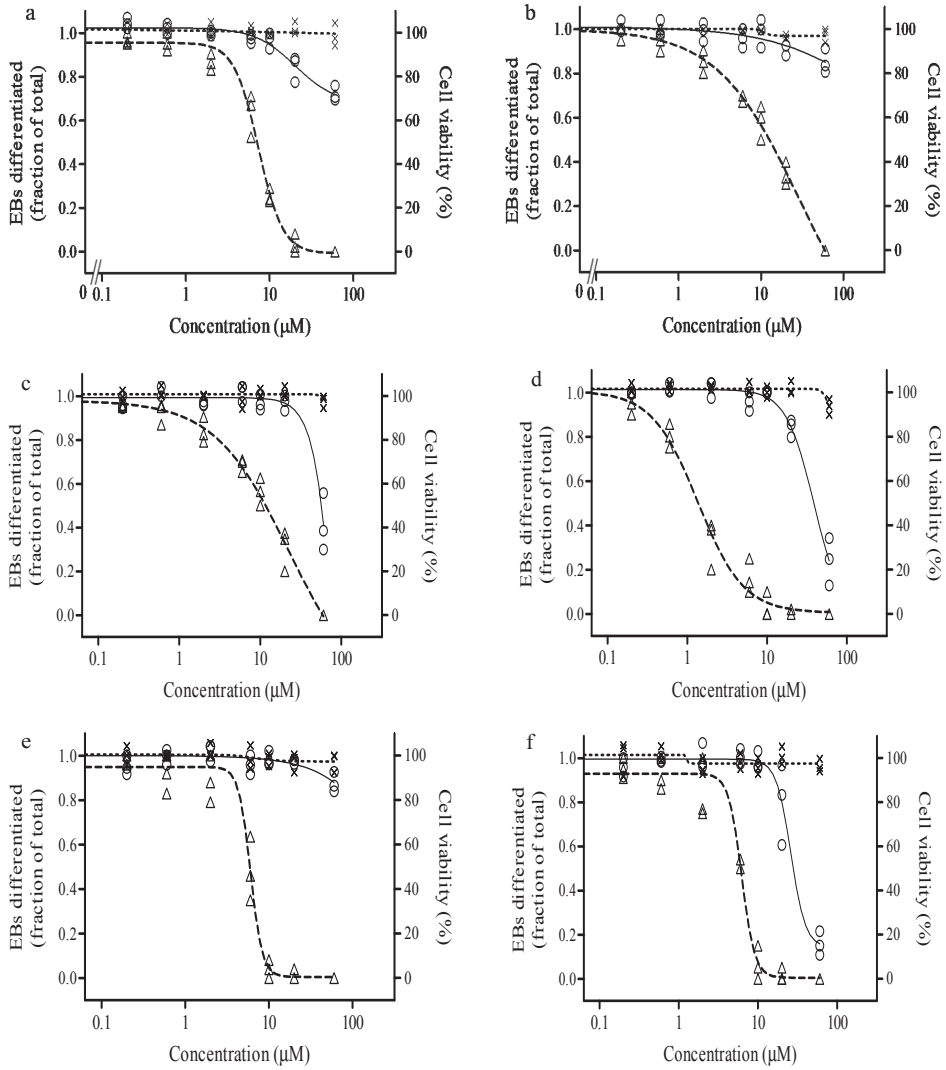

Fig. 2. Concentration-dependent effects of test compounds 0594 (a), 0595 (b), 0596 (c), 0599 (d), 0600 (e) and 0618 (f) on cell viability for one-day (×) and five-day (○) exposure and on inhibition of ES-D3 cell differentiation (Δ). Figures present data of three independent experiments.

Combination of in vitro developmental toxicity data with BeWo transport data

To combine in vitro developmental toxicity data obtained from the ES-D3 cell differentiation assay with placental transfer data obtained from the BeWo transport model, a corrected BMC_d50 value was calculated by dividing the BMC_d50 values by the relative Papp values (Table 3). After the correction, the potency ranking was altered, being 0599 > 0596 > 0600 > 0594 > 0618 > 0595.

Table 3. BMC_d50 values for in vitro developmental toxicity of test compounds in the ES-D3 cell differentiation assay and corrected BMC_d50 values obtained by combining the ES-D3 cell differentiation assay data with data on placental transfer from the BeWo transport model.

Novel triazoles	BMC_d50 (µM)	Corrected BMC_d50[a] (µM)
0594	6.9	23.3
0595	11.0	141.8
0596	11.4	18.1
0599	1.8	3.8
0600	4.2	21.0
0618	4.3	23.7

[a]corrected BMC_d50 value was calculated by dividing the BMC_d50 values by the relative Papp values

Potency ranking derivation from in vivo data

In the in vivo studies, all tested compounds showed, to some degree, a potential to cause prenatal developmental toxicity. All statistically significant and toxicologically relevant alterations relative to controls are summarized in table 4. The potency of each of the test compounds to cause prenatal developmental toxicity was assessed based on the observed fetal findings, including teratogenic effects, taking into account the present or absence of maternal toxicity at the different dose levels.

The highest potential to cause prenatal developmental toxicity, based on the results of the in vivo studies, was observed for the test compound 0599 (ranking 1). It caused teratogenic effects in 100% of fetuses, manifested in skeletal malformations on the tuberositas deltoidea and pterygoid bones as well as palate at 100 mg/kg bw/day. This dose level did not alter the overall development of the fetuses indicated by their body weight or in utero survival indicated by resorption rate. At this exposure, maternal toxicity was observed noted as decreased food consumption (74% GD 6-20), body weight

change (66% GD 6-8) and carcass weight (91%). Maternal toxicity is not considered to have contributed significantly to the fetal effects.

The second highest potential to cause prenatal developmental toxicity was observed for the test compound 0596 (ranking 2). This compound also caused teratogenic effects in all fetuses, manifested in skeletal malformations, like the test compound 0599. The malformation observed at the highest incidences were observed in skull bones (including basisphenoid) and cervical arches. But this time all fetuses were only affected at a 6-time higher dose level of 600 mg/kg bw/day. Comparable to the test substance 0599, this dose level did not affect the overall development of the fetuses indicated by their weight or in utero survival indicated by resorption rate being comparable to the control. The teratogenic effects were observed at a dose level causing almost no maternal toxicity; only visible in an increased placental weight (133%). At 200 mg/kg bw/day the pattern of treatment-related effects was comparable to 600 mg/kg bw/day, but malformations being observed in 47% of fetuses per litter.

The third highest potential to cause prenatal developmental toxicity was observed for the test compound 0600 (ranking 3). It was causing neither treatment related findings on fetuses nor on dams at 200 mg/kg bw/day. At 600 mg/kg bw/day the teratogenic potential of the test compound was manifested in an increased incidence of skeletal malformations (81% of fetuses per litter) without affecting the fetal growth or causing increased number of resorptions. The significantly increased malformation rate were observed in small or absent tuberositas deltoidea, misshapen pterygoid bones, and cleft palate. The maternal toxicity observed in lower body weight changes in the beginning of exposure (27% GD 6-8) and the increased placental weight (130%) might had enhanced the severity of teratogenic effects but could not be encountered for their occurrence only.

The third lowest potential to cause prenatal developmental toxicity was observed for the test compound 0594 (ranking 4). Like the test compound 0600, it did not caused treatment-related findings in fetuses and dams at 200 mg/kg bw/day. At 600 mg/kg bw/day only slight maternal toxicity was observed in minor alterations of food consumptions (86% GD 6-8) without any effect on the body weight development of the dams. A borderline increase of postimplantation loss was determined with 14.9% being only slightly above the spontaneous incidence of 14.7% given in the historical control data for this rat strain in our test facility. In comparison to test compound 0600, a weaker teratogenic potential of the test compound 0594 was observed manifested in an increased total skeletal malformation incidence of 12.5% fetuses per litter (bent femurs) at 600 mg/kg bw/day. This dose did not significantly affect number of fetuses alive per litter or the growth of the fetuses.

The second lowest potential to cause prenatal developmental toxicity was observed for the test compound 0618 (ranking 5). It caused slight or moderate maternal toxicity at 300 and 1000 ppm manifested in significantly decreased body (87% at 1000 ppm)

and/or decreased carcass weight (95% at 300 ppm and 83% at 1000 ppm). The highest dose level tested also caused significantly increased placental weights (162%) and postimplantation loss (30.3%). The latter was based mainly on the increased number of late resorptions (23.2%). The observed decreased fetal development manifested in the lower fetal body weight (87% at 1000 ppm) could partially be explained by the moderate maternal toxicity observed. However, the skeletal malformations observed here, low incidences of bent femur, is a rare finding and is not in the historical control data. Thereby, the test compound 0618 still demonstrated a weak teratogenic potential.

The lowest potential to cause prenatal developmental toxicity was observed for the test compound 0595 (ranking 6). It has a relatively high general toxic potential leading to poor general state in 3 out of 10 rats after 10 to 11 days of exposure to 600 mg/kg bw/day. These animals had been sacrificed moribund on GD 15 and 16. The surviving dams showed severe maternal toxicity in decreased body (74%) and carcass (89%) weight. At this dose level none of the embryos survived the *in utero* exposure leading to 100% early resorptions. At 200 mg/kg bw/day no maternal toxicity but still a significantly increase of postimplantation loss (24%) was observed mainly caused by an increase number of early resorptions (21.9%). The surviving fetuses did not show alterations on growth or malformations. Thereby, no teratogenic potential was observed for the test substance 0595 in this in vivo study.

Based on this assessment, a developmental potency ranking of the test compounds was obtained: 0599 > 0596 > 0600 > 0594 > 0618 > 0595.

Comparison of in vivo and in vitro developmental toxicity ranking

Both in vitro developmental toxicity potency rankings based on BMC_d50 alone and the placental transfer corrected BMC_d50 values were compared with in vivo potency ranking, to assess the usefulness of combining data from the ES-D3 cell differentiation assay with data from the BeWo transport model to predict in vivo potency ranking (Table 5). Based on the in vivo ranking, 0599 was the most potent compound in vivo and 0595 was the least potent one. The potency ranking of the six test compounds in the ES-D3 cell differentiation assay correlates to some extent with the in vivo ranking, with 0599 being the most potent one, however with clear differences for the less toxic ones. For 0596, especially, the relative potency is not well predicted in the ES-D3 assay, being least toxic in the ES-D3 cell differentiation assay, whereas almost most toxic in vivo. When the ES-D3 cell differentiation data are combined with the results obtained in the BeWo transport model to obtain corrected BMC_d50 values, a better correlation with the in vivo ranking was obtained with no discrepancy (Table 5). These data thus demonstrate the power of including a component of kinetics when predicting relative in vivo toxicity potencies based on in vitro toxicity data.

Table 4. Developmental data on the incidence of cleft palate or skeletal malformation in rat

Compound	Exposure route and duration	Dose	Maternal Toxicity			Placenta weight [b]	Early resorption [b]	Late resorption [b]
			No. of females (mated / pregnant)	Findings [b]	Classification			
0594	gavage GD 6-19	600 mg/kg	10/10	FC (6-8) 86%*	slight	116%	14.1%	0.8%
		200 mg/kg	10/10	no	no	110%	5.9%	1.9%
0595	gavage	600 mg/kg	10/9	mortality 30% BW 74%** CW 89%*	severe	-	100.0%**	0.0%
	GD 6-19	200 mg/kg	10/9	NAD	no	117%	21.9%*	2.0%
0596	gavage GD 6-19	600 mg/kg	10/10	NAD	no	133%**	7.7%	0.8%
		200 mg/kg	10/10	NAD	no	127%**	2.9%	0.0%
0599	gavage GD 6-19	250 mg/kg	10/10	mortality 100%**	severe	110%		
		100 mg/kg	10/10	FC(6-20) > 74%** BWC (6-8) 66%** CW 91%*	slight	116%	7.8%	0.8%
0600	gavage GD 6-19	600 mg/kg	10/10	BWC (6-8) 27%**	slight	130%**	6.0%	1.0%
		200 mg/kg	10/10	NAD	no	105%	4.0%	0.0%
0618	diet GD 6-20	1000 ppm	10/10	BW 87%** CW 83%**	moderate	162%**	7.2%	23.2%**
		300 /(2500) ppm [a]	10/10	CW 95%	slight	111%	4.6%	0.8%

Note: GD=gestation day; FC=food consumption; BW=body weight; CW=carcass weight; BWC=body weight change; CP=cleft palate; NAD=Nothing abnormal detected.
- Data not available
[a] 2500 ppm reduced to 300 ppm on GD 8 of first cohort (2 animals) and GD 7 of second cohort (8 animals)
[b] Statistics: Dunnett-test (two-sided)
[c] Statistics: Wilcoxon-test (one-sided)
* p<=0.05
** p<=0.01

	Post implantation loss [b]	Developmental Toxicity: TTotal no. of fetuses	Fetal weight [b]	No. of fetuses/ litters used in skeletal examination	Malformation [c]	3 most observed malformation [c]	Ranking
	14.9%*	98	101%	19/4	Total 12.5%	bent femur 12.5%	**4**
	7.8%	99	107%	-	-	-	
	100.0%**	0	-	-	-	-	**6**
	24.0%*	74	102%	18/4	Total 0%	-	
	8.6%	102	105%	57/10	Total 100%**	severely malformed skull bones 82.8%** misshapen cervical arch 70.2%** misshapen basisphenoid 13%	**2**
	2.9%	109	108%	54/10	Total 47%**	misshapen basisphenoid 30.6%** misshapen cervical arch 14.3%* severely malformed skull bones 5.3%	
	-	0	-		-	-	**1**
	8.6%	99	102%	21/4	CP 10% Total 100%**	Misshapen temporal bone 85.4%** misshapen basisphenoid 74.2%** small tuberositas deltoidea 66.3%**	
	7.0%	104	102%	22/4	CP 5% Total 81%**	small tuberositas deltoidea 48.1%** absent tuberositas deltoidea 44.4%** misshapen pterygoid bones 36.3%*	**3**
	4.0%	99	102%	20/4	Total 0%	-	
	30.3%**	74	87%**	57/10	Total 2.5%	bent femur 2.5%	**5**
	5.4%	110	101%	41/10	Total 1.4%	misshapen basisphenoid 1.4%	

Table 5. Comparison of the in vivo developmental toxicity ranking of test compounds with the ES-D3 cell differentiation alone or with the ES-D3 cell differentiation assay combined with the BeWo transport model.

	Toxicity ranking					
Methods	**least toxic**			→		**most toxic**
ES-D3	0596	0595	0594	0618	0600	0599
ES-D3 + BeWo	0595	0618	0594	0600	0596	0599
in vivo	0595	0618	0594	0600	0596	0599

DISCUSSION

The ES-D3 cell differentiation assay has shown to be a promising method to assess the developmental toxicity potency ranking of series of structurally related compounds in vitro, and it has been suggested that the capacity of this assay to predict in vivo potency ranking could be improved by combining kinetic information with the in vitro data [4-6]. We have previously demonstrated that the ES-D3 cell differentiation assay combined with the in vitro BeWo transport model for placental transfer, is able to better predict the in vivo developmental toxicity of a set of reference azoles, than as a stand-alone assay [8]. In this study, we extended the dataset, to validate this combined model by testing more compounds within the series of antifungal triazoles.

The in vitro BeWo transport model was used to obtain the relative transport rate in the present study, as it was previously shown to be suitable to adequately characterize relative placental transfer rates of compounds [7, 23, 24]. The six test compounds showed different transfer rates (up to 8-fold) through the placental barrier in the BeWo model, indicating that transport velocities to the fetus in vivo may differ as well. However, how this exactly translates to differences in fetal exposure is not known, due to the fact that in vivo experimentation of placental transport in humans is not feasible on a large scale for obvious ethical reasons and also because little has been published on the fetal bioavailability of antifungal compounds in vivo. We hypothesize that the extent of fetal exposure would be positively correlated to the relative transport velocity in the BeWo model. Therefore, we investigated whether the correlation between in vitro BMC_d50 values-based potency ranking and in vivo developmental toxicity potency ranking would improve when we corrected the in vitro BMC_d50 values for differences in placental transfer, by dividing these BMC_d50 values for the six test compounds in the ES-D3 cell differentiation assay by relative Papp values obtained in the BeWo model.

With the ES-D3 cell differentiation assay alone, the predicted toxic concentrations among the six test compounds were within the same order of magnitude, but the combined assays resulted in a different ranking, with the most potent compound being about

30 times more toxic than the least potent one. The results obtained reveal that the ranking according to the corrected BMC_d50 values correlated better than the uncorrected ones with the ranking that could be derived from in vivo developmental toxicity data. This shows that combining the ES-D3 cell differentiation assay results with placental transfer kinetic data improved the ability of this in vitro assay to predict in vivo developmental toxicity potencies of the tested compounds. It should be noted that other important in vivo kinetic processes than placental transfer, such as intestinal absorption, maternal metabolism and placental metabolism, were not taken into account, and that these will contribute to the amount of compound available in the fetus. Therefore, incorporating more kinetic information is likely to further improve the predictive value of the ES-D3 cell differentiation assay. Besides, the BeWo cell model may not be suitable to determine the absolute transport rates of compounds, given that the BeWo cell model is a simplification of the in vivo placental transfer system. In those cases where it is important to use exact fetal bioavailability values, physiologically based kinetic (PBK) models, which describe the in vivo absorption, distribution, metabolism, and excretion processes of a compound should be used for the translation of in vitro toxicity data to the in vivo situation [25-27].

We acknowledge that the increase in placental weight, noted for 0596 (at 200 and 600 mg/kg bw/day), 0599 (at 600 mg/kg bw/day) and 0618 (at 1000 ppm) in the rat studies cannot be reflected in the ES-D3 assay, and therefore these effects were not taken into account when making the comparison among the different triazoles. The increased placental weight in pregnant rat may be the result of inhibition of aromatase enzyme activities [11]. The contribution of placental changes induced by azole compounds to fetal development, and its relevance to humans is currently under investigation [28].

In this study, intracellular accumulation of test compounds in the placental cells was investigated in the BeWo cell model. Triazoles 0595, 0600 and 0618 showed a high percentage of accumulation, up to 79% of the total mass added, in the BeWo cells. If this accumulation in placental cells is also occurring in vivo, the accumulated compounds may potentially affect the development or function of the placenta, given that the placenta is an entry organ to the fetus and vulnerable to the adverse effects of many toxicants. Structural or functional damage to the placenta can lead to adverse effects, such as abortion, birth defects, stillbirth, etc. We observed that triazole 0595, which showed the highest accumulation in the BeWo cells, induced the highest level of post-implantation loss, making it tempting to speculate that there might be a link between the BeWo cellular accumulation and the in vivo resorption rate. However, as BeWo cells only represent part of the placental tissue and since placental tissue changes over pregnancy, it is difficult to translate this in vitro finding to the exact in vivo situation. Results on placental accumulation of the six triazoles in vivo are not available, but it has been elucidated that some pesticides were found to accumulate in placental tissue [29, 30]. Therefore, it might be of interest to pay attention to possible placental toxicity, for which the in vitro BeWo

cell model might be useful for prioritization because as shown in the present study the model is able to provide information on the intracellular accumulation of compounds.

The ES-D3 cell differentiation assay is considered to represent the fetal component of developmental toxicity. However, the (azole) antifungal compounds are found to have the ability to inhibit cytochrome enzymes (Marotta and Tiboni 2010), and in particular may inhibit estrogen biosynthesis through CYP19 aromatase inhibition, reducing the conversion of androgens to estrogens and exerting endocrine disrupting effects [31]. This process takes place in the maternal organism, and the phenotypic analysis of the inhibition of cardiac differentiation in the ES-D3 cell differentiation assay cannot take this part of developmental toxicity into account. Therefore, other relevant in vitro assays, such as the steroidogenesis assay [31, 32] may be needed. In addition, although we have proven in the previous and the present study that the ES-D3 cell differentiation assay combined with the BeWo transport assay shows to be an appropriate in vitro toxicological system to achieve an enhanced predictivity of the relative developmental toxicity for antifungal compounds, one should still note that one single alternative testing approach is not likely able to predict for the entire scope of developmental toxicants. To obtain information on mechanisms underlying developmental toxicity, the ES-D3 cell differentiation assay could be combined with transcriptomics analyses, which can be used to group compounds in classes based on toxicity mechanisms [33]. A tool box which integrates different relevant in vitro assays into an integrated testing strategy may provide the best possible strategy to characterize both the toxicological profile and the relevant mechanistic information for human chemical risk assessment.

When it comes to risk assessment, it should be remembered that many antifungal products contain mixtures of active substances, for example, for broad-spectrum disease control or for resistance management to obtain the broadest benefits possible [34]. Though current risk assessment approaches are predominantly based on individual azole fungicides, human exposure is in general to complex mixtures of pesticides. It is therefore of interest to investigate the developmental potency of the mixture of antifungal compounds in the future research.

In conclusion, in the present study we provided further evidence that the ES-D3 cell differentiation assay, combined with the in vitro BeWo transport model, is able to better predict the in vivo developmental toxicity ranking of the antifungal compounds, than as a stand-alone assay. At this stage the applicability domain of this combined in vitro toxicodynamics and toxicokinetics approach is not known. Other series of compounds from different chemical classes should be tested to further evaluate the capacity of this approach in predicting developmental toxicity. Since the method uses only in vitro assays to predict in vivo developmental toxicity ranking of chemicals, it can contribute to the 3Rs (replacement, reduction and refinement) of animal testing. The predictive ability of the ES-D3 cell differentiation assay can most likely be further enhanced if it is combined with more kinetic data and PBK modeling.

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NOTES

The authors declare that they have no conflict of interest. The compounds studied in the assays where produced by BASF SE, Germany, but none of these are in commercial use.

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CHAPTER 5

Use of the ex ovo assay of chicken embryos to predict the in vivo developmental toxicity of antifungal compounds

Hequn Li, Burkhard Flick, Ivonne M. C. M. Rietjens, Jochem Louisse,
Martina Dammann, Bennard van Ravenzwaay

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ABSTRACT

We investigated the applicability of the ex ovo assay of chicken embryos to predict the teratogenic potential of five antifungal compounds and investigated if the combination of the assay with placental transfer information would improve its predictive capacity. The ex ovo assay assesses prenatal developmental toxic potential of compounds based on morphological endpoints. Each compound produced a characteristic pattern of alterations. Data on the relative placental transport velocity were taken from an earlier study, using the BeWo transport model. When the toxicity data from the ex ovo assay were combined with the relative transport rates from the BeWo model, the correlation with the in vivo data were better. In conclusion, the ex ovo assay of chicken embryos is able to assess the teratogenic potential of antifungal compounds, and, when combined with the in vitro BeWo transport model, is able to better predict the relative in vivo prenatal developmental toxicity.

INTRODUCTION

The implementation of the REACH legislation results in the increased use of animals for toxicity testing, especially for developmental toxicity testing [1, 2]. Therefore, there is an urgent need to develop and validate in vitro and in silico alternative methods for in vivo developmental toxicity studies [3]. Several in vitro methods have been developed to evaluate the developmental toxic potential of chemicals, such as the embryonic stem cell test (EST), the rodent whole embryo culture test (WEC) and the ex ovo assay of chicken embryos [4-6]. Among these tests only the EST does not require the use of primary animal tissues, since it uses the blastocyst-derived mouse embryonic stem cell line D3 (ES-D3) that spontaneously differentiates into contracting cardiomyocytes. We have previously shown that the ES-D3 cell differentiation assay is able to predict the relative in vivo developmental toxicity potency of a series of selected antifungal compounds [7, 8].

The in vitro models mentioned above that are used to predict developmental toxicity do not contain a placental compartment. The placenta is an important organ required for the development of the embryo or fetus and it separates the maternal blood circulation from the fetal circulation. Different compounds transfer through the placenta at different rates, and this may influence the developmental toxicity outcome. Placental transfer can be studied in vitro using the BeWo transport model. The BeWo cell transport model, consisting of BeWo b30 cells grown on transwell inserts and mimicking the placental barrier, can be used to determine the relative placental transport velocity. We have previously shown that this model is suitable to adequately characterize relative placental transfer rates of chemicals [9]. We have also shown that the predictive ability of the ES-D3 cell differentiation assay was improved when it was combined with kinetic information on placental transfer determined using the BeWo transwell model [7, 8].

Although the ES-D3 cell differentiation assay, combined with the BeWo transport model, has been shown to be useful to predict the relative developmental toxicity potency of antifungal compounds, the ES-D3 cell differentiation assay is limited to the evaluation of only one endpoint, i.e. the differentiation of embryonic stem cells into contracting cardiomyocytes. Prenatal developmental toxicity studies in vivo have shown that many teratogenic compounds have specific manifestation patterns of adverse outcomes for different compound classes. Some antifungal compounds may induce specific morphological alterations [10]. The mechanism of action of antifungal compounds on skeletal development has been reported to result from alterations during hindbrain segmentation and branchial arches formation [11]. The phenotypic endpoint of cardiomyocytes differentiation, which is used as a sensitive readout for general embryotoxicity in the ES-D3 cell differentiation assay, cannot distinguish between different manifestation patterns of teratogenic compounds with different modes of action

by showing different phenotypes after exposure. Thus, the ES-D3 cell differentiation assay cannot indicate the specific type of effects that may potentially be induced. The ex ovo assay of chicken embryos assesses chemicals with regard to their prenatal developmental toxic potential based on morphological changes [12]. The culture duration of the chicken embryos covers major parts of organogenesis, offering the assessment of various morphological endpoints including specific development of different organ anlagen, i.e. head region with different brain -, eye -, and ear anlagen and branchial arches. Therefore, the ex ovo assay of chicken embryos is a suitable tool to study the teratogenic potential of antifungal compounds to gain additional information about the embryotoxic potential of test compounds.

The aim of the present study was to investigate the teratogenic potential of five antifungal compounds, being ketoconazole, tebuconazole, propiconazole, prothioconazole and fenarimol, using the ex ovo assay of chicken embryos. The results obtained were compared to the in vivo teratogenic potential and to the developmental toxic potencies derived from the ES-D3 cell differentiation assay. Furthermore, it was investigated whether the combination of the ex ovo assay of chicken embryos with the BeWo model would improve its predictive capacity of the relative in vivo developmental toxicity of the test compounds.

MATERIALS AND METHODS

Chemicals

Ketoconazole, tebuconazole, propiconazole, prothioconazole, fenarimol (with purity for the five compounds all >98%) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Munich, Germany).

Ex ovo assay of chicken embryos

The assay was performed according to a modified protocol of the ex ovo assay of chicken embryos described by Nagai et al.[12].

Preparation of the embryo

Specific pathogen free fertilized chicken eggs were obtained from either Valobiomedia (Osterholz-Scharmbeek, Germany) or Charles River (Sulzfeld, Germany). Their development was stopped immediately after laying by cooling to 12-16 °C. The embryonic development was restarted by a stepwise heating up procedure. Eggs were incubated in a humidified atmosphere at 38 °C for 38-40 hours to reach the developmental stage of 5-8 somites in the embryo (Fig. 1A). During transportation and incubation, the eggs were positioned with the embryos facing upwards. Embryo preparation was carried out in the Pannett-Compton solution (Quartett, Berlin, Germany) using a sterilized dissection kit (spoons, pincette and scissors), filter paper ring and pipette tips. The eggs were carefully

cracked open into a petri dish with the vitelline membrane being intact, covering the embryo facing upwards. Thin albumin was collected into a container to be kept for later use in the culture medium. A filter paper ring was placed on the vitelline membrane encircling the embryo (Fig. 1A). Then the vitelline membrane was cut along the outside edge of the filter paper to free the assembly. When cut free, both the membrane and paper along the edge of the assembly were picked up and transferred into Pannett-Compton solution in a petri dish. The yolk was flushed off by carefully lifting up the assembly in the Pannett-Compton solution. Subsequently, under the stereomicroscope (Stemi 2000, Zeiss), the blastoderm was peeled off the vitelline membrane (Fig. 1B). The free edge of the blastoderm was folded inwards and gently pulled until the blastoderm forms a fairly flat semicircle surface (Fig. 1C). Then the edge of the blastoderm was trimmed with scissors with care to obtain a closed semicircular blastoderm with the embryo lining along the diameter (Fig. 1D).

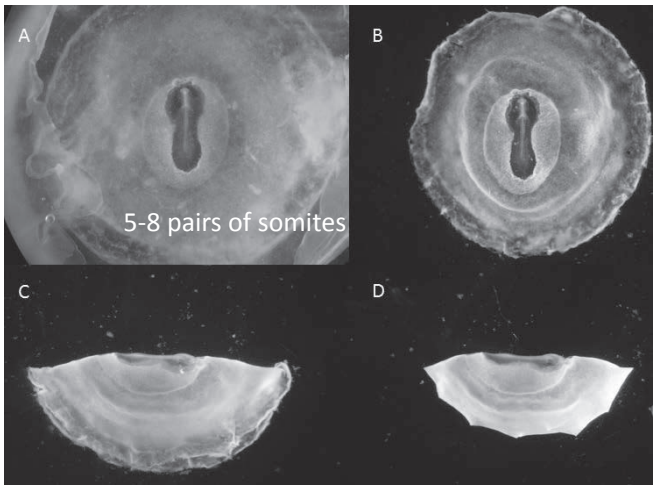

Fig. 1. Preparation of the chicken embryo. (A) Embryo with 5-8 pairs of somites covered by the filter ring, (B) isolated blastoderm peeled off the vitelline membrane, (C) flat semicircle surface formed by folding the free edge of the blastoderm inwards and gentle pulling, and (D) closed semicircular blastoderm with the embryo lining along the diameter.

Exposure of the embryo to the test compounds

After the semicircular blastoderm was prepared, it was placed to stand in Pannett-Compton solution for one hour to allow better adherence. Then the semicircular blastoderm disc was transferred to a six-well plate with 5 ml of culture medium per well containing the required concentration of the test compound or DMSO (solvent control), with one semicircular blastoderm per well. The culture media consisted of thin

albumin and Pannett-Compton solution at a 2:1 ratio, supplemented with antibiotics and antimycotics solution, with the final dilution of antibiotics and antimycotics solution in the culture media being 1:300 of the stock concentration (15240-062, Life technologies, Carlsbad). Test compounds were added to the culture media from 1000 times concentrated stock solutions in DMSO. Operated embryos were incubated at 38°C and 5% CO_2 in a humidified atmosphere for 48 hours. At the end of the incubation period the embryos that had developed normally reached a developmental stage showing 25-30 somites.

Range finding experiments were conducted prior to the main experiments to find the concentration range not causing embryonic death (data not shown). Embryos were exposed to the test compounds, ketoconazole, tebuconaozle, propicoanzole, prothiocoanzole and fenarimol, at 3 to 4 concentrations for 48 hours to determine concentration-dependent effects. A solvent control of 0.1% DMSO was included in each single experiment.

Morphological evaluation of the embryonic development

After 48 hours exposure, the embryos were transferred into a petri dish with Pannett-Compton solution and the morphological changes of embryos were examined under the stereomicroscope. The evaluation was carried out based on a scoring system developed for rat embryos of Klug et al. [13] to assess the morphological parameters of chicken embryos during organogenesis (the scoring table is presented in the Supplementary data file 1). The evaluation included morphological endpoints representing differentiation endpoints, i.e. somite number, head-, ear-, and eye anlagen, branchial arches, neurulation, fore and hind limb, caudal part of trunk (referred to as tail), as well as representing growth parameters, i.e. crown-rump length and vessel-tail length. The embryos were considered to be viable when a beating heart and blood flow through the inflated yolk sac vessels were observed. The viable embryos were scored as 0 and the dead ones as -2. The development of different primordia during culture was scored with a number corresponding to the developmental stage. By adding the single score values of the different organ anlagen, the total morphological score of the embryo was obtained. The growth parameters were measured using an ocular micrometer. The somite numbers were counted. Dysmorphogenesis was distinguished from general embryotoxicity following the terminology of in vitro toxicology summarized in Flick et al. [14]: dysmorphogenesis was defined as disproportional development of single organ anlagen in comparison to the whole embryo development. A pattern of dysmorphogenesis in all tissues and organ anlagen, being rather homogeneously affected, indicated an unspecific general embryotoxicity of a compound rather than a specific, target-tissue related developmental effect. The pictures were taken with the stereomicroscope mounted with a Canon camera.

Data analysis

Ex ovo assay of chicken embryos

Statistical analysis was performed using the Jonkheere-Terpstra - asympt test (one-sided) for morphological endpoint scores and total morphological scores. The Dunnett test (one-sided) was used for analysis of growth parameter scores. The level of significance was set at $p < 0.05$. The lowest test concentration at which the results were significantly different from those for the solvent control was determined as the lowest observed adverse effect concentration (LOAEC).

Different dichotomous concentration–response models were fit to the morphological evaluation data on different endpoints obtained from the ex ovo assay of chicken embryos to calculate benchmark concentrations (BMC) using the Environmental Protection Agency benchmark dose (BMD) software version 2.4. For each test compound, the BMC50, representing the concentration for a 50% increase in the incidence of malformed embryos, was derived. Models included in the evaluation were the gamma, logistic, log logistic, probit, log probit, multistage, Weibull and the quantal-linear model. Goodness-of-fit of the models was evaluated to accept or decline a model, based on the p-values, the scaled residuals and the graphical displays obtained. The lowest BMC50 value was chosen from the accepted models.

To combine in vitro developmental toxicity data obtained from the ex ovo assay of chicken embryos with placental transfer data obtained from the BeWo transport model, corrected BMC50 and corrected LOAEC values were calculated by dividing the BMC50 and LOAEC values by the relative permeability coefficient (Papp) values, obtained from the BeWo transport model as previously determined [8].

In vivo data

A literature study was performed to determine the in vivo developmental toxicity potencies of each test compound. Search terms included the name of the test compound together with combinations of the search terms teratogenicity, developmental toxicity, malformation, embryotoxicity, skeletal malformation and cleft palate. For the purpose of this paper, the results of studies performed in rats were used, mostly published in reports of the Environmental Protection Agency (EPA), European Commission (EC) and the European Food Safety Authority (EFSA) or in published papers on developmental toxicity (Table 1). A study was selected if it included at least one control group and two dose groups, which enables analysis by the BMD approach to derive a BMD value. A benchmark response (BMR) for the BMD for each compound was defined as a 10% extra incidence of structural alterations or of changes in the incidence of variations. The BMD10 was derived and chosen using dichotomous concentration–response models as described for the in vitro data.

Table 1. Literature data on developmental toxicity induced by antifungal compounds in rats with oral administration.

Compound	Dose (mg/kg bw/day)	Exposure duration	Fetal effects	References
Ketoconazole				
	25, 75	PI 6-17	Increased incidence of cleft palate	[15] [a]
	80	GD 6-15	Increased embryolethality, decrease of fetal body weights, and increase of fetal defects including cleft palate, frontal, parietal and interparietal incomplete ossification, limb anomalies, absent fibula and pubis, short femur, tibia and pubis.	[16] [b] [17] [b]
Tebuconazole				
	30, 60, 120	GD 6-15	Increased incidences of skeletal variations.	[18] [a]
	10, 30, 100	PM 6-15	Facial alteration dysplacia of scapula and long bones, exencephaly + spinia bifida, encephalomeningocele + macroglossia and other external alterations.	[19] [a]
Propiconazole				
	30, 90, 300	GD 6–15	Increased incidence of rudimentary ribs, cleft palate, unossified sternebrae, as well as increased incidence of shortened and absent renal papillae.	[20] [a]
Prothioconazole				
	20, 80, 750	PC 6-19	Marginal increase in the incidence of fetal supernumerary rudimentary (comma-shaped) ribs.	[21] [a]
	80, 500, 1000	PC 6-19	Increased incidence of microphthalmia.	[21] [a]
Fenarimol				
	5, 13, 35	GD 6-15	Increased hydronephrosis without maternal toxicity (This minor effect of delayed development is not of particular concern).	[22] [a]
	150, 300	GD 15–21	Increased neuromuscular and behavioral deficits in nursing pups.	[23] [b]

Note. PI=post-insemination; GD=gestational day; PC=post coitum; PM=post mating
- Data not available
[a] Study used for BMD analysis
[b] Study not used for BMD analysis because not suitable for BMD analysis

If data were available on more than one endpoint, all available endpoints were analyzed to obtain, if possible, each an acceptable BMD10 value. Then from all accepted values for different endpoints, lowest BMD 10 values were chosen, and were used for the comparison with in vitro data. For tebuconazole, propiconazole and prothioconazole, the

lowest BMD10 values were related to endpoints of variation with high incidence number in the control treatment. In the available papers of the tested antifungal compounds no historical control data were reported that would enable the assessment of those variations in the control groups and potential dose-dependent increase of incidences as treatment-related or not. If these variations are not treatment-related in reality, the lowest BMD10 values may overestimate toxicity. Therefore, besides the lowest BMD10 values, we also included the median BMD10 values for the comparison with in vitro data. The median BMD10 values were calculated from all accepted BMD10 values for different endpoints.

RESULTS

Embryotoxic effects induced by the tested antifungal compounds

Table 2 shows the evaluation of chicken embryos on differentiation endpoints given in the morphological score, the somite number, as well as death and growth parameters, vessel-tail length and crown-rump length, after exposure to the five antifungal compounds. At the tested concentrations, the compounds did not induce significantly increased mortality. Besides, the vessel-tail length and crown-rump length of the embryos were not significantly influenced at the tested concentrations, indicating there was no disturbance on the normal growth of the embryos. However, all test compounds induced concentration-dependent effects on the differentiation of the chicken embryos, with significant influence on neurulation, head, ear, eye, branchial arch, fore limb, hind limb development and/or somite number. For ketoconazole, propiconazole, prothioconazole and fenarimol, the LOAEC values were determined to be 0.1, 3, 3 and 3 μM, respectively, as there was no adverse effect shown at the next lower concentration tested. However, for tebuconazole, the tested concentrations were insufficient to determine the LOAEC, as even the lowest concentration tested, 3 μM, caused dysmorphogeneses on branchial arch differentiation. The LOAEC value for tebuconazole was therefore determined as ≤3μM. All LOAEC values are presented in Table 4.

As the test compounds did not induce general embryotoxic effects in the chicken embryos at the LOAEC levels, it was of interest to know whether these compounds induced a test substance specific dysmorphogenesis, or if there was a common pattern of morphological changes caused by this chemical class of antifungal compounds. The number of structurally altered embryos after treatment with the test compounds is shown in Table 3. Based on these data, BMC50 values of each endpoint induced by each compound were derived and the endpoint giving the lowest BMC50 value, was selected and is highlighted (bold) in Table 3. The lowest BMC50 values are presented in Table 4. Representative examples of the alterations representing the most affected endpoints are shown in Fig 2.

Ketoconazole at the higher concentrations 0.1 and 0.3 μM induced specific dysmorphogenses on neurulation, head, eye, ear, branchial arch and hind limb. As

Table 2. Total morphological scoring (mean values) of chicken embryos exposed to antifungal compounds.

	Concentration (μM)	N	Dead[a]	Neu[a]	Head[a]	Eye[a]	Ear[a]	Bran[a]	FL[a]	HL[a]	S[a]	GSL[b]	CRL[b]	Score[a]
Ket	0	11	0.0	4.9	2.4	4.6	4.3	2.4	2.7	2.4	27.3	1.4	4.1	36.1
	0.03	13	0.0	4.9	2.4	4.7	3.8	2.2	2.6	2.3	26.8	1.7	4.1	35.3
	0.1	12	0.0	4.0	1.8*	3.9*	2.9*	2.3	2.4	2.3	25.9	1.7	4.2	31.8
	0.3	15	-0.1	3.3*	1.6**	3.7*	3.0*	2.3	2.6	2.2	23.2*	1.5	4.4	30.7*
Teb	0	12	-0.2	4.8	2.3	4.6	3.8	2.5	2.6	2.3	25.5	1.4	4.1	35.0
	3	16	-0.1	5.0	2.5	4.9	3.8	1.9**	2.5	2.7	23.8	1.6	4.1	35.6
	10	11	0.0	4.3	1.8	4.2	3.4	1.5**	2.3	2.2	25.6	1.6	4.2	32.6
	30	14	0.0	4.1	1.9*	3.6**	2.7	1.6**	2.3	2.2	24.8	1.5	4.1	31.6
	50	10	0.0	3.4*	1.3**	3.4**	3.3	1.4**	2.6	2.5	23.8	1.4	3.8	31.2
Prp	0	11	-0.2	3.7	2.6	4.6	4.4	2.4	2.8	2.7	28.4	1.5	4.2	35.2
	1	12	0.0	4.0	2.4	4.7	4.8	2.6	3.1	2.8	30.1	1.6	4.3	36.8
	3	11	-0.4	3.8	1.6**	4.2	4.1	2.6	2.8	2.3	27.4	1.7	4.2	34.4
	10	12	-0.2	2.3*	1.7**	3.1**	2.5*	2.4	2.4*	2.1*	25.8	1.5	4.1	28.4**
Prt	0	11	-0.2	3.4	2.2	4.6	3.8	2.7	2.5	2.2	27.1	1.4	4.2	32.8
	1	11	0.0	4.2	2.5	4.8	4.7	3.0	2.9	2.8	30.0	1.9	4.2	37.0
	3	11	0.0	3.7	2.0	3.7	3.8	2.1*	2.5	2.5	26.8	1.5	4.1	32.6
	10	10	-0.2	3.7	1.8	3.6*	2.8	2.6*	2.3	1.8	23.4	1.5	4.2	29.9
	30	10	0.0	3.0	1.6*	2.2**	2.2**	2.4*	2.1	2.1	25.8	1.5	4.1	27.4
Fen	0	11	-0.4	4.0	2.1	5.0	4.1	2.7	2.6	2.0	25.9	1.4	4.1	34.1
	0.3	12	-0.2	3.9	2.3	4.6	4.3	2.5	2.4	2.2	29.5	1.4	4.2	34.1
	1	11	0.0	3.8	1.7	4.8	3.3	2.4	2.4	2.0	25.2	1.6	4.2	32.3
	3	11	-0.4	2.8	1.3**	4.1	3.2*	2.2*	2.4	1.9	23.9*	1.3	4.1	29.9*
	10	11	-0.2	2.2*	1.5**	3.5**	2.5**	2.2**	2.1	2.1	22.2**	1.3	3.9	28.0**

Note. Ket=ketoconazole, Teb=tebuconazole, Prp=propiconazole, Prt=prothioconazole, Fen=fenarimol, Neu=neurulation; Bran=branchial arch; FL=fore limb; HL=hind limb; S=somites number; GSL=vessel-tail length (mm); CRL=crown-rump length (mm)

[a] Jonkheere-Terpstra - asympt (one-sided): * p≤ 0.05 ** p≤0.01

[b] Dunnett test (one-sided): * p≤0.05 ** p≤0.01

shown in Fig. 2b, as the most sensitive endpoint, the ear structure was disproportionally smaller than that in control embryos, and affected by ketoconazole at a concentration that did not influence any other endpoint. Some dysmorphogeneses were also found on the head region, with the metencephalon altered and the closure of neural tube at the cranial section delayed (Fig. 2c).

Table 3. Incidence of the number of malformed chicken embryos upon exposure to antifungal compounds

	Concentration (μM)	N	Neu	Head	Eye	Ear	Bran	FL	HL
Ket	0	11	0	1	1	**0**	1	0	2
	0.03	13	0	1	0	**2**	0	0	4
	0.1	12	3	4	2	**4**	3	0	3
	0.3	15	6	7	4	**6**	4	0	4
Teb	0	12	0	0	1	1	**2**	0	2
	3	16	0	2	0	2	**3**	0	2
	10	11	2	3	2	4	**7**	1	3
	30	14	3	5	4	6	**8**	0	2
	50	10	4	7	3	3	**7**	1	2
Prp	0	11	3	**2**	1	1	1	0	1
	1	12	3	**3**	1	0	1	0	1
	3	11	2	**5**	1	2	3	1	2
	10	12	7	**7**	5	7	5	2	5
Prt	0	11	4	4	2	3	**2**	0	4
	1	11	2	2	0	0	**1**	0	1
	3	11	3	5	2	3	**3**	0	1
	10	10	3	5	3	5	**5**	1	5
	30	10	5	6	7	7	**6**	4	4
Fen	0	11	**1**	2	0	1	2	0	4
	0.3	12	**3**	3	1	2	1	0	4
	1	11	**3**	5	0	3	2	0	4
	3	11	**5**	6	2	3	2	1	4
	10	11	**7**	7	3	5	3	3	4

Note. Ket=ketoconazole, Teb=tebuconazole, Prp=propiconazole, Prt=prothioconazole, Fen=fenarimol, Neu=neurulation; Bran=branchial arch; FL=fore limb; HL=hind limb; Dys=dysmorphogenesis

Tebuconazole at the higher concentrations 10, 30 and 50 μM induced specific dysmorphogeneses on neurulation, head, eye, ear and branchial arch. As shown in Fig. 2d, the size of the three branchial bars were normal, but the upper lining tissue of the second branchial bar was severely ruptured, as compared to embryos of the control treatment.

Propiconazole at the higher concentrations 3 and 10 μM induced specific dysmorphogeneses on neurulation, head, eye, ear, branchial arch, fore limb and hind limb. The head region was mostly affected by propiconazole, manifested by the malformed mesencephalon and metencephalon, as compared to control embryos (Fig. 2e).

Prothioconazole at the higher concentrations 10 and 30 µM induced specific dysmorphogeneses on neurulation, head, eye, ear, branchial arch and fore limb. The most sensitve dysmorphogenesis was found in the branchial arch. In contrast to tebucoanzole, prothiocoanzole induced a fusion between the second and the third branchial arch (Fig. 2f), as compared to embryos from the control treatment.

Fenarimol at the higher concentrations 1, 3 and 10 µM induced specific dysmorphogeneses on neurulation, head, eye, ear, branchial arch and fore limb. As compared to embryos from the control treatment, fenarimol induced delayed closure of the neural tube in the cranial region, being the most sensitive endpoint (Fig. 2g).

Each compound produced a characteristic pattern of alterations at the tested concentrations that were shown to be not generally toxic. We observed that all antifungal compounds tested significantly altered the development of neurulation, head, eye, ear and branchial arch reflecting some common dysmorphogeneses.

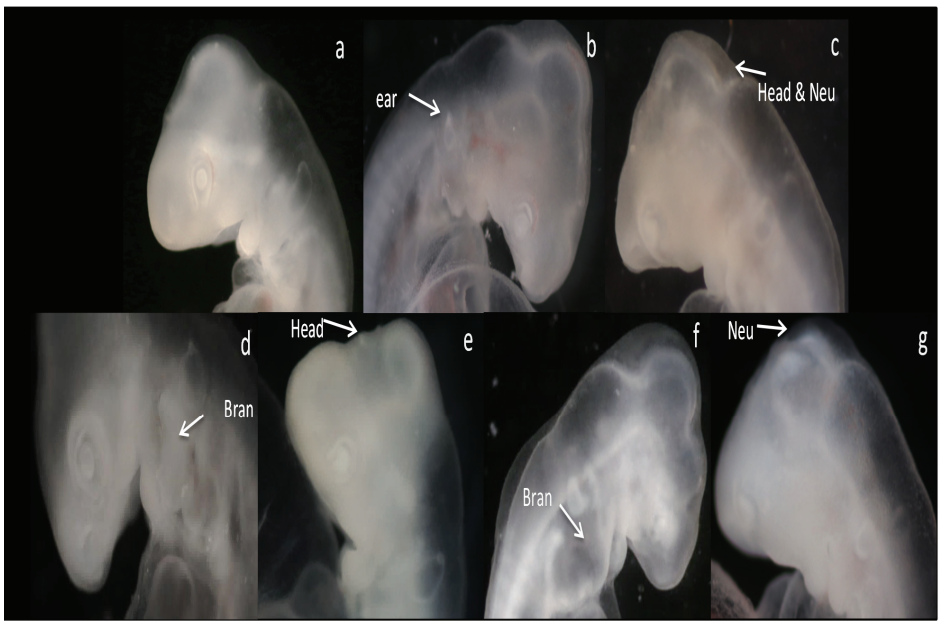

Fig. 2. Morphology of a chicken embryo exposed to solvent control (a) and morphology of chicken embryos exposed to different antifungal compounds showing the most affected malformation endpoints, being ear, head and neurulation affected by ketoconazole (b and c), branchial arch by tebuconazole (d) head by propiconazole (e), branchial arch by prothiocoanzole (f) and neurulation by fenarimal (g). Note. Neu=neurulation; Bran=branchial arch.

BMD derivation from in vivo data

A literature study on in vivo developmental toxicity was performed to obtain developmental toxicity potencies of the test compounds in vivo (Table 1) (the BMD10 values derived from different endpoints are presented in the Supplementary data file 2). Table 4 shows that lowest and median BMD10 values obtained for tebuconazole, propiconazole and prothioconazole. For ketoconazole and fenarimol, BMD10 values for only one endpoint were derived, as only data for one endpoint were available. Both lowest and median (if available) values were used for the comparison with in vitro toxicity potency values.

Comparison of antifungal compound-induced relative developmental toxicity potencies in vivo with the ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos

The BMC50 and the LOAEC values, obtained from the ex ovo assay of chicken embryos, as well as the BMC_d50 values, obtained from the ES-D3 cell differentiation assay of our earlier study [8], were compared with in vivo BMD10 values to evaluate the predictive ability for developmental toxicity of the two alternative assays. Fig. 3a, c and e show the correlation curves between in vivo and the in vitro assays when placental transfer was not taken into account. It illustrates that reasonable correlations were found when correlating the BMC_d50 from the ES-D3 cell differentiation assay to the in vivo lowest and median BMD10 values ((Fig. 3a; R^2=0.53 and R^2=0.52, respectively), as well as when correlating the BMC50 from the ex ovo assay to the median BMD10 values (Fig. 3c; R^2=0.50).

To assess the usefulness of taking into account BeWo transport data for placental transfer, a correction was made on the in vitro effect concentrations, by dividing these values by the relative permeability coefficient (relative Papp) values obtained from the BeWo transport model (Table 4) [9]. When the ES-D3 cell differentiation data are combined with the results obtained in the BeWo transport model, a better correlation of the corrected BMC_d50 values from the ES-D3 cell differentiation assay with the in vivo BMD10 values was obtained indicated by an increase of the R^2 from 0.53 to 0.95 for the in vivo lowest BMD10 values and from 0.52 to 0.79 for the in vivo median BMD10 values (Fig. 3b). A similar increase in the R^2 was found for the corrected BMC50 (Fig. 3d) and the corrected LOAEC (Fig. 3f) from the ex ovo assay of chicken embryos. For the corrected BMC50, R^2 increased from 0.19 to 0.67 and from 0.50 to 0.88 for the in vivo lowest and median BMD10 values, respectively, compared to the correlations obtained for the non-corrected values (Fig. 3d). For the corrected LOAEC values, the R^2 increased from 0.23 to 0.76 and from 0.23 to 0.58, when it correlated to the lowest and median BMD10 values, respectively (Fig. 3f), as compared to non-corrected values. These data thus demonstrate the power of including a component of kinetics when predicting relative in vivo toxicity potencies based on in vitro toxicity data. In addition, it is noteworthy

Table 4. Summary of in vitro toxicokinetic and toxicodynamic data and in vivo developmental toxicity data applied in the present study. Relative Papp values of the five antifungal compounds in the BeWo model, as well as the BMC50 and corrected BMC50 values (μM) in the ES-D3 cell differentiation assay as previously reported [8]. BMC50, corrected BMC50, LOAEC and corrected LOAEC values (μM) in the ex ovo assay of chicken embryos of the present study. Lowest and median BMD10 values (μmol/kg bw/day) for in vivo developmental toxicity of the test compounds in rats.

	BeWo	**ES-D3**	**ES-D3+BeWo**	**Ex ovo**	**Ex ovo+BeWo**	**Ex ovo**	**Ex ovo+BeWo**	**In vivo**	**In vivo**
	Relative Papp value[a]	BMC_d50[a]	Corrected BMC_d50[a]	BMC50	Corrected BMC50	LOAEC	Corrected LOAEC	Lowest BMD10	Median BMD10
Ketoconazole	0.79	10.6	13.5	0.3	0.4	0.1	0.1	20.1[b]	20.1[b]
Tebuconazole	0.86	18.9	21.9	21.8	25.4	≤3.0	≤3.5	27.6	275.8
Fenarimol	0.55	24.3	44.1	6.0	11.0	3.0	5.5	88.5[b]	88.5[b]
Propiconazole	0.70	38.6	55.2	9.3	13.2	3.0	4.3	84.6	386.7
Prothioconazole	0.40	35.4	88.6	20.4	51.1	3.0	7.5	212.4	917.8

[a] Data from [8]

[b] for ketoconazole and fenarimol, only one value for lowest and median BMD10 was derived, as only data for one endpoint were available

- Data not available

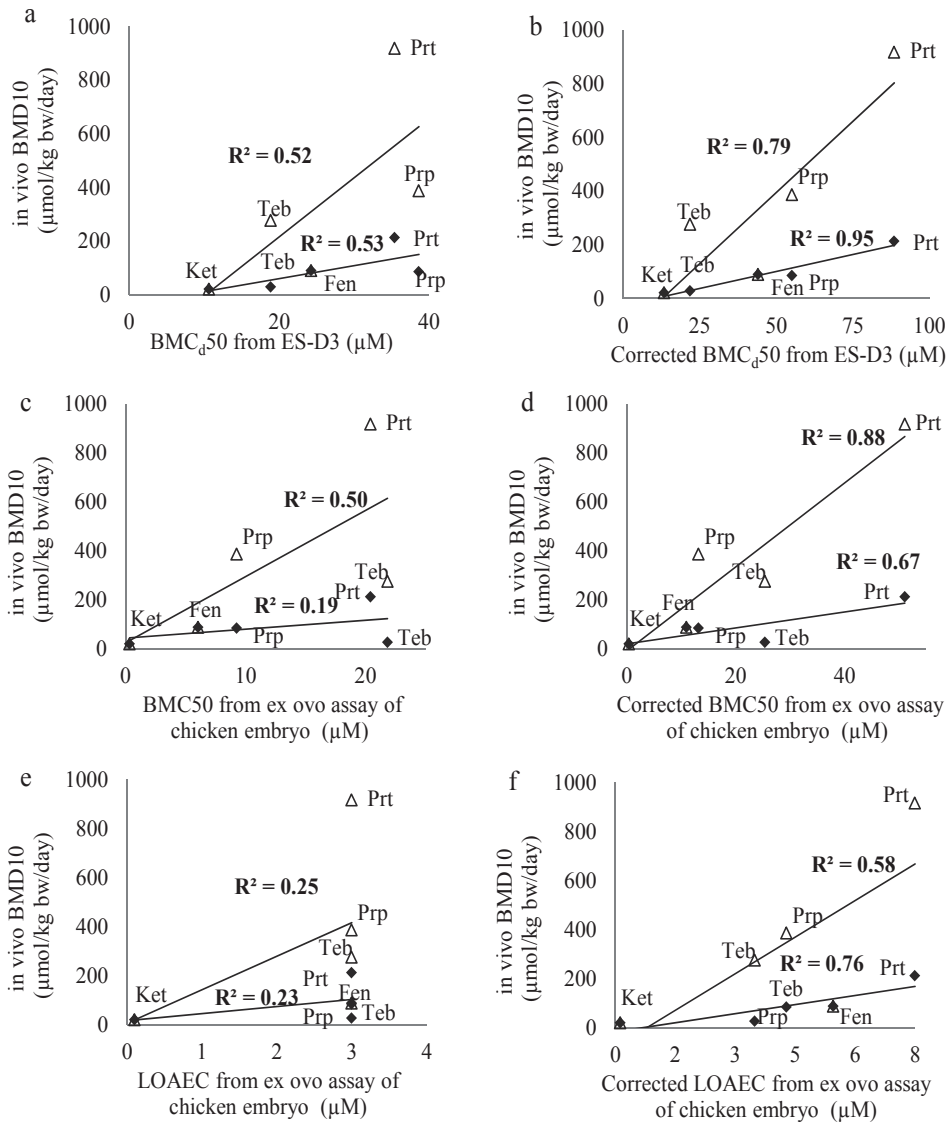

Fig. 3. Correlation between in vivo lowest (♦) and median (△) BMD10 values of the five test compounds ketoconazole (Ket), tebuconazole (Teb), propiconazole (Prp), prothioconazole (Prt) and fenarimol (Fen), as derived based on data reported in the literature, with (a) BMCd50 values, obtained from the ES-D3 cell differentiation assay, (c) BMC50 and (e) LOAEC, obtained from the ex ovo assay of chicken embryos, as well as with (b) the corrected BMC$_d$50, obtained from the ES-D3 differentiation assay with relative Papp values from the BeWo transport model, (d) corrected BMC50 and (f) corrected LOAEC values, obtained from the ex ovo assay of chicken embryos with relative Papp values from the BeWo transport model.

that better correlation was obtained for the corrected BMC_d50 and LOAEC values to the lowest BMD10 values than to the median BMD10 values; whereas, the corrected BMC50 values correlated better to the median BMD10 values than to the lowest BMD10 values.

DISCUSSION

Developmental toxicity is of high concern and currently a number of animal free methods to evaluate developmental toxicity have been developed. Both the ES-D3 cell differentiation assay of the EST and the ex ovo assay of chicken embryos have been used to investigate the developmental toxic potential of compounds. For the ES-D3 cell differentiation assay, we have previously shown its usefulness in predicting relative in vivo developmental toxicity potencies of five antifungal compounds, especially when combined with kinetic data on placental transfer. However, as the ES-D3 cell differentiation assay only focuses on the effect of compounds on a single endpoint, namely cardiomyocyte differentiation, as a sensitive indicator for general developmental toxicity [24], it may not be always suitable to assess specific teratogenic effects of compounds. In teratology, multiple and often interrelated events on the complex cells and tissues are thought to ultimately lead to dysmorphogenesis. These complex mechanisms may not be discovered in the ES-D3 cell differentiation assay when characterising only one differentiation endpoint. Therefore, in the present study, the ex ovo assay of chicken embryos was applied to evaluate the teratogenic potential of the same set of compounds, as previously tested in the ES-D3 cell differentiation assay, enabling a comparison of the predictive ability on developmental toxic potencies in the two assays.

Antifungal compounds have shown to induce specific alterations, including craniofacial changes and inter alia cleft palate [10], with the mechanism of action of antifungal compounds on affecting skeletal development related to alterations during hindbrain segmentation and branchial arches formation [11]. The ex ovo assay of chicken embryos may be a suitable tool to study this characteristic of the teratogenic potential of the antifungal compounds as it assesses chemicals with regard to their developmental toxic potential based on morphological changes including changes of neural tube closure at the head region. As shown in Table 1, ketoconazole, tebuconazole, propiconazole and prothioconazole all induced skeletal defects in rats. The results of our ex ovo assay of chicken embryos support the critical effect expected for these antifungal compounds as all four compounds caused significant alterations on branchial arch and neurulation endpoints.

Unlike these four compounds, fenarimol did not produce significant branchial arch dysmorphogenesis, although it exerted severe alteration on neurulation and head anlage. This is in accordance with the in vivo data that fenarimol may be developmental neurotoxic as increased neuromuscular and behavioral deficits in nursing pups after exposure at GD 15-21 was observed [23].

It is noteworthy that in chicken embryos of the concurrent control treatment for prothioconazole, higher incidences of alterations (neurulation, head and hind limb) were observed than for the control embryos for other compounds. This phenomenon of variable incidences of alterations in controls, although unusual, can happen in the ex ovo assay of chicken embryos and thus indicates the need to have substantial experience with this assay to interpret study results. Clearly it is preferable to have a low rate of alterations in the control group to increase sensitivity to have a neat picture of the type of alterations induced. Whenever unusually high rates of alterations are observed in control groups, evaluation of the data should be performed with utmost care to avoid an incorrect assessment of the developmental toxicity potential of a chemical.

The power of including a component of kinetics, i.e. placental transfer, when predicting relative in vivo developmental toxicity potencies based on in vitro toxicity data, was also investigated in the present study. The results obtained reveal that the corrected BMC_d50 of the ES-D3 cell differentiation assay, and the BMC50 and LOAEC values of the ex ovo assay of chicken embryos correlated better with the in vivo BMD10 values than the uncorrected ones. This indicates that combining the in vitro toxicity data with placental transfer kinetics improved the ability of the ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos to predict relative in vivo prenatal developmental toxicity potencies of the selected antifungal compounds. Other important in vivo kinetic processes than placental transfer, such as intestinal absorption, maternal metabolism and placental metabolism, were not taken into account in the present study. Therefore, incorporating more kinetic information is likely to further improve the predictive value of the two assays. It should be noted that the data from the BeWo cell model were used in a relative way. The assay is not suitable to determine absolute transport rates of compounds, given that the BeWo cell model is a simplification of the in vivo placental transfer system. In those cases where it is important to use exact fetal bioavailability values, physiologically based kinetic (PBK) models which describe the in vivo absorption, distribution, metabolism, and excretion processes of a compound should be used. Furthermore, in order to translate the in vitro concentration-response curves to in vivo dose-response curves, a so-called reverse dosimetry approach could be used. In this approach, in vitro toxic effect concentrations are considered as surrogate tissue (fetal) or blood concentrations that would cause adverse effect in the in vivo situation. The in vivo toxic dose levels can be predicted using a PBK model to calculate the doses that are needed to reach these internal effect concentrations. This PBK-model based reverse dosimetry approach will enable definition of reference values for risk assessment, e.g. BMD10 and BMDL10 values [25-28].

BMC50 and LOAEC values were obtained from the ex ovo assay of chicken embryos in the present study. The BMC50 values, in both original and corrected form, correlated

better to the in vivo BMD10 values, than the LOAEC values, indicating that the BMD approach is a scientifically more advanced method to the LOAEL approach for deriving a reference point, also because it makes full use of the available dose-response data and provides a quantification of the uncertainties in the dose-response data.

In risk assessment, lowest BMD10 values derived from in vivo data were used as reference values from a conservative point of view. However, when the lowest BMD10 values are given by the endpoints with high incidence number in control group and historical control data are not available, it is questionable if these endpoints are treatment-related. When the endpoint is not treatment-related, it may result in a false BMD10 value and the toxic potency of the compound is overestimated. Therefore, it is highly recommended, whenever possible, to take historical control data into consideration when doing the BMD analysis of in vivo data. In the present study, the historical control data were not available for some endpoints for which a high incidence is noted in the control group. Therefore we included also median BMD values for comparison with in vitro toxic concentrations. It was shown that the in vitro concentrations, obtained from the ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos, when corrected for placental transfer data, correlated well to both lowest and median BMD values, showing a good predictive ability of both assays.

The ex ovo assay of chicken embryos showed higher sensitivity than the ES-D3 cell differentiation assay to toxic antifungal compounds, indicated by the higher BMC_d50, obtained from the ES-D3 assay, as compared to the BMC50 and LOAEC values obtained from the ex ovo assay of chicken embryos (Table 4). It is interesting to note that both assays have shown their own advantages and limitations. Advantages of the ex ovo assay of chicken embryos include higher sensitivity, shorter experimental procedure, the capability to evaluate more specific teratogenic endpoints and good predictivity of the toxic potency. However the assay is more labor intensive and requires special equipment and a well trained staff. Advantages of the ES-D3 differentiation assay are that it is animal free, cheaper, and showing good predictivity of the toxic potency. The assay is however not able to identify specific manifestation patterns or is less able to distinguish potential modes of action of different teratogenic compounds underlying the embryotoxicity. Based on the results of the present and our previous studies [7, 8] it can be suggested that a combined and tiered testing strategy based on the two assays could be used to predict teratogenic potential and developmental toxicity. As a simpler assay, the ES-D3 cell differentiation assay can be used in a first tier to predict toxicity potency of chemicals and to detect the compound(s) of high concern, i.e. showing high toxic potency. Then the ex ovo assay of chicken embryos can be applied to test chemical(s) of high concern in a second tier enabling identification of a more specific pattern of morphological changes, giving thereby more information on the structures affected by the developmental toxicant.

CONCLUSION

In conclusion, the present study showed that the ex ovo assay of chicken embryos was able to detect the teratogenic potential and to predict relative developmental toxic potencies of antifungal compounds. When combined with the in vitro BeWo transport model, both the ex ovo assay of chicken embryos and the ES-D3 cell differentiation assay appeared to better predict the relative in vivo developmental toxicity potencies of the antifungal compounds. In combination, the three assays could provide a valuable tiered alternative approach for prenatal developmental toxicity screening and prioritization. More compounds with different modes of action should be tested to further evaluate the capacity of this approach in predicting the teratogenic potential and the developmental toxicity potency of chemicals. Since the method uses only in vitro assays to predict in vivo prenatal developmental toxicity ranking of chemicals, it can contribute to the 3Rs of animal testing. The predictive ability of the assays can most likely be even further enhanced when combined with more kinetic data and PBK modeling.

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SUPPLEMENTARY MATERIAL 1

Table 1. Evaluation Scheme for calculating the morphological scores (modified according to Klug et al. [13]).

Primordium	Evaluation
Posture of the embryo	5 = spiral bend 4 = U-shaped curve 3 = caudal trunk or tail portion are positioned at the right side of the embryo or embryo is stretched 2 = bend over the backs 1 = abnormality
Neurulation	5 = neuropore closed completely 4 = only caudal neuropore open 3 = cranial and caudal neuropore open 2 = only cranial neuropore open 1 = abnormality
Head	4 = rombencephalon with incipient division into hemispheres by media-nelongitudinal furrow 3 = separated by transverse furrow telencephalon from the diencephalon 2 = no transverse furrow between telencephalon and diencephalon smooth transition between peak and neck hump 1 = abnormality
Eye	5 = lens bag 4 = invagination of the optic vesicle to the eye cup; cleft of the eye column 3 = optic vesicle 2 = no eye system 1 = abnormality
Ear	5 = endolymphatic duct visible 4 = bladder 3 = mine ears 2 = no ear system 1 = abnormality
Heart	4 = starting septa; heart appears as four chambers 3 = S-shaped heart loop with thickened endocardial cushion between primitive atrium (atrium primitivum) and primitive main chamber (ventricle primitivum) 2 = U-shaped bulboventricular loop 1 = abnormality
Branchial arches	2 = two branchial arches 1 = one branchial arch 0 = none / abnormality
Fore/hind limb	4 = limb stump, in which the height is ≥ the width of the base 3 = limb stump, in which the height is < the width of the base 2 = no cusp extremities 1 = abnormality

Table 1. Evaluation Scheme for calculating the morphological scores (modified according to Klug et al. [13]).

Primordium	Evaluation
Caudal part of trunk	5 = tail bud, length > width (stretch) 4 = length > width 3 = length ≤ width 2 = no structure caudal to the hind limb system 1 = abnormality

SUPPLEMENTARY MATERIAL 2

Table 1. BMD10 values derived from the incidence of malformation(s) in rats exposed to ketoconazole [15]. The lowest and the median (if possible) BMD10 values used for the comparison with in vitro data are presented in Table 4.

	Dose (mg/kg bw/day)			
	Control	**25**	**73**	**BMD10(mg/kg bw/day)**
No. litters examined	22	22	3	
Cleft palate	0	3	3	10.7

Table 2. BMD10 values derived from the incidence of variation(s) in rats exposed to tebuconazole [18]. The lowest and the median (if possible) BMD10 values used for the comparison with in vitro data are presented in Table 4.

	Dose (mg/kg bw/day)				
	Control	**30**	**60**	**120**	**BMD10(mg/kg bw/day)**
No. pups examined	144	137	127	116	
Cervical vertebra 1	18	21	24	48	48.1
Cervical vertebra 2	29	40	38	48	39.3
Cervical vertebra 3	9	10	12	16	134.5
Cervical vertebra 4	0	5	2	13	118.8
Cervical vertebra 5	3	3	3	10	131.2
Cervical vertebra 6	1	2	2	6	168.8
Vertebral arch 6, left	1	2	3	14	114.7
Vertebral arch 6, right	0	2	6	13	100.3
Vertebral arch 7, left	50	53	49	66	34.9

Table 2. BMD10 values derived from the incidence of variation(s) in rats exposed to tebuconazole [18]. The lowest and the median (if possible) BMD10 values used for the comparison with in vitro data are presented in Table 4.

	Dose (mg/kg bw/day)				
	Control	**30**	**60**	**120**	**BMD10(mg/kg bw/day)**
Vertebral arch 7, right	45	53	51	65	30.8
Sternebra 2	4	3	3	15	118.5
Sternebra 6	0	0	1	4	150.6
Supernumerary ribs, left	14	20	18	26	90.0
Supernumerary ribs, right	15	23	19	24	113.6
Digit 2 proximal phalanx (l)	27	36	37	42	52.1
Digit 3 proximal phalanx (l)	0	3	5	9	133.5
Digit 4 proximal phalanx (l)	2	7	7	11	138.6
Metacarpal 5 (l)	0	0	2	4	157.5
Digit 2 proximal phalanx (r)	27	33	39	40	54.5
Digit 3 proximal phalanx (r)	0	3	4	8	141.0
Digit 4 proximal phalanx (r)	1	6	6	11	131.3
Metacarpal 5 (r)	0	0	2	5	147.4
Metatarsal (l)	18	24	18	31	84.9
Toe 2 proximal phalanx (l)	107	92	96	103	40.3
Toe 3 proximal phalanx (l)	81	70	78	87	23.6
Toe 4 proximal phalanx (l)	79	65	72	85	50.6
Metatarsal (r)	18	24	20	32	76.4
Toe 2 proximal phalanx (r)	110	96	105	105	37.6
Toe 3 proximal phalanx (r)	86	75	76	92	46.4
Toe 4 proximal phalanx (r)	81	71	74	91	45.3
No. litters examined	24	24	22	24	
Cervical vertebra 2	14	17	17	20	8.5
Sacral vertebral arch 6 (r)	0	2	6	8	55.8
Digit 2 proximal phalanx (l)	14	14	16	18	45.8
Digit 3 proximal phalanx (l)	0	3	5	4	83.1
Digit 3 proximal phalanx (r)	0	3	4	4	91.3
Digit 4 proximal phalanx (r)	1	4	6	6	83.4

Table 3. BMD10 values derived from the incidence of external alteration(s) in rats exposed to tebuconazole [19]. The lowest and the median (if possible) BMD10 values used for the comparison with in vitro data are presented in Table 4.

	Dose (mg/kg bw/day)				
	Control	**10**	**30**	**100**	**BMD10(mg/kg bw/day)**
No. pups examined	174	144	202	168	
External alterations	3	1	4	12	126.3

Table 4. BMD10 values derived from the incidence of malformation(s) or of altered variation(s) in rats exposed to propiconazole [20]. The lowest and the median (if possible) BMD10 values used for the comparison with in vitro data are presented in Table 4.

	Dose (mg/kg bw/day)				
	Control	**30**	**90**	**300**	**BMD10(mg/kg bw/day)**
No. pups examined	141	148	156	148	
Cleft palate	0	0	1	2	551.8
Renal papilla short	32	27	40	57	123.0
No. pups examined	129	136	146	137	
Renal papilla missing	4	4	8	16	318.2
rudimentary ribs	0	1	4	53	141.7
sternebrae not ossified	49	54	83	99	29.0
No. litters examined	22	21	22	22	
cleft palate	0	0	1	2	303.4
Renal papilla missing	4	3	3	12	62.2
rudimentary ribs	0	1	4	16	31.1

Table 5. BMD10 values derived from the incidence of malformation(s) or of altered variation(s) in rats exposed to prothioconazole [21]. The lowest and the median (if possible) BMD10 values used for the comparison with in vitro data are presented in Table 4.

	Dose (mg/kg bw/day)				
	Control	**20**	**80**	**750**	**BMD10(mg/kg bw/day)**
No. litters examined	25	25	25	25	
Rudimentary ribs (punctiform)	23	19	22	24	73.1
Rudimentary (comma-shaped)	13	16	9	17	268.8
Extra (full-size)	8	4	3	10	337.8

Table 6. BMD10 values derived from the incidence of malformation(s) or of altered variation(s) in rats exposed to prothioconazole [21]. The lowest and the median (if possible) BMD10 values used for the comparison with in vitro data are presented in Table 4.

	Dose (mg/kg bw/day)				
	Control	**80**	**500**	**1000**	**BMD10(mg/kg bw/day)**
No. litters examined	26	25	22	24	
Microphthalmia	0	6	3	11	306.5

Table 7. BMD10 values derived from the incidence (percentage) of malformation(s) or of altered variation(s) in rats exposed to fenarimol [22]. The lowest and the median (if possible) BMD10 values used for the comparison with in vitro data are presented in Table 4.

	Dose (mg/kg bw/day)				
	Control	**5**	**13**	**35**	**BMD10(mg/kg bw/day)**
Hydronephrosis	9%	0	0	30%	29.3

CHAPTER 6

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

Hequn Li, Mengying Zhang, Jacques Vervoort, Ivonne M. C. M. Rietjens,
Bennard van Ravenzwaay, Jochem Louisse

In preparation

ABSTRACT

Toxicological hazard and risk assessment largely rely on animal testing. For economic and ethical reasons, the development and validation of reliable alternative methods for these animal studies, such as in vitro assays, are urgently needed. In vitro concentration-response curves, however, need to be translated into in vivo dose-response curves for the risk assessment purposes. In the present study, we translated in vitro concentration–response data of the antifungal compound tebuconazole, obtained in the ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos, into predicted in vivo dose–response data for developmental toxicity using physiologically based kinetic (PBK) modeling-facilitated reverse dosimetry. The predicted in vivo dose–response data were used to derive BMD10 values for developmental toxicity in rat, which were compared with BMD10 values derived from toxicity data in rats as reported in the literature. The results show that the BMD10 values from predicted dose–response data from both assays are in concordance with BMD10 values derived from in vivo data (within 5-fold difference). It is concluded that PBK modeling-facilitated reverse dosimetry of in vitro toxicity data is a promising tool to predict in vivo dose-response curves and could be used to define a point of departure for deriving safe exposure limits in risk assessment.

INTRODUCTION

The determination of safe human exposure levels of chemicals in toxicological risk assessments largely relies on animal toxicity data. In these toxicity studies, the majority of the animals are used for reproductive and developmental toxicity testing [1]. For economic and ethical reasons, there is large interest in the development of in vitro test systems as alternatives for the animal studies. A number of in vitro methods have been developed to screen compounds for potential developmental toxicity, using primary cultures of embryonic cells or embryonic stem cell lines, such as the embryonic stem cell test (EST), as well as test methods using whole embryos, such as the ex ovo assay of chicken embryos [2-4].

Among these alternative test systems for the assessment of developmental toxicity, the EST does not require the use of primary animal tissues and can therefore be considered as an animal-free test. The differentiation assay of the EST has been proven useful in the prediction of the in vivo potency ranking of structurally related compounds, such as glycol ethers, retinoids and phenols [5-7]. This assay uses the blastocyst-derived embryonic stem cell line D3 (ES-D3) that spontaneously differentiates into contracting cardiomyocytes when cultured as embryoid bodies, and it determines the concentrations of test compounds that inhibit this process as a measure of in vitro developmental toxicity. Recently, we have demonstrated that the ES-D3 cell differentiation assay is useful to predict the relative developmental toxicity potencies of antifungal compounds in vivo [8, 9]. Furthermore, we showed that the correlation between the in vitro effect concentrations and the in vivo effect doses improves when kinetic data on placental transfer are taken into account [8, 9].

A limitation of the ES-D3 cell differentiation assay is that it is an assay for general developmental toxicity and may not be predictive for specific morphological changes, since it assesses the effect of the chemical on the differentiation of stem cells into cardiomyocytes. The ex ovo assay of chicken embryos assesses chemicals with regard to their developmental toxic potential based on morphological changes [10]. The culture duration of the chicken embryos covers major parts of the organogenesis, thereby offering the assessment of various morphological endpoints including specific development of different organ anlagen, i.e. head region with different brain -, eye -, and ear anlagen and branchial arches. Therefore, the ex ovo assay of chicken embryos is a more suitable tool to study in a more specific way the potential interference of compounds with the embryo and fetal development. We have previously shown that the ex ovo assay of chicken embryos was able to predict the developmental toxicity potential of antifungal compounds, and, when combined with the in vitro BeWo transport model, was also able to better predict the relative in vivo developmental toxicity potencies of antifungal compounds than when the ex ovo assay of chicken embryos was used as a stand-alone assay [11].

Although both the ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos, in combination with data on placental transfer from the BeWo model, were shown to correctly predict relative in vivo potencies of antifungal compounds, these data cannot be used as such for risk assessment. An important reason is that the in vitro and ex ovo assays provide in vitro concentration–response curves on adverse effects on the cells and on the embryo, respectively, whereas in vivo dose–response curves are required for setting safe exposure levels in risk assessment. The application of so-called reverse dosimetry enables the translation of in vitro concentration–response curves into in vivo dose–response curves using physiologically based kinetic (PBK) models, providing a platform to use in vitro toxicity data for risk assessment [4, 12-14]. PBK models quantitatively describe absorption, distribution, metabolism, and excretion processes of a compound in the body, and can relate external (toxic) doses to internal (toxic) concentrations [15]. In reverse dosimetry approaches aiming to predict in vivo toxic dose levels, in vitro toxic effect concentrations are considered as surrogate tissue or blood concentrations that would cause adverse effect in the in vivo situation. The in vivo toxic dose levels can be predicted using a PBK model to calculate the doses that are needed to reach these internal effect concentrations. PBK modeling-facilitated reverse dosimetry is the only method that enables the quantitative translation of in vitro data to the in vivo situation. Therefore, there is increased interest in using the PBK modeling-facilitated reverse dosimetry approach to derive points of departure (PoDs) for risk assessment, such as Benchmark Dose (BMD) values, based on the translation of in vitro concentration–response data obtained from in vitro toxicity assays [16, 17]. Our group has shown that reverse dosimetry of developmental toxicity data obtained in the ES-D3 cell differentiation assay resulted in the correct prediction of in vivo dose–response curves of glycol ethers, phenol and retinoic acid [4, 12, 13]. No proof-of-principles of chemicals from other chemical categories are available so far.

In the present study, we assessed whether the reverse dosimetry approach correctly predicts in vivo developmental toxicity of tebuconazole, a chemical belonging to the category of antifungal compounds. To this end we developed a PBK model for tebuconazole in the rat, based on in vitro- and in silico-derived parameter values, and used this model to translate data on tebuconazole-induced developmental toxicity obtained in the ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos to predicted in vivo dose-response curves for developmental toxicity in rat. The predicted in vivo dose–response data were used to derive BMD10 values for rats, and were compared with BMD10 values derived from toxicity data in rats as reported in the literature, in order to evaluate the predictions made.

MATERIALS AND METHODS

Chemicals

Tebuconazole was purchased from Sigma-Aldrich (Steinheim, Germany). Dimethyl sulfoxide (DMSO) was purchased from Acros Organics (Geel, Belgium). Penicillin, streptomycin, L-glutamine, non-essential amino acids, phosphate-buffered saline (PBS) and Hank's balanced salt solution (HBSS) were obtained from Invitrogen (Breda, the Netherlands).

High-performance liquid chromatography (HPLC) analysis

HPLC analysis was performed to quantify the amount of the test compound tebuconazole and its metabolites. The HPLC system used consisted of a Waters (Milford, MA) 600 controller and a 600 pump, equipped with a photodiode array detector set to record absorption of wavelengths between 200 and 400 nm. A Waters 717 plus autosampler was used for sample injection. The temperature of the autosampler was kept at 7 °C.

For analysis of all compounds, 50 µl sample was injected to a C18 5 µm reverse-phase column (150 mm × 4.6 mm I.D.) with a guard column (7.5 mm × 4.6 mm I.D.) (Alltech, Bergen op Zoom, the Netherlands). The mobile phase used for analysis consisted of (A) 0.1% trifluoroacetic acid in nanopure water and (B) HPLC-grade acetonitrile. Elution was at a flow rate of 0.8 ml/min, starting at 22% B with a linear increase to 100% B in 8 min. Subsequently, the gradient returned linearly to the initial condition in 10 min and remained 2 min at this condition prior to the next injection.

PBK modeling-facilitated reverse dosimetry approach

The PBK modeling-facilitated reverse dosimetry approach to predict in vivo developmental toxicity based on in vitro toxicity data (Figure 1) consists of six steps, being (1) the determination of in vitro concentration–response data for tebuconazole in the ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos, (2) the development of a PBK model for tebuconazole in rat, (3) the evaluation of the predictions made by the PBK model, (4) the translation of in vitro concentration–response data into in vivo dose–response data using the PBK model, (5) the derivation of a BMD value based on the predicted dose–response data and (6) the evaluation of the approach by comparison of the predicted BMD value with a BMD value derived from in vivo developmental toxicity data obtained from the literature.

1. Determination of in vitro concentration–response data in the ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos

The in vitro concentration–response data on the tebuconazole-induced inhibition of ES-D3 cell differentiation into contracting cardiomyocytes were obtained from our earlier study [8]. The data of three independent experiments of this study are presented in Fig. 1a.

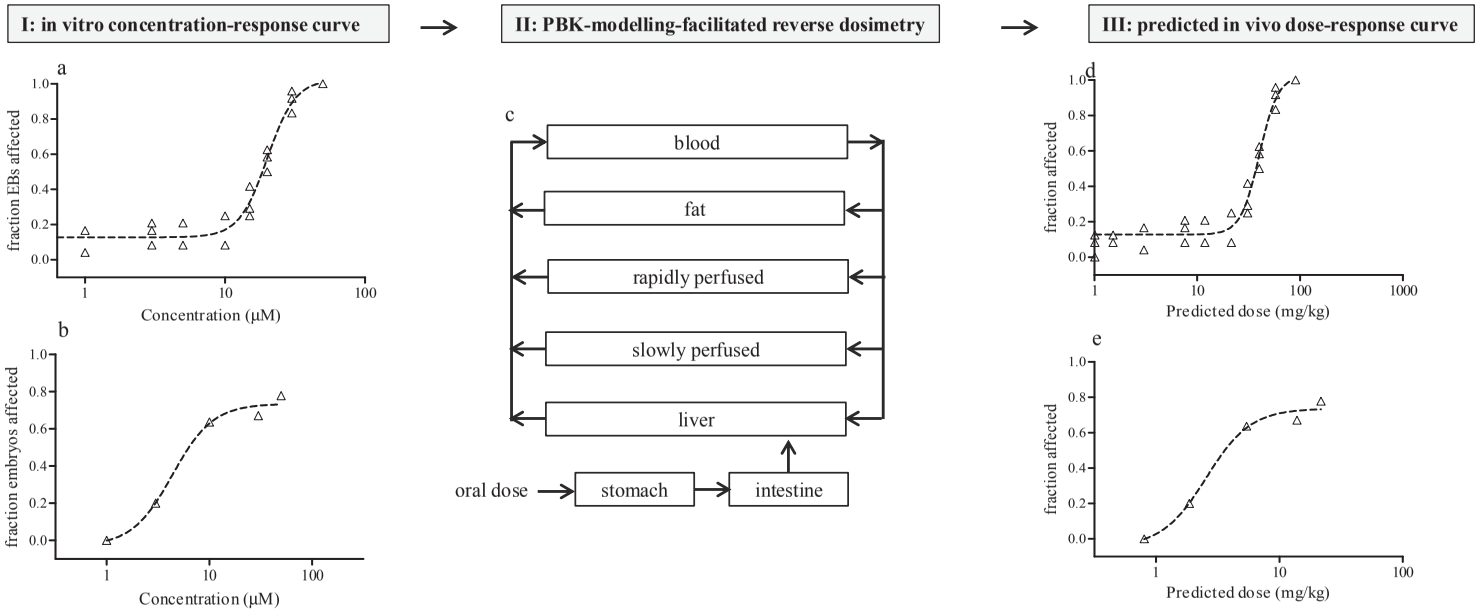

Fig. 1 A schematic representation of the reverse dosimetry approach used in the present study. I: in vitro concentration-response curves obtained from two assays. a: Concentration-dependent inhibition of ES-D3 cell differentiation by tebuconazole. Graph represents data from three independent experiments and were taken from our earlier study [8]. b: Concentration-dependent increase of alterations of chicken embryos by tebuconazole. Graph represents data taken from one experiment from our earlier study [8] II: Schematic representation of the PBK model for tebuconazole in rat used for reverse dosimetry (c). III: Hypothetically predicted dose-response curves for developmental toxicity of tebuconazole using PBK modelling-facilitated reverse dosimetry based on data from the ES-D3 cell differentiation assay (d) and from the ex ovo assay of chicken embryos (e) (the real-predicted curves are presented in Fig. 6).

The in vitro concentration-response data on the tebuconazole-induced branchial arch alteration (most sensitive endpoint for tebuconazole in the ex ovo assay), were obtained from another previous study [11]. The data of this study are presented in Fig. 1b.

2. Development of a PBK model for tebuconazole in rat

The conceptual structure of the PBK model developed for tebuconazole is shown in Fig. 1c. Values for anatomical and physiological parameters are presented in supplementary material 1 Table 1 and were taken from literature [18]. The processes of stomach emptying (half-life in rat, liquid, 15 min) [19] and the small intestinal transition time (complete transition in rat, 1.5 h) [20] are included in the model. To describe small intestinal transition, the intestinal compartment was divided in 7 sub-compartments [12]. Values for kinetic parameters, including (1) intestinal absorption, (2) partitioning into tissues and (3) hepatic clearance, were determined in the present study using data obtained from in vitro and in silico methods as described below. The PBK model describes only the kinetics of tebuconazole and not of its metabolites, since we assume that the developmental toxicity is caused by the parent compound. The PBK model was coded and numerically integrated in Berkeley Madonna 8.3.18 (UC Berkeley, CA, USA), using the Rosenbrock's algorithm for stiff systems. The PBK model code is presented in the supplementary material 3.

2.1 Determination of model parameter value for intestinal absorption

The parameter value for the intestinal absorption of tebuconazole was determined by using the following equation: absorption rate (μmol h^{-1}) = Papp coefficient in vivo (cm h^{-1}) × surface area of the rat small intestine (cm^2) × luminal concentration of tebuconazole (mM) [21]. The value for the surface area of the rat small intestine was calculated to be 94 cm^2 (radius of 0.18 cm and a small intestine length of 83 cm [22]). The small intestinal volume was calculated to be 8.4 ml. The luminal concentration of tebuconazole is calculated by the model by dividing the amount of tebuconazole by the small intestinal volume (ml).

The Papp coefficient in vivo was estimated based on in vitro transport studies using Caco-2 cell layers in a transwell set-up. Sun et al. (2002) described the correlation between Papp values obtained in such Caco-2 studies and that obtained from ex vivo studies using human jejunum tissue ($Papp_{,in\ vivo}$): $\text{Log}(Papp_{,in\ vivo}) = 0.6836 \times \text{Log}(Papp_{,Caco\text{-}2}) - 0.5579$. Caco-2 transport studies were performed as described below to determine the $Papp_{,Caco\text{-}2}$, which was used to estimate the $Papp_{,in\ vivo}$. It was assumed that the estimated $Papp_{,in\ vivo}$ value for humans is the same as the $Papp_{,in\ vivo}$ value for rats.

For the Caco-2 transport studies, Caco-2 cells (P35-P40, obtained from ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Breda, the Netherlands, cat. no. 61965), supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS, HyClone-Perbio, Etten-Leur, the Netherlands), 10,000 U/ml penicillin, 10 mg/ml streptomycin, 1 % non-essential amino acids and 2 mM L-glutamine. The cells

were seeded at a density of 2.24 x 10^5 cells/cm^2 on transwell® polycarbonate membranes (12 mm diameter, 0.4 µm pore size) (VWR International BV, Amsterdam, the Netherlands) and cultured in a humidified incubator at 5 % CO_2 and 37 °C. Medium (0.5 ml apical compartment, 1.5 ml basolateral compartment) was changed every 2-3 days. Transport studies were performed at day 20 post-seeding. The barrier forming capacity of the Caco-2 cell layers was evaluated by measuring the transepithelial electrical resistance (TEER) of the cell layer using a Millicell ERS-2 Volt-Ohm Meter (Millipore, USA). The TEER value was determined after replacing culture medium with HBSS, showing TEER values between 700-900 Ω/cm^2.

Transport experiments were initiated by adding 0.5 ml of transport buffer HBSS containing 50 µM tebuconazole, added from a 200 times concentrated stock solution in DMSO (final solvent concentration 0.5% DMSO) to the apical compartment and 1.5 ml HBSS to the basolateral compartment. Subsequently, the plate was incubated in a humidified atmosphere with 5 % CO_2 at 37 °C. After 15, 30, 60 and 90 min, a sample of 0.2 ml was taken from the basolateral compartment and replaced by an equal volume of HBSS. At the end of each experiment, a 0.2 ml sample was also taken from the apical compartment. Subsequently, the filters with the Caco-2 cell layers were washed 3 times with PBS, cut out of the insert, dissolved in 0.25 ml 65 % (v/v) methanol and sonificated for 15 min in a Bandelin Sonorex RK100 (Berlin, Germany) in order to determine the amount of compound accumulated in the cells and enabling mass-balance calculations. All samples were stored at –20 °C until HPLC analysis. In each transport study, amoxicillin was included as a control for monolayer integrity since it is a compound known to be transported only to a limited extent in the Caco-2 cell model [23] and antipyrine was included as a control for optimal transport [24]. The transport of tebuconazole, amoxicillin and antipyrine was also determined through the permeable membrane of the transwell filter in the absence of Caco-2 cells. It was shown to be equally fast among these compounds (data not shown), ensuring that any differences observed in the transport studies with Caco-2 cells were related to the Caco-2 cell layer and not to the filter.

For the test compound, the linear appearance rate in the basolateral compartment was determined to calculate the apparent permeability coefficient $Papp_{,Caco\text{-}2}$ ($Papp_{,Caco\text{-}2}$ (cm/s) = $(\Delta Q/\Delta t)/(A \cdot C_0)$), where ΔQ is the amount of test compound (nmol) transported to the receiver chamber in a certain time span (Δt (s)), A is the cell surface area (cm^2) and C_0 is the initial concentration of the test compound in the donor compartment (µM).

2.2 Determination of model parameter values for tissue/blood partitioning

Tissue/blood partition coefficients for tebuconazole were calculated using the algorithm reported in literature [25], requiring information on fraction unbound (f_{ub}) in blood, the pKa and log Kow of the compound as sole input parameters. The log Kow (at pH 7.4) reported for tebuconazole is 3.7 [26] and the pK_a 5.0 [27]. The f_{ub} of tebuconazole in rat blood was determined using an approach described in literature [28] to determine protein

binding of chemicals. In short, protein binding parameters were estimated based on the assessment of the increase of EC_{50} values for tebuconazole cytotoxicity with increasing albumin concentrations, determined as described before [28] with slight modifications. A description of the cytotoxicity study is presented in the supplementary material 2. Obtained EC_{50} values of tebuconazole were determined from concentration–response relationships obtained in independent experiments with increasing concentrations of albumin and were plotted against the albumin concentration present in the exposure medium. A linear correlation was obtained between the EC_{50} values and the albumin concentration. Linear regression ($Y = aX + b$, $Y = EC_{50}$, X= albumin concentration) was obtained with the slope (a) and intercept (b) for the best fit. The f_{ub} to albumin was estimated by using the following equation: $f_{ub} = b / EC_{50}$ where b is the intercept of the linear regression equation, and EC_{50} is the value in the presence of a certain concentration of albumin [28]. Based on these equations, the f_{ub} in rat blood was determined, using albumin concentration in rat blood being 293 µM [29].

2.3 Determination of model parameter values for hepatic clearance

Tebuconazole body clearance was described by hepatic clearance of the parent compound. To determine parameter values for description of the hepatic clearance, the maximum enzyme reaction rate (V_{max}) and the Michaelis–Menten constant (K_m) for microsomal metabolism of tebuconazole were determined using incubations with female rat microsomes in Eppendorf vials. Conditions were optimized to obtain linear reaction rates with respect to incubation time and microsome concentration (data not shown). Final studies for the determination of the kinetic constants were performed using incubation mixtures consisting of 0.1 M Tris-HCl (pH 7.4), containing (final concentrations) 2 mM NADPH (Roche Diagnostics, Mannheim, Germany) and 1 mg/ml pooled female Wistar rat liver microsomes (XenoTech, Kansas, USA). The incubations were started, after a 1-min pre-incubation at 37 °C, by the addition of the substrate tebuconazole from a 100 times concentrated stock solution in DMSO and left in a shaking water bath of 37 °C for 7 min. The reactions were terminated by the addition of ice-cold acetonitrile (20 % v/v). Subsequently, the Eppendorf vials were put on ice. All samples were centrifuged for 5 min at 15000 rpm using a microcentrifuge (CT15RE, VWR, Leuven, Belgium), and the supernatant was stored at –20 °C until HPLC analysis. Blank incubation mixtures were included, in which NADPH was omitted. The intact masses of metabolites were identified by liquid chromatography–mass spectrometry (LC-MS) analysis as described below.

To obtain the kinetic parameters V_{max} and K_m, the in vitro data on the concentration dependent formation of metabolites were fit to the Michaelis–Menten equation using GraphPad Prism 5.0 software (San Diego, CA, USA). The in vitro-derived V_{max} values (µmol/min/mg protein) were scaled to the in vivo situation using a microsomal protein concentration of 35 mg/g liver [30]. The in vivo K_m values were assumed to equal the in vitro K_m values.

2.4 Liquid chromatography–mass spectrometry (LC-MS) analysis

Separation and purification of the three major tebuconazole metabolites were performed by collecting the eluate of the HPLC column for the respective metabolite peaks. Then LC-MS analysis of each metabolite was conducted using a micrOTOF MS (Bruker) coupled to an Agilent LC (1200 Series) equipped with an Altima C18 column (150 x 4.6 mm, 3 µm). The mobile phase used consisted of (A) nanopure water with 0.1 % formic acid and (B) HPLC-grade acetonitrile with 0.1 % formic acid. Elution was at a flow rate of 0.8 ml/min, starting at 22 % B with a linear increase to 100 % B in 30 min. Subsequently, the gradient returned linearly to the initial condition in 2 min and remained 13 min at this condition prior to the next injection.

3. PBK model evaluation

In order to assess the performance of the PBK model, the predicted blood concentrations of tebuconazole were compared with reported blood concentrations from an in vivo kinetic study as reported in the literature [31]. The model was also evaluated by comparison of the predicted cumulative excretion of metabolites (in % of the administered dose) to in vivo data on the cumulative excretion (urine and feces) of tebuconazole and its metabolites [32]). These in vivo kinetic data were not used to optimize model performance, but were solely used for model evaluation.

Sensitivity analysis

Normalized sensitivity coefficients (SC) were determined to identify parameters that largely influence the prediction of the maximal blood concentrations (C_{max}) by the PBK model, using the equation: $SC = (C' - C)/(P' - P) \times (P/C)$, where P and P' are the initial and modified parameter values respectively, and C and C' are the initial and modified values of the model output resulting from an increase in parameter value, respectively [33]. A 5% increase in parameter value was chosen to analyze the effect of a change in parameter on the C_{max} of tebuconazole for 10-day exposure. Doses of 2, 12.5, and 25 mg/kg bw/day were used for the sensitivity analysis as these cover the range of doses applied in the in vivo kinetic studies that were used for model evaluation. Each parameter was analyzed individually, keeping the other parameters to their initial values.

4. Translation of in vitro concentration–response data into predicted in vivo dose–response data using PBK-modeling facilitated reverse dosimetry

In the reverse dosimetry approach for the prediction of in vivo developmental toxicity, the in vitro effect concentrations of tebuconazole inducing inhibition of ES-D3 cell differentiation or inducing alterations of chicken embryos, were considered as surrogate in vivo concentrations in blood that could result in adverse effect of embryonic/fetal development. The PBK model is used to determine the oral dose levels that would result in

these concentrations in the blood. Differences may be expected in the f_{ub} of tebuconazole in the culture medium in the two assays compared with the f_{ub} in rat blood, because rat blood contains different protein levels than the culture medium. As it is assumed that the f_{ub} of the chemical is most likely to cause toxicity, corrections were made to take the differences in the f_{ub} in the culture medium compared with the rat blood into account. A description of the procedure for this correction is presented in the supplementary material 2.

Subsequently, translation of the in vitro concentrations into in vivo doses was made by relating the toxic effect (both in vitro and in vivo) to the C_{max} of tebuconazole in blood. Then, it was determined which oral doses are required in the PBK model to reach the C_{max} of tebuconazole in the blood compartment that would result in developmental toxicity. By performing this exercise for each concentration used in the in vitro assay, all in vitro concentration–response data were translated into predicted in vivo dose–response data.

5. Derivation of a BMD value on the predicted dose–response data

BMD modeling was applied on the predicted in vivo dose–response data using all models for dichotomous data of the Environmental Protection Agency (EPA)'s Benchmark Dose Software (BMDS) version 2.4. BMD10 values that correspond with a 10% extra effect on the fraction of EBs inhibited or the fraction of chicken embryos with alterations were derived. Models included in the evaluation were the gamma, logistic, loglogistic, probit, logprobit, multistage, weibull and the quantal-linear model. Goodness of fit of the models was evaluated to judge if a model was accepted, using the p values with $p>0.05$, the scaled residuals and the graphical displays obtained as the criteria [34]. All models that met the requirements for acceptance of the model fit were considered for the determination of BMD10 values. Of the models that passed the criteria for model fit, the lowest BMD10 value was selected as the BMD10 value for developmental toxicity of tebuconazole. These values were used for comparison with BMD10 values derived from data of rat developmental toxicity studies reported in the literature.

6. Evaluation of the predictive value of the approach

To evaluate the potential of the in vitro–in silico approach to obtain a PoD for risk assessment, the predicted BMD10 values for rat were compared with BMD10 values derived from data from rat developmental toxicity studies reported in the literature. To this end, a literature study was performed, with search terms including tebuconazole together with combinations of the search terms teratogenicity, developmental toxicity, (skeletal) alteration, (skeletal) variation, and embryotoxicity. The results of studies performed in rats were used. A study was selected if it included at least one control group and two dose groups, which enables BMD modeling to derive a BMD10 value. BMD10 values that correspond with a 10% extra incidence of developmental toxicity above background levels were derived. The BMD10 values were calculated as described above for the predicted dose-response data.

RESULTS

Development of a PBK model for tebuconazole in rat

The PBK model code is presented in the supplementary material 3.

Determination of the model parameter value for intestinal absorption

Intestinal absorption was determined by multiplying the $Papp_{,in\ vivo}$ for intestinal absorption by the surface area of the intestine and the luminal concentration in the intestine. The $Papp_{,in\ vivo}$ coefficient was estimated based on Papp coefficients obtained in Caco-2 transport studies ($Papp_{,Caco\text{-}2}$) using the formula reported by Sun et al. (2002) that relates $Papp_{,Caco\text{-}2}$ to $Papp_{,in\ vivo}$ values. The obtained $Papp_{,Caco\text{-}2}$ was 3.8×10^{-5} cm s^{-1}, which resulted in a $Papp_{,in\ vivo}$ of 3.3×10^{-4} cm s^{-1} (equivalent to 1.2 cm h^{-1}).

Determination of model parameter values for tissue/blood partitioning

Tissue/blood partition coefficients (supplementary material 1 Table 2) were obtained based on the algorithm reported in literature [25] using information on the f_{ub} in blood, the pKa and the logP. The f_{ub} in rat blood was estimated to be 0.34 (for more details on the calculation see the supplementary material 2).

Determination of model parameter values for hepatic clearance

Upon incubation of tebuconazole with female rat liver microsomes and NADPH, three metabolites (here called A, B and C) were detected (Fig. 2). Quantification of the formed metabolites was carried out by using the calibration curve for tebuconazole at a wavelength of 225 nm, which was shown to have the same UV spectrum for two of the three metabolites (metabolite B and C). The UV spectra of the three metabolites, as well as that of the parent compound tebuconazole are presented in the supplementary material 1 Fig. 1. The intact masses of the three metabolites were identified by LC-MS analysis and shown to amount in positive mode to mass values (m/z) of 324.16, 338.14 and 324.16, for A, B and C, respectively, with the expected isotopic pattern for a single chlorine containing molecule. According to the reported in vivo metabolic profile of tebuconazole, three main phase I metabolites are formed in rat, being tebuconazole-o-hydroxy, tebuconazole-1-hydroxy and tebuconazole–carboxylic acid, with exact mass values (m/z) of 324.16, 324.16 and 338.14, respectively [32]. The structures of tebuconazole and the three metabolites are presented in Fig. 2. Based on this information and the mass values obtained it is concluded that metabolite A and C represent the two hydroxylated compounds and that metabolite B represents the carboxylic acid. As the UV spectrum of metabolite A is different from that of tebuconazole, we tentatively identified metabolite A as tebuconazole-o-hydroxy, given that its hydroxyl group is attached to the benzene ring and therefore likely to modify the UV absorption spectrum. This implies that metabolite C corresponds to tebuconazole-1-hydroxy, which can be further metabolized to metabolite B (tebuconazole–carboxylic acid).

Fig. 3 shows the formation rate of the metabolites with increasing concentration of tebuconazole, indicating that the reactions follow Michaelis–Menten kinetics. In the PBK model, the clearance of the parent compound tebuconazole was described by modeling its metabolic conversion to the primary metabolites A and C. Given that metabolite C was partly further metabolized to form metabolite B, the amount of metabolite B was added to that of metabolite C for the determination of the kinetic parameter values for the formation of metabolite C (Fig. 3b). The apparent K_m and V_{max} values for formation of A and B+C are presented in Table 1 and were scaled to parameter values for rat liver in the PBK model as described in the Materials and Methods section.

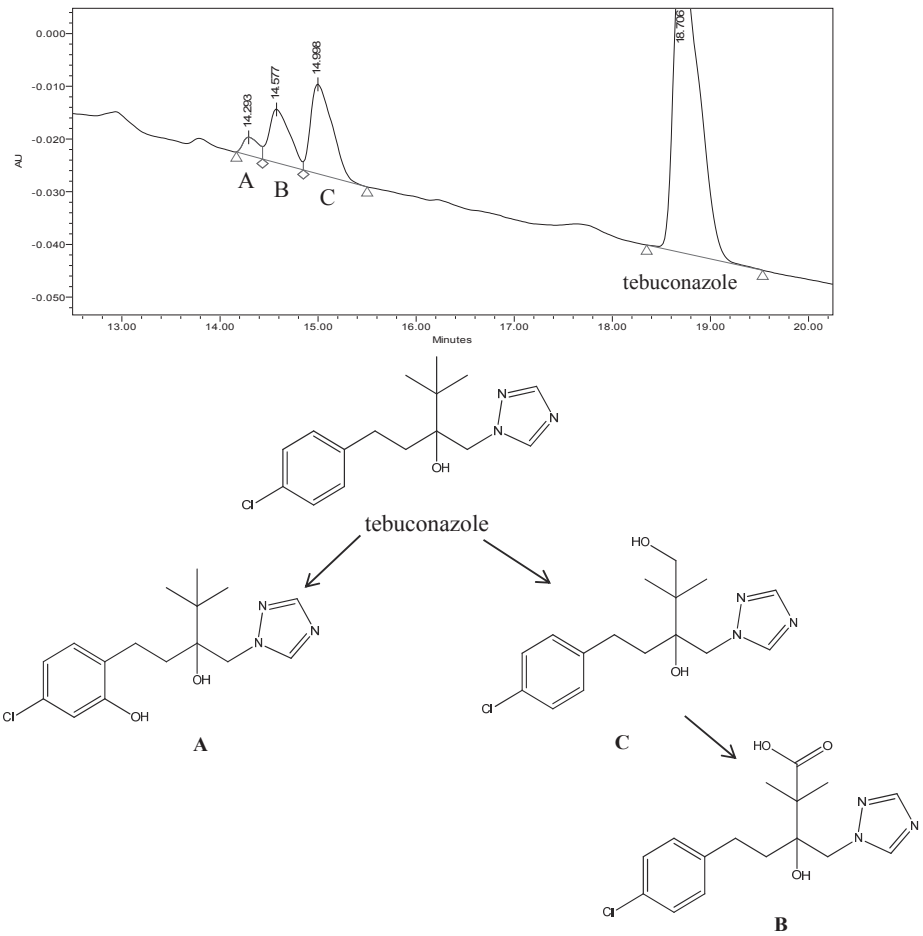

Fig. 2. HPLC chromatogram (upper) and structures (lower) of tebuconazole and metabolites A (tebuconazole-o-hydroxy), B (tebuconazole–carboxylic acid) and C (tebuconazole-1-hydroxy).

Table 1. Apparent K_m and V_{max} values for the formation of metabolite A and B+C using female Wistar rat microsomes.

	Metabolite A	Metabolite B+C
V_{max} (nmol/min/mg microsomal protein)	0.028	0.25
K_m (μM)	3.27	8.12

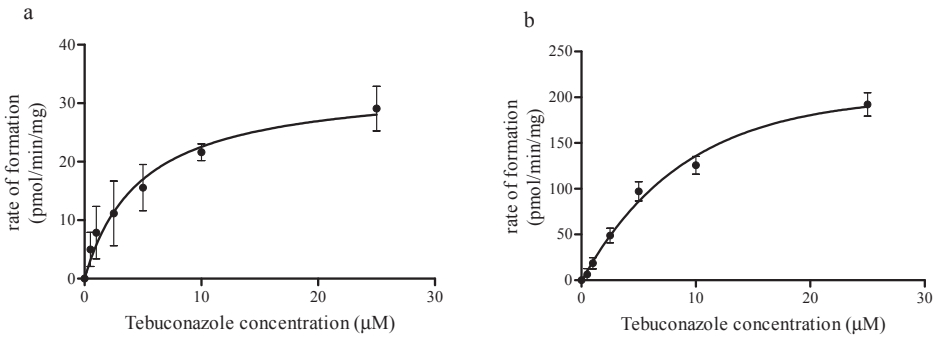

Fig. 3. Tebuconazole concentration-dependent rate of formation of metabolite A (a) and B+C (b) in incubations with female rat liver microsomes. Results represent data of three independent experiments.

PBK model evaluation

To evaluate the performance of the PBK model, which was developed without the use of in vivo data, model predictions were compared with kinetic data from the literature on blood concentrations of tebuconazole and excretion of tebuconazole and its metabolites. Hass *et al.* (2012) reported tebuconazole blood concentrations in rats that were orally exposed to 12.5 or 25 mg/kg body weight (bw) of tebuconazole in a mixture with other compounds. Blood samples were taken from two or three female rats 1-5 hrs after tebuconazole exposure. The blood concentrations of tebuconazole for the two dams in the lower dose group was reported to be between 1.7 and 2.7 μM, and for the three dams in the higher dose group between 3.7 and 25 μM [31] (Table 2). Table 2 shows that the predicted blood concentrations of tebuconazole upon a dose of 12.5 and 25 mg/kg bw were between 1.0-4.4 μM and 2.5-10.4 μM , respectively, and thus close to the in vivo data reported by Hass et al. (2012).

In vivo data on the cumulative excretion (urine and faeces) of tebuconazole and its metabolites by female Wistar rats that were orally exposed to 2 or 20 mg tebuconazole/kg bw, was also used for model evaluation [32]. We predicted the cumulative excretion of tebuconazole and its metabolites by assuming that tebuconazole metabolites are

excreted immediately and that unchanged tebuconazole is not excreted and compared the predicted cumulative excretion with the reported in vivo data (Fig. 4). Fig. 4 shows that for both doses, the in vivo excretion after 24 hours was well predicted with the PBK model (within 1.2-fold difference), whereas the excretion at earlier time points was 2- to 7-fold over-predicted.

Table 2. Tebuconazole blood concentration range between 1 and 5 hours after dosing 12.5 mg/kg bw or 25 mg/kg bw tebuconazole, reported by Hass et al. (2012) and predicted by the PBK model.

	12.5 mg/kg bw	**25 mg/kg bw**
reported blood concentration (μM)	1.7-2.7	3.7-25
predicted blood concentration (μM)	1.0-4.4	2.5-10

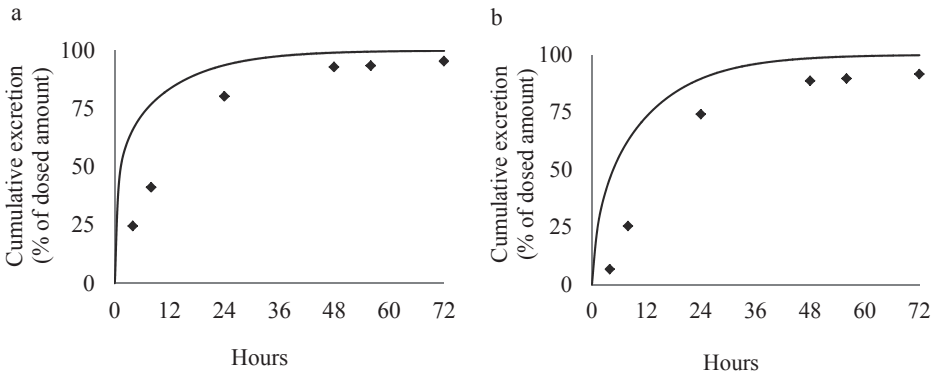

Fig.4. Cumulative excretion (% of dosed amount) in rat predicted with the PBK model (solid line) and reported in vivo (filled diamonds) after a single oral dose of 2 (a) or 20 (b) mg/kg bw [32].

Sensitivity analysis

Figure 5 shows the most sensitive model parameters as indicated by the normalized sensitivity analysis. It shows that the predictions of C_{max} in the PBK model is most sensitive to the stomach emptying rate (ksto) and, at low doses, also to the fraction of liver tissue (VLc) and the K_m and V_{max} values for the reaction of the formation of metabolites B+C (K_m B+C and V_{max} B+C).

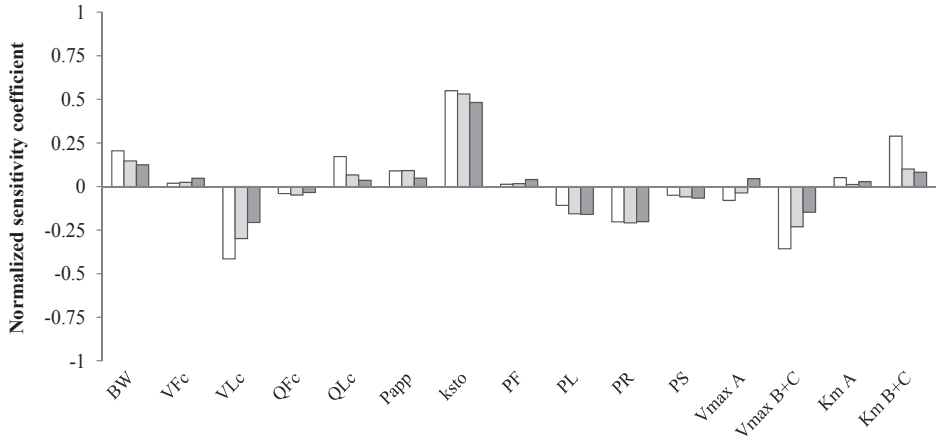

Fig. 5. Normalized sensitivity coefficients of PBK model parameters for the predicted C_{max} of tebuconazole in blood after oral administration to 2 (white bars), 12.5 (light grey bars) or 25 (dark grey bars) mg/kg bw tebuconazole. All model parameters with normalized sensitivity coefficients smaller than -0.03 and larger than 0.03 are shown. BW=body weight, VFc=fraction of fat tissue, VLc=fraction of liver tissue, QFc=fraction of blood flow to fat, QLc=fraction of blood flow to liver, ksto=stomach emptying rate, PF=fat/blood partition coefficient, PL=liver/blood partition coefficient, PR=rapidly perfused tissue/blood partition coefficient, PS=slowly perfused tissue/blood partition coefficient.

Translation of the in vitro concentration–response data into predicted in vivo dose–response data, application of BMD modeling on predicted dose–response data, and evaluation of the predictive value of the approach

The f_{ub} of tebuconazole in ES-D3 culture medium and in the ex ovo chicken embryo culture medium was calculated to be 1.6 times higher and 3.0 times lower than that in rat blood, respectively. Corrections were made to take this difference into account when applying reverse dosimetry. Fig. 6 shows predicted dose–response data for rat by applying reverse dosimetry on in vitro concentration-response data from the ES-D3 cell differentiation assay or from the ex ovo assay of chicken embryos. The predicted BMD10 values obtained from these predicted dose-response data are 11.4 and 1.9 mg/kg bw/day, respectively (Table 4).

To evaluate the predictive value of the approach, BMD10 values determined from predicted dose-response data were compared with BMD10 values determined from in vivo dose-response data as reported in two studies (Table 3). Developmental toxicity data on the incidence of skeletal variations or fetal external alterations following tebuconazole exposure were used to derive BMD10 values. For each endpoint, the lowest value of the models that passed the criteria for model fit was selected as the BMD10 value for

developmental toxicity of tebuconazole. These values are presented in the supplementary material 1, Tables 3 and 4. Table 4 shows the range of in vivo BMD10 values derived for all reported endpoints, based on litter incidence or fetus incidence. From the data on tebuconazole-induced alterations as reported in European Commission (2007b), one BMD10 value was obtained, being 126 mg/kg bw/day, which is based on fetus incidence. From the data on skeletal variations reported by EPA (1992), BMD10 values ranging from 23.6-169 mg/kg bw/day were derived when considering effects on individual fetuses. When BMD10 values were derived from these data based on litter incidences, BMD10 values ranged from 8.5-91.3 mg/kg bw/day. The lowest BMD10 value determined from all available data was 8.5 mg/kg bw/day (relating to increased variations, based on litter incidences), which was used to compare with the BMD10 values derived from the predicted dose-response data. The dose–response data for this endpoint are presented in Fig. 6 for comparison with our predicted dose-response data.

Table 3. Developmental toxicity studies of tebuconazole in rat upon oral exposure.

Ref.	Exposure	Dose (mg/kg bw/day)	Developmental endpoint
[32] [a]	PM 6-15	10, 30, 100	Increased incidences of external alterations
[35] [b]	GD 6-15	30, 60, 120	Increased incidences of skeletal variations

GD=gestational day, PM=post mating day
[a] see Supplementary Table 3 for details of toxicity data
[b] see Supplementary Table 4 for details of toxicity data

Table 4. BMD10 values for developmental toxicity of tebuconazole in rat derived from predicted in vivo data applying reverse dosimetry of ES-D3 cell differentiation assay data and ex ovo assay of chicken embryos data, based on C_{max}, and BMD10 values derived from in vivo data in rat.

		BMD10
		(mg/kg bw/day)
Predicted using reverse dosimetry of data from the ES-D3 cell differentiation assay		11.4
Predicted using reverse dosimetry of data from the ex ovo assay of chicken embryos		1.9
In vivo		
	based on fetus incidence [a]	126
	based on litter incidence [b]	8.5[c]-91.3
	based on fetus incidence [b]	23.6-169

[a] Based on data reported in [32]. Only data on fetus incidence are available.
[b] Based on data reported in [35]. See Supplementary Table 4 for all BMD10 values obtained for different endpoints.
[c] the full BMD analyses and all BMD10 values obtained by different model fits were reported in [8]

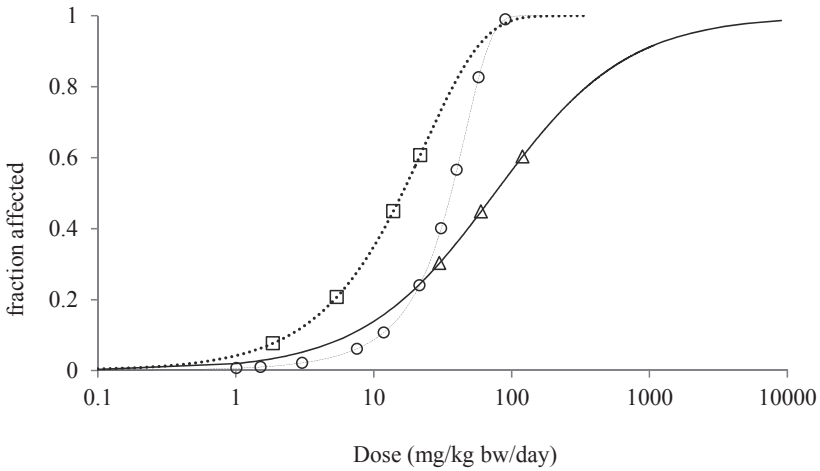

Fig. 6. Predicted (squares and circles) and reported (triangles) dose-response data for developmental toxicity of tebuconazole in rats with fitted dose-response curves obtained with BMDS. Predicted dose-response data were obtained using PBK modelling-facilitated reverse dosimetry of data from the ES-D3 cell differentiation assay (circles) and from the ex ovo assay of chicken embryos (squares). Reported dose–response data were on the most sensitive endpoint as reported by EPA (1992).

The BMD10 value determined from predicted dose-response data based on the ES-D3 cell differentiation assay was 1.3-fold higher compared to the lowest BMD10 value obtained from the in vivo data. The BMD10 value determined from predicted dose-response data based on the ex ovo assay of chicken embryos was 4.5-fold lower compared to the lowest BMD10 value obtained from the in vivo data. These results indicate that the differences between BMD10 values based on our predicted data and BMD10 values based on in vivo data were smaller than 5-fold, indicating that we could quite accurately predict in vivo developmental toxicity dose levels of tebuconazole using the combined in vitro-in silico approach. The results also indicate that lower BMD10 values were obtained when applying reverse dosimetry on data from the ex ovo assay of chicken embryos than when applying reverse dosimetry on data from the ES-D3 cell differentiation assay (Table 4).

DISCUSSION

The aim of the present study was to assess whether in vivo developmental toxicity can be predicted by PBK modeling-facilitated reverse dosimetry of in vitro toxicity data obtained in the ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos.

We have previously shown that with this approach based on the ES-D3 cell differentiation assay, dose levels causing developmental toxicity could be predicted within one order of magnitude for glycol ethers, retinoic acid and phenol [4, 12, 13]. In the present study we show that for the antifungal compound tebuconazole the BMD10 value derived from predicted developmental toxicity dose levels differed less than 5-fold compared to BMD10 values obtained from in vivo studies, indicating that the use of our combined in vitro-in silico approach is also promising to predict developmental toxicity of chemicals belonging to other chemical categories.

It is of interest to note that the PBK model developed in the present study that was used for the reverse dosimetry is solely based on kinetic parameter values derived from in silico and in vitro experimental data, without adjustment to fit the in vivo data. The PBK model-predicted tebuconazole blood concentrations upon dosing of 12.5 and 25 mg/kg bw tebuconazole were within the range of blood concentrations reported in an in vivo kinetic study (Table 2), indicating a good prediction of tebuconazole blood concentrations by the PBK model. The PBK model was also evaluated by comparing the predicted cumulative excretion of tebuconazole metabolites with reported in vivo data on the excretion of tebuconazole metabolites in female rats. The model over-predicted the total excretion throughout 72 hours, especially in the first 24 hours (Figure 4). This may be explained by the fact that in vivo mainly phase II metabolites are excreted [32] and that the formation of phase II metabolites is not described in the model. For our reverse dosimetry-based predictions, lack of the description of phase II metabolism is not considered a problem, assuming that the parent compound causes the toxicity. Therefore, in such cases, description of phase I metabolism is sufficient to describe the body clearance of the parent compound.

For the prediction of developmental toxicity by PBK modeling-facilitated reverse dosimetry, we assumed that the concentrations in the embryo/fetus are the same as the concentrations in the mother, and did not take the possible effects of the placental transfer on fetal concentrations into account. Placental transfer may play an important role in fetal bioavailability of chemicals and may thereby affect the developmental toxicity outcome of a chemical. We have previously demonstrated that we better predicted relative developmental toxicity potencies of antifungal compounds based on in vitro data from the ES-D3 cell differentiation assay or the ex ovo assay of chicken embryos, by combining these data with information on placental transfer in the in vitro BeWo model, a useful model to predict relative placental transfer rates of compounds [8, 9]. This suggests that differences in placental transfer of the antifungal compounds may play an important role in their differences in developmental toxicity potencies in vivo. We have shown that the placental transport rate of tebuconazole as determined in the BeWo model is almost as high as the transport rate of antipyrine, a model compound known to easily cross the placenta [8]. Therefore, we assumed that the placenta does not

affect the levels of tebuconazole that reach the fetus, and we assumed that tebuconazole levels in fetal blood equal those in maternal blood, so we did not include a specific fetal compartment in the PBK model. However, this assumption cannot be made for the chemicals that have a low placental transfer rate. Therefore, for such compounds a fetal compartment should be incorperated in the PBK model when using it for reverse dosimetry of in vitro developmental toxicity data.

The present study shows that PBK modeling-facilitated reverse dosimetry of both the ES-D3 cell differentiation assay data and the ex ovo assay of chicken embryos data closely predicted in vivo developmental toxicity data of tebuconazole, indicating that both assays are useful to predict developmental toxicity of antifungal compounds. However, each assay has its own advantages and limitations. For instance, the ES-D3 cell differentiation assay is a relatively easy assay, whereas the ex ovo assay of chicken embryos is more labor intensive and requires special equipment and a well trained staff. However, unlike the ex ovo assay of chicken embryos, the ES-D3 cell differentiation assay cannot be used to study specific alterations induced by chemicals. Since both assays have their advantages and limitations, a combined and tiered testing strategy could be followed. The ES-D3 cell differentiation assay combined with PBK-modeling facilitated reverse dosimetry could be regarded in a first tier to define compounds of high concern. In the second tier, the ex ovo assay of chicken embryos could be applied to detect specific alterations that may be induced. Such a testing strategy may provide valuable early information for prioritization and potentially also for risk assessment.

In conclusion, we showed that PBK modeling-facilitated reverse dosimetry allows translation of in vitro concentration-response curves of tebuconazole obtained in the ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos to in vivo dose-response curves for developmental toxicity, enabling the derivation of a PoD, such as BMD10 values, for risk assessment without the use of in vivo studies. Since the method uses only in vitro assays and in silico models to predict the in vivo developmental toxicity of chemicals, it contributes to the 3Rs (replacement, reduction and refinement) of animal testing.

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NOTES

The authors declare that they have no conflict of interest.

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SUPPLEMENTARY MATERIAL 1

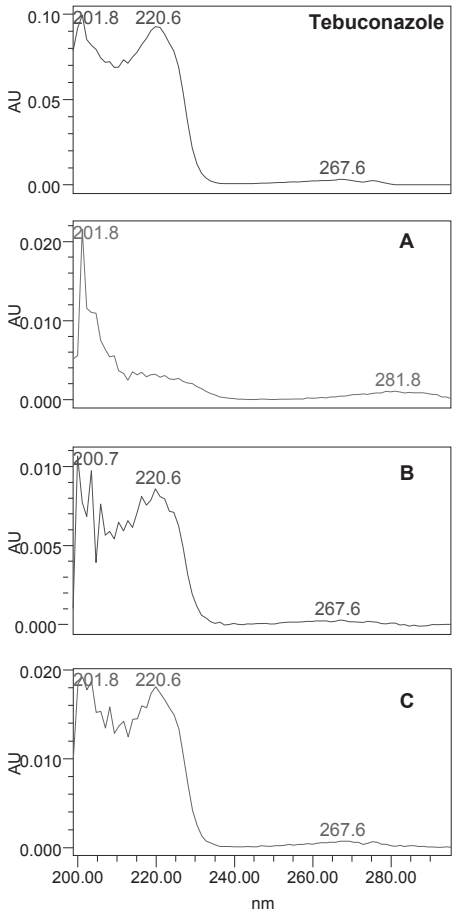
Supplementary Table 1. Physiological parameter values used for the rat PBK model [18].

Physiological parameters	**Values**
Tissue volumes (percentage of body weight)	
liver	3.4
fat	7.0
richly perfused tissue	5.6
slowly perfused tissue	67.6
blood	7.4
Cardiac output ($L \cdot h^{-1} \cdot kg\ bw^{-0.74}$)	15.0
Tissue blood flows (percentage of cardiac output)	
liver	25.0
fat	7.0
richly perfused tissue	51.0
slowly perfused tissue	17.0

Supplementary Table 2. Rat tissue/blood partition coefficients.

	partition coefficients
liver	10.1
fat	131.6
richly perfused tissue [a]	10.1
slowly perfused tissue [b]	5.7

[a] partition coefficient of liver was used for richly perfused tissue
[b] partition coefficient of muscle was used for slowly perfused tissue



Supplementary Fig. 1. UV spectra of tebuconazole and its metabolites A, B, C.

Supplementary Table 3. Incidences of external alterations in rats exposed to tebuconazole, including BMD10 values derived from the data reported in [32].

	Dose (mg/kg bw/day)				
	Control	**10**	**30**	**100**	**BMD10 (mg/kg bw/day)**
No. pups examined	174	144	202	168	
External alterations	3	1	4	12	126.3

Supplementary Table 4. Incidences of skeletal variations in rats exposed to tebuconazole, including BMD10 values derived from the data reported [35].

	Dose (mg/kg bw/day)				BMD10 (mg/kg bw/day)
	Control	**30**	**60**	**120**	
No. pups examined	144	137	127	116	
Cervical vertebra 1	18	21	24	48	48.1
Cervical vertebra 2	29	40	38	48	39.3
Cervical vertebra 3	9	10	12	16	134.5
Cervical vertebra 4	0	5	2	13	118.8
Cervical vertebra 5	3	3	3	10	131.2
Cervical vertebra 6	1	2	2	6	168.8
Vertebral arch 6, left	1	2	3	14	114.7
Vertebral arch 6, right	0	2	6	13	100.3
Vertebral arch 7, left	50	53	49	66	34.9
Vertebral arch 7, right	45	53	51	65	30.8
Sternebra 2	4	3	3	15	118.5
Sternebra 6	0	0	1	4	150.6
Supernumerary ribs, left	14	20	18	26	90.0
Supernumerary ribs, right	15	23	19	24	113.6
Digit 2 proximal phalanx (l)	27	36	37	42	52.1
Digit 3 proximal phalanx (l)	0	3	5	9	133.5
Digit 4 proximal phalanx (l)	2	7	7	11	138.6
Metacarpal 5 (l)	0	0	2	4	157.5
Digit 2 proximal phalanx (r)	27	33	39	40	54.5
Digit 3 proximal phalanx (r)	0	3	4	8	141.0
Digit 4 proximal phalanx (r)	1	6	6	11	131.3
Metacarpal 5 (r)	0	0	2	5	147.4
Metatarsal (l)	18	24	18	31	84.9
Toe 2 proximal phalanx (l)	107	92	96	103	40.3
Toe 3 proximal phalanx (l)	81	70	78	87	23.6
Toe 4 proximal phalanx (l)	79	65	72	85	50.6
Metatarsal (r)	18	24	20	32	76.4
Toe 2 proximal phalanx (r)	110	96	105	105	37.6
Toe 3 proximal phalanx (r)	86	75	76	92	46.4
Toe 4 proximal phalanx (r)	81	71	74	91	45.3

Supplementary Table 4. Incidences of skeletal variations in rats exposed to tebuconazole, including BMD10 values derived from the data reported [35]. (*Continued*)

	Dose (mg/kg bw/day)				BMD10 (mg/kg bw/day)
	Control	**30**	**60**	**120**	
No. litters examined	24	24	22	24	
Cervical vertebra 2	14	17	17	20	8.5
Sacral vertebral arch 6 (r)	0	2	6	8	55.8
Digit 2 proximal phalanx (l)	14	14	16	18	45.8
Digit 3 proximal phalanx (l)	0	3	5	4	83.1
Digit 3 proximal phalanx (r)	0	3	4	4	91.3
Digit 4 proximal phalanx (r)	1	4	6	6	83.4

SUPPLEMENTARY MATERIAL 2

Cytotoxicity study used to estimate the fraction unbound

In order to estimate the tissue/blood partition coefficients, the fraction of unbound tebuconazole (f_{ub}) in rat blood was determined using an approach developed by Gülden et al. (2002) to determine protein binding of chemicals. The f_{ub} was estimated based on the assessment of the increase of the EC_{50} value for tebuconazole cytotoxicity with increasing albumin concentrations, as described by [28] with slight modifications. To this end, cytotoxicity assays using BeWo cells were performed. The BeWo cell line (clone b30) was kindly provided by the Institute of Public Health of the Faculty of Health Sciences of the University of Copenhagen (Denmark) with permission from Dr. Alan Schwartz (Washington University, St. Louis, MO). BeWo b30 cells were cultured in DMEM (D5671, Sigma-Aldrich, Zwijndrecht, the Netherlands) supplemented with 10% (v/v) heat-inactivated FCS, 10,000 U/ml penicillin, 10 mg/ml streptomycin, and 2 mM L-glutamine. The cells were maintained in polystyrene cell-culture flasks (Corning, USA), under a humidified atmosphere of 5% CO_2 at 37 °C. The cells were harvested by exposing to a 0.05% trypsin-EDTA solution and transferred to 96-well plate (Greiner bio-one).

Experiments were performed with passages 26–33. For the experiments, cells were seeded at concentrations of 20 x 10^4 cells/ml in 100 µL culture medium into the inner 60 wells of the 96-well plate and incubated for one day at 37°C and 5% CO_2 in a humidified atmosphere to allow cell adherence. Then the cells were exposed to tebuconazole at concentrations up to 1000 µM (final solvent concentration: 0.2% DMSO) for one day, by dissolving tebuconazole in exposure medium (DMEM supplemented with 1% (v/v) heat-inactivated FCS, 10,000 U/ml penicillin, 10 mg/ml streptomycin, and 2 mM L-glutamine) alone or in exposure medium supplemented with bovine serum albumin (BSA, molecular weight/ 66500) at concentrations of 2, 5, 7.5, 10 or 12.5 mg/ml. After incubation of one day, 20 µL MTT-1 reagent (Sigma-Aldrich, the Netherlands) was added to each well and the plates were incubated for another 3 hours. Then absorbance was measured at 450 nm using a SpectraMax M2 (Molecular Devices, Sunnyvale, USA). Three wells were used per treatment in each independent experiment. 1% Triton X-100 served as a positive control in all cytotoxicity assays. The cell viability was expressed as percentage of the solvent control (0.2% DMSO), with the solvent control set at 100% viability. Reproducible results were obtained from the treatments of negative and positive controls in all of the cytotoxicity assays. EC 50 values were determined with Graphpad Prism 5 using a four-parameter logistic model.

Fig. 1. shows that EC50 value of tebuconazole-induced cytotoxicity increases with increasing albumin concentrations. A linear correlation was obtained between the EC50 value and the albumin concentration described by Y=1.7838X+267.74 (Y = aX + b, with Y=EC50 and X=albumin concentration, and values given for the slope (a) and the intercept (b) being the best fit). The fraction of tebuconazole unbound to

albumin (f_{ub}) was estimated by using the following equation: f_{ub} = b / EC50 where b is the intercept of the linear regression equation, and EC50 is the value in the presence of a certain concentration of albumin [28], in this case described by Y=1.7838X+267.74, with X=albumin concentration. Based on the equation f_{ub} = b / EC50, which can also be written as f_{ub} = 267.74 / (1.7838X+267.74) with X being the albumin concentration in μM, the f_{ub} in rat blood ($f_{ub,\ rat\ blood}$) was estimated, using albumin concentration in rat blood being 293 μM [29]. The $f_{ub,\ rat\ blood}$ was estimated to be 0.34, which was used as input for the calculation of partition coefficients parameters.

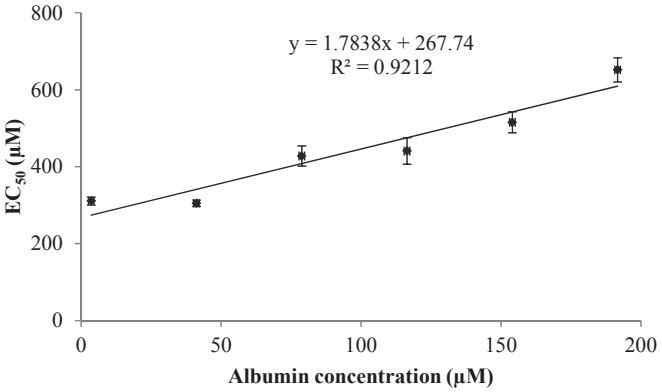


Fig. 1 Relation between albumin concentration and EC50 value obtained in cytotoxicity assays with tebuconazole. Results represent EC50 values determined from at least three independent experiments (average ± SD are shown).

Corrections made to take the differences between the fub in culture medium compared with the fub in rat blood into account

The unbound concentration in the in vitro culture medium ($C_{ub,\ in\ vitro}$) was estimated by using the following equation: $C_{ub,\ in\ vitro} = C_{,\ in\ vitro} \times f_{ub,\ in\ vitro}$, where the $C_{,\ in\ vitro}$ is the nominal concentration applied in the in vitro assay and $f_{ub,\ in\ vitro}$ is the f_{ub} of tebuconazole in the culture medium, which was derived using the equation f_{ub} = b / EC50, which can also be written as f_{ub} = 267.74 / (1.7838X+267.74) with X being the albumin concentration in μM. The albumin concentration was 134 μM in the in vitro ES-D3 cell culture medium and 1277 μM in the ex ovo chicken embryo culture medium, as determined using the Pierce™ BCA protein assay kit (Thermo, Rockford, the USA). The $f_{ub,\ in\ vitro}$ in ES-D3 cell culture medium was to be 0.53 and the $f_{ub,\ in\ vitro}$ in ex ovo assay of chicken embryo culture medium was calculated to be 0.11. Then the effect concentration in rat blood ($C_{,\ rat\ blood}$), used for reverse dosimetry, was estimated by using the following equation: $C_{,\ rat\ blood} = C_{ub,\ in\ vitro} / f_{ub,\ rat\ blood}$.

SUPPLEMENTARY MATERIAL 3

PBK Model for tebuconazole, built with in vitro and in silico data; Species: Rat

```
;=====================================================================
;Physiological parameters
;=====================================================================
;tissue volumes
BW = 0.250 {Kg}      ; body weight rat (variable, dependent on study)
VFc = 0.07           ; fraction of fat tissue              reference: [18].
VLc = 0.034                  ; fraction of liver tissue                    reference: [18].
VBc = 0.074                  ; fraction of blood                reference: [18].
VRc = 0.09-VLc               ; fraction of richly perfused tissue          reference: [18].
VSc=0.746-VFc                ; fraction of slowly perfused tissue          reference: [18].

VF = VFc*BW                  {L or Kg}   ; volume of fat tissue (calculated)
VL = VLc*BW                  {L or Kg}   ; volume of liver tissue (calculated)
VB = VBc*BW                  {L or Kg}   ; volume of blood (calculated)
VR = VRc*BW                  {L or Kg}   ; volume of richly perfused tissue (calculated)
VS = VSc*BW                  {L or Kg}   ; volume of slowly perfused tissue (calculated)
;---------------------------------------------------------------------------------------------------------------
;blood flow rates
QC = 15*BW^0.74/60{L/min}    ; cardiac output                                      reference: [18].
QFc = 0.07           ; fraction of blood flow to fat                       reference: [18].
QLc = 0.25                   ; fraction of blood flow to liver                     reference: [18].
QRc = 0.76 - QLc     ; fraction of blood flow to richly perfused tissue    reference: [18].
QSc = 0.24 - QFc             ; fraction of blood flow to slowly perfused tissue    reference: [18].

QF = QFc*QC                  {L/min}     ; blood flow to fat tissue (calculated)
QL = QLc*QC                  {L/min}     ; blood flow to liver tissue (calculated)
QS = QSc*QC                  {L/min}     ; blood flow to slowly perfused tissue (calculated)
QR = QRc*QC                  {L/min}     ; blood flow to richly perfused tissue (calculated)
;---------------------------------------------------------------------------------------------------------------
; Intestinal lumen volumes, surfaces, absorption rates, transfer rates

Papp  = 2E-3                 ; apparent intestinal permeability coefficient in vivo {dm/min}
ksto  = 0.047                ; stomach emptying rate {/min}

Vdd   = 0.0012               ; duodenum volume {L}
SAdd  = 0.134                ; surface area {dm2}
```

```
kabdd  = Papp*SAdd              ; absorption rate constant {L/min}
kin   =  0.0695                 ; transfer rate to jj1 {/min}
kdd = kin

Vjj1  = 0.0012                  ; jejunum 1 volume {L}
SAjj1 = 0.134                   ; surface area {dm2}
kabjj1  = Papp*SAjj1            ; absorption rate constant {L/min}
kjj1  = kin                     ; transfer rate to jj2 {/min}

Vjj2  = 0.0012                  ; jejunum 2 volume {L}
SAjj2 = 0.134                   ; surface area {dm2}
kabjj2  = Papp*SAjj2            ; absorption rate constant {L/min}
kjj2  = kin                     ; transfer rate to jj3 {/min}

Vjj3  = 0.0012                  ; jejunum 3 volume {L}
SAjj3 = 0.134                   ; surface area {dm2}
kabjj3  = Papp*SAjj3            ; absorption rate constant {L/min}
kjj3  = kin                     ; transfer rate to jj4 {/min}

Vjj4  = 0.0012                  ; jejunum 4 volume {L}
SAjj4 = 0.134                   ; surface area {dm2}
kabjj4  = Papp*SAjj4            ; absorption rate constant {L/min}
kjj4  = kin                     ; transfer rate to jj5 {/min}

Vjj5  = 0.0012                  ; jejunum 5 volume {L}
SAjj5 = 0.134                   ; surface area {dm2}
kabjj5  = Papp*SAjj5            ; absorption rate constant {L/min}
kjj5  = kin                     ; transfer rate to il {/min}

Vil   = 0.0012                  ; ileum volume {L}
SAil  = 0.134                   ; surface area {dm2}
kabil  = Papp*SAil     ; absorption rate constant {L/min}
kil   = kin                     ; transfer rate to co {/min}

kfe = 0.00033                   ; transfer rate to feces {/min}
;=====================================================================
;Partition Coefficients
;=====================================================================
```

PF = 131.56 ; fat/blood partition coefficient calculated using QSAR of: [25]
PL = 10.14 ; liver/blood partition coefficient calculated using QSAR of: [25]
PR = 10.14 ; richly perfused tissue/blood partition coefficient calculated using QSAR of: [25]
PS = 5.69 ; richly perfused tissue/blood partition coefficient calculated using QSAR of: [25]
;===
;Kinetic parameters
;===
;Metabolism liver

;metabolites of tebuconazole, scaled maximum rate of metabolism
VmaxAc = 0.028 {nmol/min/mg} ; data derived from incubation experiment with female rat microsomes
VMaxA = VMaxAc/1000*35*VL*1000 {umol/min}

VMaxBCc = 0.25 {nmol/min/mg} ; data derived from incubation experiment with female rat microsomes
VMaxBC = VMaxBCc/1000*35*VL*1000 {umol/min}

;metabolites of tebuconazole, affinity constants (umol/L)
KmA = 3.27 ; data derived from incubation experiment with female rat microsomes
KmBC = 8.115 ; data derived from incubation experiment with female rat microsomes

;===
;Run settings
;===

;Molecular weight
MW = 307.82 ; Molecular weight tebuconazole

;oral dose
ODOSEmg = 12.5 {mg/kg bw} ; ODOSEmg = given oral dose in mg/kg bw
ODOSEumol2 = ODOSEmg*1E-3/MW*1E6 {umol/ kg bw} ; ODOSEumol2 = given oral dose recalculated to umol/kg bw
ODOSEumol=ODOSEumol2*BW; ; ODOSEumol = umol given oral

;time

```
Starttime = 0                    ; in min
Stoptime = 14400                 ; in min

;=====================================================================
;Model calculations
;=====================================================================
;stomach
;ASt  = amount in stomach
Ast' = -ksto*Ast+pulse(ODOSEumol, 0, 1440) - pulse(ODOSEumol,Tstop, 1440) ; Tstop stop time of dosing
(min)
Tstop=14400
Init ASt =0

;-----------------------------------------------------------------------------------------------------
;intestines, divided in 7 compartments
;Add  = Amount tebuconazole in duodenum compartment (umol)
Cdd  = Add/Vdd
Add' = ksto*Ast - kdd*Add - kabdd*Cdd
Init Add  =  0

;Ajj1 = Amount tebuconazole in jejunum compartment 1 (umol)
Cjj1  = Ajj1/Vjj1
Ajj1' = kdd*Add - kjj1*Ajj1 - kabjj1*Cjj1
Init Ajj1  = 0

;Ajj2 = Amount tebuconazole in jejunum compartment 2 (umol)
Cjj2  = Ajj2/Vjj2
Ajj2' = kjj1*Ajj1 - kjj2*Ajj2 - kabjj2*Cjj2
Init Ajj2  = 0

;Ajj3 = Amount tebuconazole in jejunum compartment 3 (umol)
Cjj3  = Ajj3/Vjj3
Ajj3' = kjj2*Ajj2 - kjj3*Ajj3 - kabjj3*Cjj3
Init Ajj3  = 0

;Ajj4 = Amount tebuconazole in jejunum compartment 4 (umol)
Cjj4  = Ajj4/Vjj4
Ajj4' = kjj3*Ajj3 - kjj4*Ajj4 - kabjj4*Cjj4
Init Ajj4  = 0
```

```
;Ajj5 = Amount tebuconazole in jejunum compartment 5 (umol)
Cjj5  = Ajj5/Vjj5
Ajj5' = kjj4*Ajj4 - kjj5*Ajj5 - kabjj5*Cjj5
Init Ajj5  = 0

;Ail = Amount tebuconazole in ileum compartment (umol)
Cil  = Ail/Vil
Ail' = kjj5*Ajj5 - kil*Ail - kabil*Cil
Init Ail  = 0

;Aco = Amount tebuconazole in colon (umol)
Aco' = kil*Ail - kfe*Aco
Init Aco  = 0
ACco' = kil*Ail
Init ACco  = 0    ; cumulative amount reaching colon
;-----------------------------------------------------------------------------------------------------
;feces
;AFA = amount tebuconazole in feces (umol)
AFe' = kfe*Aco
Init AFe  = 0
;-----------------------------------------------------------------------------------------------------
;liver compartment

;AL = Amount tebuconazole in liver tissue, umol
AL' =  QL*(CB - CVL) + kabdd*Cdd + kabjj1*Cjj1 + kabjj2*Cjj2 + kabjj3*Cjj3 + kabjj4*Cjj4 + kabjj5*Cjj5 +
kabil*Cil - AMA'  - AMBC'
Init AL = 0
CL = AL/VL
CVL = CL/PL

;AMA = amount tebuconazole metabolized to metabolite A
    AMA' = VmaxA*CVL/(KmA + CVL)
    init AMA = 0

;AMBC = amount tebuconazole metabolized to metabolite B+C
    AMBC' = VmaxBC*CVL/(KmBC + CVL)
    init AMBC = 0
;liver compartment
```

```
CL = AL/VL
CVL = CL/PL

;formed metabolites
AMtotal= AMA+AMBC                                    ; total metabolites formed
PAMtotal = AMtotal/ODOSEumol*100      ; percentage of total metabolites formed
;-----------------------------------------------------------------------------------------------------------
;fat compartment

;AF = Amount tebuconazole in fat tissue (umol)
    AF' = QF*(CB-CVF)
    Init AF = 0
    CF = AF/VF
    CVF = CF/PF
;-----------------------------------------------------------------------------------------------------------
;tissue compartment richly perfused tissue

;AR = Amount tebuconazole in richly perfused tissue (umol)
    AR' = QR*(CB-CVR)
    Init AR = 0
    CR = AR/VR
    CVR = CR/PR
;-----------------------------------------------------------------------------------------------------------
;tissue compartment slowly perfused tissue

;AS = Amount tebuconazole in slowly perfused tissue (umol)
    AS' = QS*(CB-CVS)
    Init AS = 0
    CS = AS/VS
    CVS = CS/PS
;-----------------------------------------------------------------------------------------------------------
; blood compartment

;AB = Amount tebuconazole in blood (umol)
    AB' = (QF*CVF +  QL*CVL  + QS*CVS + QR*CVR - QC*CB)
    Init AB = 0
    CB = AB/VB
    AUC' = CB          ;umol*min/L
    Init AUC = 0
```

;===

;Mass balance calculations

;===

Total = ODOSEumol

Calculated = ASt + Add + Ajj1 + Ajj2 + Ajj3 + Ajj4 + Ajj5 + Ail + Aco+ AFe + AL + AMA + AMBC + AF + AS + AR + AB

CHAPTER 7

General discussion, future perspectives
and conclusions

MAIN FINDINGS

Determination of safe human exposure levels of chemicals in toxicological risk assessments largely relies on animal toxicity data. In these toxicity studies, the highest number of animals are used for reproductive and developmental toxicity testing [1]. Because of economic and ethical reasons, there is large interest in the development of in vitro and/or in silico test systems as alternatives for the animal studies. The aim of the present thesis was to evaluate the applicability of combined in vitro approaches taking toxicokinetic and toxicodynamic aspects into account, as well as of an integrated in vitro and in silico approach for prediction of developmental toxicity using a series of antifungal compounds as the model compounds.

Transplacental transfer of compounds is highly likely to play an important role in developmental toxicity and should therefore be taken into account when predicting in vivo developmental toxicity based on in vitro toxicity data. In **Chapter 2**, we focused on the development and validation of an in vitro model to obtain kinetic information on placental transfer. Placental transfer of compounds has been studied using the human ex vivo placental perfusion model. However, this method is laborious and dependent on the availability of fresh human placenta, hampering its use for the assessment of large numbers of compounds. An in vitro placental barrier model using BeWo b30 cells was assessed as an alternative to the ex vivo system to predict placental transfer. To this end, BeWo b30 cells, derived from a human choriocarcinoma, were grown on transwell inserts to form a cell layer, separating an apical maternal compartment from a basolateral fetal compartment. For a set of nine selected model compounds, including the reference compound antipyrine, the transport velocity from the apical to the basolateral compartment was determined. Relative transport rates obtained were compared with the transfer indices (a measure for the transport relative to antipyrine) of these compounds obtained in ex vivo placental perfusion studies as reported in the literature. The relative transport rates in the in vitro BeWo model were in very good correlation ($R^2 = 0.95$) with the transfer indices reported for the ex vivo placental model. This demonstrated that the BeWo model could be a valuable in vitro model to predict relative placental transfer of compounds.

In **Chapter 3**, we investigated the applicability of the ES-D3 cell differentiation assay combined with the in vitro BeWo transport model to predict the relative in vivo developmental toxicity potencies of five selected model antifungal compounds. To this end, the in vitro developmental toxicity of the five antifungal compounds was investigated by determining the effect concentrations that inhibit the differentiation of ES-D3 cells into beating cardiomyocytes. The BeWo transport model, consisting of BeWo b30 cells grown on transwell inserts and mimicking the placental barrier, was used to determine the placental transport velocity of the selected antifungal compounds relative to that of the reference compound antipyrine. In a next step the ES-D3 cell differentiation data were first compared to benchmark doses (BMDs) for in vivo developmental toxicity as derived

from data reported in the literature. Correlation between the benchmark concentration for 50% effect (BMC_d50 values), obtained in the ES-D3 cell differentiation assay, with in vivo BMD10 values for developmental toxicity showed a reasonable correlation (R^2 = 0.57). When the ES-D3 cell differentiation data were combined with the relative transport rates obtained from the BeWo model, the correlation with the in vivo data increased profoundly (R^2 = 0.95). This shows that the ES-D3 cell differentiation assay is able to better predict the in vivo developmental toxicity potencies of antifungal compounds when combined with the BeWo transport model, than as a stand-alone assay.

To validate this combined in vitro approach to predict in vivo developmental toxicity, we combined ES-D3 cell differentiation data of six novel triazole antifungal compounds with their relative transport rates obtained from the BeWo model and compared the obtained ranking to the developmental toxicity ranking as derived from in vivo data (**Chapter 4**). The data obtained show that the combined in vitro approach provided a correct prediction for the relative in vivo developmental toxicity, whereas the ES-D3 cell differentiation assay as stand-alone did not. It was concluded that these data validated the combined in vitro approach for developmental toxicity, previously developed with a set of reference azoles, for a set of six novel triazoles. We suggest that this combined model, which takes both toxicodynamic and toxicokinetic aspects into account, should be further validated for other chemical classes of developmental toxicants.

Although the ES-D3 cell differentiation assay, when combined with the BeWo transport model, has been shown to be useful to predict the relative developmental toxicity potency of the investigated compounds, it is limited to the evaluation of one phenotypic endpoint, i.e. the differentiation of embryonic stem cells into contracting cardiomyocytes, and can therefore not be used to detect specific structural alterations induced by chemicals. In **Chapter 5,** we investigated the applicability of the ex ovo assay of chicken embryos to predict the specific structural alterations induced by the five antifungal compounds that were used in Chapter 3 and investigated if the combination of the assay with placental transfer information would improve its predictive capacity. The ex ovo assay assesses developmental toxic potential of compounds based on well-defined morphological endpoints. Each compound produced a characteristic pattern of alterations. The BMC50 values for the in vitro effect were compared with the in vivo BMD10 values to assess the potential of the ex ovo assay of chicken embryos to predict in vivo developmental toxicity. When the toxicity data from the ex ovo assay were combined with the relative transport rates from the BeWo model, the correlation with the in vivo data were better than when the toxicity data from the ex ovo assay were used without taking differences in placental transfer rates into account. This revealed that the ex ovo assay of chicken embryos is able to assess the teratogenic potential of antifungal compounds, and, when combined with the in vitro BeWo transport model, is able to better predict the relative in vivo prenatal developmental toxicity.

With the help of the in vitro assays that assess kinetic processes, such as the BeWo cell transport model, the predictive value of in vitro developmental toxicity tests can be improved. However, the challenge of predicting developmental toxicity using in vitro assays still remains that the effect concentrations obtained from the in vitro assays cannot be used for risk assessment, because risk assessment requires in vivo dose response curves from which points of departure to define safe levels of exposure for humans can be derived. Therefore, in vitro concentration-response curves need to be translated into in vivo dose-response curves to enable the use of in vitro toxicity data in risk assessment. In **Chapter 6**, we translated in vitro concentration–response data of the antifungal compound tebuconazole, obtained in the ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos, into predicted in vivo dose–response data using physiologically based kinetic modelling-facilitated reverse dosimetry. The predicted in vivo dose–response data were used to derive BMD10 values for rat, which were compared with BMD10 values derived from toxicity data in rats as reported in the literature. The results show that the BMD10 values from predicted dose–response data from both assays are in concordance with BMD10 values derived from in vivo data (within 5-fold difference). This revealed that physiologically based kinetic modeling is a promising tool to predict in vivo dose-response curves based on the results of in vitro toxicity assays, and may therefore be used to set a point of departure for deriving safe exposure limits in risk assessment.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The present thesis shows, for antifungal compounds, the importance of taking placental transfer of compounds into account when predicting developmental toxicity with in vitro methods. The thesis also provided the proof-of-principle that for the selected antifungal compound tebuconazole, in vitro concentration-response curves can be converted into in vivo dose-response curves for developmental toxicity, in principal suitable for risk assessment, using PBK modeling with a reverse dosimetry approach. These results show the feasibility of combined in vitro approaches taking toxicokinetic and toxicodynamic aspects into account, and the potential of the integrated in vitro-in silico approach for predicting developmental toxicity of chemicals belonging to the antifungal class. The outcomes of the present thesis can be discussed in a wider perspective on what would be topics of interest to include and consider when applying the combined in vitro approaches and the integrated in vitro-in silico approach in predicting developmental toxicity for other chemicals of diverse nature, including both existing and new chemicals, in toxicity testing and risk assessment. The topics that are of relevance and importance to discuss in this respect in some more depth include 1) the in vitro developmental toxicity assay used, 2) the role of the placenta in contributing to developmental toxicity, 3) the use and development of PBK models, 4) the in vivo data available and their use for validation purposes and 5) future perspectives and conclusions. In the following sections these aspects are discussed in more detail.

Ad 1) The in vitro developmental toxicity assay used

In the present thesis two in vitro developmental assays were applied, i.e. the ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos. The possibilities and limitations of the two assays for developmental toxicity testing are discussed in the present section. Besides, other in vitro assays that may be considered for characterizing developmental toxicity are presented.

The ES-D3 cell differentiation assay

In the present thesis, the ES-D3 cell differentiation assay of the EST was used to determine the developmental toxicity potency of antifungal compounds. The EST is the only validated animal-free assay among in vitro tests for developmental toxicity that does not require the use of primary animal tissues. The readout parameter in this assay is the inhibition of the differentiation of ES-D3 cells into beating cardiomyocytes by tested chemicals, while in vivo studies in rats and mice have shown that antifungal compounds induce skeletal alterations of fetuses [2]. This indicates that the determination of inhibition of differentiation into beating cardiomyocytes may not be a direct endpoint for predicting developmental toxicity of antifungal compounds. However, it is not the intention of the ES-D3 cell differentiation assay to specifically predict concentrations affecting cardiac development, but rather to use this endpoint as a sensitive in vitro

readout parameter for developmental toxicity [3]. The present thesis shows the usefulness of chemical-induced inhibition of ES-D3 cell differentiation into beating cardiomyocytes as the readout parameter when predicting in vivo adverse effect concentrations of antifungal compounds. Based on the obtained in vitro adverse effect concentrations, the relative in vivo developmental toxicity potencies of series of antifungal compounds and even the in vivo developmental toxic dose level of the antifungal compound tebuconazole were well predicted. The usefulness of the ES-D3 cell differentiation assay using this classical readout parameter in the prediction of the in vivo developmental toxicity has also been reported for other classes of chemistry, such as valproic acid, glycol ethers, retinoids and phenols [4-10]. The results of the present thesis provide further support for the conclusion that the in vitro ES-D3 cell differentiation assay may also be appropriate to predict developmental toxicity of other classes of chemicals of diverse nature.

One may argue that other developmental toxicity endpoints such as developmental neurotoxicity, developmental immune toxicity or developmental skeletal toxicity might not be well predicted using the inhibition of cardiomyocyte differentiation as readout parameter. Therefore, for the compounds that are known to induce other developmental toxicity endpoints, it could be evaluated whether in vivo potency rankings or in vivo toxic dose levels could be better predicted based on in vitro effect concentrations in in vitro assays characterizing other adverse endpoints [11-13]. However, for new compounds for which no upfront knowledge on the type of adverse effects is available, the investigation on the classical endpoint in the ES-D3 cell differentiation assay would provide a good starting point for predicting in vivo developmental toxicity.

Other than the phenotypic endpoints, such as the contraction of the cardiomyocytes, the ES-D3 cell differentiation assay has also been used to evaluate the effect of chemicals on the expression of specific genes for neuronal differentiation [14-16], osteoblast differentiation [17, 18], endothelial differentiation [18] or cardiac differentiation [19]. These readouts may be useful to predict the influence on specific developmental pathways, such as neurodevelopment, bone formation, vasculogenesis and/or angiogenesis, or heart development, respectively. When using transcriptome approaches, one has to take into consideration, however, that not all gene expression changes necessarily lead to an effect, deleterious or beneficial. One study compared the effect of a developmentally toxic compound, flusilazole, on gene expression regulation and on the ES-D3 cells differentiation into cardiomyocytes. Although transcriptomic profiling identified effects already at lower concentrations, reflecting a higher sensitivity than the phenotypic endpoint, it is difficult to establish adversity of a compound-induced effect based on gene expression alone [19], as the identification of regulation of gene expression does not necessarily represent an adverse biological response. Thus, an important challenge in the use of toxicogenomics for hazard characterization is to determine adversity of the observed compound-induced effect. Nevertheless, to obtain information on mechanisms

underlying developmental toxicity, the ES-D3 cell differentiation assay could be combined with transcriptomics analyses, which can be used to group compounds in classes based on toxicity mechanisms [20]. This may facilitate screening of compounds for possible developmental toxicity when no data are available.

The ex ovo assay of chicken embryos

The second in vitro assay used in the thesis was the ex ovo assay of chicken embryos. In this assay, compound-induced specific structural alterations can be detected. Neural crest cells (NCCs) are the cells that migrate from the neural primordia throughout the embryo and contribute to morphogenesis. Malfunction of NCCs leads to dysmorphologies which are considered to result in in vivo skeletal alterations, such as cleft palate or other craniofacial changes [21]. Using chicken embryos, the investigation on NCCs and their migratory behavior in the avian embryos can be performed, which makes the assay suitable for studying developmental toxicity of compounds that my induce skeletal alterations, such as the antifungal compounds of the present thesis. In the present thesis, we have demonstrated that with the ex ovo assay of chicken embryos, we could discover different alteration patterns of different antifungal compounds, with good correlation to the in vivo findings.

The exposure of chicken embryos can be conducted by injecting through the eggshell (in ovo) [23-27] or in a culture medium outside an egg (ex ovo). It has been reported that when performing toxicity assays using chicken eggs in ovo, there is limited visibility of the embryo and solubility of the tested chemicals may be a problem [28]. The exposure position in the egg is a critical factor and when the exposure is done by injection into the egg, the concentration of the chemical to be studied may not be evenly distributed. It has been shown that the toxicity of thiram injected in the yolk was two-times less than when it was injected in the air chamber, which is probably caused by a difference in distribution [27]. Therefore, the exposure route has been shifted to a so-called shell-less (ex ovo) system in which embryos are exposed to a culture medium outside an egg [28-30]. When cultured ex ovo, in a developmental context, normal growth was shown with regard to weight and size as well as cartilage, bone, and myelinated nerves of embryos [28]. However, although the ex ovo technique used in the present assay is a more advanced method for exposure, it has also its limitations. In the present thesis, test compounds were added to the culture media from 1000 times concentrated stock solutions in DMSO (final DMSO concentration 0.1%). This DMSO concentration is 2.5 times less than its concentration in most other in vitro assays, such as the ES-D3 cell differentiation assay. Using this final concentration of DMSO the concentrations we could reach for most antifungal compounds were above 300 μM, while it was 50 μM for tebuconazole, and in the present thesis these concentrations were high enough to allow the detection of adverse effects. However, for compounds that are less toxic and need higher concentrations to induce toxicity, this assay may appear inappropriate. To find

a solution to this issue, it should be studied whether a higher concentration of solvent has any influence on the normal growth of the embryo and can be used.

Different studies have reported the usefulness of the chicken embryos test for detecting structural alterations of chemicals. Most available studies applied in ovo exposure by injecting through the eggshell, as the shift of exposure route to a shell-less system has only been introduced for a relatively short time. Salzgeber and Salaun (1965) and Jurand (1966) established that thalidomide produces in the chicken embryos in an in ovo assay essentially the same kind of limb alterations as it does in human and rabbit embryos [23, 24]. Another in ovo study has shown captopril to exert angiogenesis and vascular alterations in a chicken embryo model, and this model has been suggested to be a useful system to quantify the anti-angiogenic activity of compounds [31]. In addition, Verrett et al. investigated the toxicity of 80 chemicals that are used as food additives upon their administration to developing chicken embryos in ovo. Results obtained indicated that the chicken embryo test is capable of demonstrating the teratogenic potential of these compounds and that it is selective and does not respond nonspecifically to any agent introduced during development [25]. Given that the ex ovo system supports normal growth of chicken embryos as good as the in ovo system, with regard to weight and size as well as cartilage, bone, and myelinated nerves of embryos [28], it can be concluded that the ex ovo assay may be very likely to be able to observe the above mentioned structural alterations of the chemicals, although it may predict different toxicity potencies than the in ovo assay due to difference in solubility of chemicals in the two systems. Support for the ex ovo assay can be found in the results from a study showing that a variety of structural alterations such as retarded growth, abnormal heart development, macrosomia, exencephaly, etc. observed in chicken embryos exposed to glucose in an ex ovo assay, were similar to those reported in mammalian embryos as a consequence of diabetic pregnancy [29]. Based on these findings, the ex ovo assay of chicken embryo seems to be a valuable tool to predict developmental toxic and teratogenic potential of chemicals of diverse nature not being limited to the class of antifungal compounds.

Other in vitro assays that may be considered for characterizing developmental toxicity

Given that in vitro assays represent only part of the complexity of the whole developing conceptus and its maternal environment, one may question the possibility to develop an in vitro assay that adequately captures all aspects of this important endpoint in toxicity studies. Although the present thesis focused on two toxicity assays, i.e. ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos, and proved their usefulness and applicability for predicting developmental toxicity, one should be aware that these two assays may not be the most relevant to study all characteristics that may relate to developmental toxicity. The applicability of a test may vary with the class of compounds tested, depending e.g. on the mechanism of toxic action and the window of exposure during which in a pregnancy the adverse effect is induced.

An important mode of action underlying developmental toxicity can be endocrine disruption. Awareness about the long-term health consequences associated with exposure to endocrine disrupting chemicals (EDCs) during intrauterine life has been raised [32]. During particularly vulnerable stages of development, the course of development of many tissues is regulated by endogenous steroid hormones along with other endocrine and paracrine factors [32]. EDCs are likely to influence the critical steps of hormonal development of the fetus [33]. Besides, some EDCs are capable of altering the hormonal balance in the placenta. For instance, endosulfan has a slight inhibiting effect, while methomyl, pirimicarb, propamocarb, and iprodion have inducing effects on placental aromatase activity [34], and 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD) increases oestradiol secretion, which suggests that it also affects aromatase activity in placental tissue cultures [35]. The antifungal compounds used in this thesis are known for their abilities to inhibit cytochrome enzymes [2], and in particular may reduce estrogen biosynthesis through CYP19 aromatase inhibition. As estrogens are important in placental development, particularly in rats, reducing the conversion of androgens to estrogens may induce inappropriate development of the placenta [36, 37]. Moreover, some medicinal products to which pregnant women are commonly exposed affect placental steroidogenesis [38]. Smoking also affects placental steroidogenesis [39]. In animal and human studies offsprings affected by EDCs are not only born with congenital abnormalities but may also suffer from several health and behavioral problems throughout their lifespan, such as demonstrated for diethylstilbestrol (DES) and polychlorinated biphenyls (PCBs) [40].

A battery of different in vitro testing approaches have been established by the OECD, including receptor binding and transactivation assays, minced testis and aromatase assays [41], aiming to assess the risks associated with chemicals that have endocrine disrupting properties. These assays are not specifically developed for predicting developmental endocrine disruption during pregnancy, but these tests could be taken into consideration when considering in vitro assays for developmental toxicity [42].

Ad 2) The role of the placenta in contributing to developmental toxicity

A second topic to consider in some more detail when applying the combined in vitro approaches and the integrated alternative approach of the present thesis is the role of the placenta and how that can be best taken into account. The placenta is a key organ in fetal growth and development because it controls the exchange of nutrients and hormones between the mother and the fetus. It also interferes with compound delivery to the fetus by expressing active membrane transporters and xenobiotic metabolism enzymes. Besides, chemical-induced toxicity in the placenta may contribute to the occurrence of developmental adverse effects [43]. Therefore, understanding the effect of xenobiotics on the placenta and also the effect of the placenta on the xenobiotics

increases insight in prediction of developmental toxicity. In the present thesis we have demonstrated the importance of including placental transfer information in predicting in vivo developmental toxicity using in vitro techniques. In this section, characteristics of i) placental transfer, ii) placental metabolism and iii) placental toxicity are discussed that may need future attention when developing novel and improved assays for in vitro and in silico toxicity testing of developmental toxicity.

Placental transfer

In the present thesis, we have proven that the BeWo transport model is capable of predicting relative placental transfer rates for a set of 9 heterogeneous compounds. Subsequently, for two groups of antifungal compounds, the usefulness of including relative placental transfer rates obtained in the BeWo transport model for predicting developmental toxicity based on in vitro assays was demonstrated. The BeWo transport model can be used as an appropriate model for placental transfer and can provide valuable information for improving the prediction of developmental toxicity, its use is limited, however, by the fact that it only predicts transport rates in a relative, but not an absolute way. It should also be noted that the BeWo transport model is a simplification of the in vivo placental transfer system, with several characteristics being different from the in vivo situation, e.g. circulation, protein binding, flow rate and the volumes of maternal and fetal compartments. As a result, the BeWo cell model may not be suitable to determine the absolute transport rates of compounds. The relative Papp value seems to be a "rough approximation" that can be used as an initial approach to estimate the relative placental transport and resulting fetal bioavailability. Placental transfer is a complex process, and a more sophisticated method may be needed for a more accurate quantitative determination of fetal bioavailability. However, our intention was to demonstrate, in a simple model and in a relative and not an absolute way, that placental transfer is important and that taking it into account can improve the quality of the predictions for the relative developmental toxicity based on in vitro assays. In the present thesis we, for the first time, combined the in vitro developmental toxicity values with relative placental transfer data obtained in the BeWo model, and showed that in this way adequate predictions could be made.

Given the good correlation obtained between the placental transfer rates as determined from the BeWo transport model and as reported in the literature based on ex vivo human placental perfusion method that uses term placenta, it may be concluded that the BeWo model is especially relevant to model the last period of pregnancy. Since many structural abnormalities are induced at early stages of pregnancy [44], it seems important to predict placental transfer at these early stages. In this respect it must be noted that it is not clear how well the BeWo model and the ex vivo placental model predict placental transfer at early stages of pregnancy. Therefore, knowledge is needed on whether placental transfer in early stages of pregnancy differs from placental transfer

in the last period of pregnancy, in order to determine how well the BeWo model predicts placental transfer in these critical stages of pregnancy.

The placenta expresses a number of transporter proteins. In the syncytiotrophoblast of the placenta, various transporters have been found both in the brush-border (apical membrane) facing maternal blood and the basolateral membrane close to fetal capillaries. Differential distribution of transporters between the maternal and fetal sides of the placental membrane might have profound effects on the behavior of compounds in transplacental transfer [45]. Multiple studies in several species including human suggest that several ABC (ATP binding cassette) transporters, and in particular P-glycoprotein (P-gp), multidrug resistance proteins (MRPs) and breast cancer resistance protein (BCRP), decrease fetal exposure to xenobiotics [46-49]. It has also been shown that transporters mediating the transfer of amino acids, glucose (GLUTs) and fatty acids (FATPs) are expressed in plasma membranes of the syncytiotrophoblast. and mediate efflux of specific nutrients from the placenta into the fetal circulation [50]. Several of these transporters are also expressed in BeWo cells. Inhibition studies reported that MRP1 and BCRP are expressed in these cells [51, 52]. BeWo cells also express the transporter of neutral amino acids including several essential amino acids as seen in human placental tissue [53]. One study showed that accumulation of P-gp substrates by BeWo cells was significantly enhanced in the presence of a typical P-gp inhibitor, cyclosporine, indicating the presence of P-gp in BeWo cells [54]. On the contrary, another study demonstrated that in BeWo cells the activity of P-gp transporter seems to be limited [51]. Obviously further studies are needed with a wider selection of substrates to further evaluate the transporter activity or expression in the BeWo cell model. Nevertheless, the BeWo cell model seems to exhibit several relevant transporter activities. Although in the present thesis it was not intended to determine transporters involved but rather to define relative Papp values for transport from the maternal to the fetal side, to be used to adjust the BMC50 values obtained from the toxicity assays, it would be of importance to characterise to a further extent whether the BeWo cell model is a suitable model to study the relative transport rate of compounds that are substrates of transporters.

Another factor that may influence placental kinetics and thus developmental toxicity is protein binding. Compounds that are protein bound, usually to albumin or α 1-acid glycoprotein, at the maternal or fetal side do not cross the placenta [55]. Therefore, plasma protein gradients between mother and fetus can also influence transfer. One example is propofol, which is highly protein bound, and in a human perfused placental system, the total propofol concentration in the fetal vein increased significantly with increasing albumin concentration in the fetal circulation, with the concentration of free propofol remaining unchanged [57]. Although the BeWo

transport model, as an in vitro system, seems suitable for studying the influence of protein binding on transfer by applying different albumin concentrations in the apical and basolateral chambers, we did not find a good correlation between relative transport rates in the BeWo model and in the ex vivo perfused placenta system when the same concentrations of albumin were used in both systems [58]. This may be related to the fact that the in vitro BeWo model is a static system, without flow of perfusates, whereas the ex vivo model is a dynamic system including flows of maternal and fetal perfusates controlled by peristaltic pumps. For antifungal compounds, we have shown that the BeWo transport model, with the absence of protein in the transport buffer, provided useful information on relative placental transfer, with which the predictive value of in vitro assays for developmental toxicity could be improved. However, for the compounds of other classes, one should be aware that protein binding may have impact on the transfer.

Placental metabolism

In addition to transfer, metabolism of compounds may occur in the placenta, which would alter the concentrations of the parent xenobiotic and its metabolites to which the fetus is exposed [45, 59]. Therefore, we investigated the metabolic activity of the placenta for antifungal compounds as a potential further modifier of fetal exposure, which should be viewed as a further step to include the toxicokinetic aspects in in vitro models for developmental toxicity. Placental metabolism depends on the activities of the xenobiotic-metabolizing enzymes localized in the placenta. Although several studies have addressed the question whether such enzymes are expressed in the placenta of humans and experimental animals [59-61], quantitative data on the activities of placental xenobiotic-metabolizing enzymes are rare. We have therefore conducted a systematic quantification of seven major xenobiotic-metabolizing enzymes in the near-term placenta of untreated Wistar rats and also in the liver of untreated adult male Wistar rats for comparison [62]. Commonly accepted assays were used to quantify the enzyme activities, paying special attention to high sensitivity in order to be able to measure low activities. For some enzymes consisting of multiple isoforms, the activities of isoform families were determined. Moreover, the metabolic turnover of testosterone and four reference antifungal compounds, being ketoconazole, tebuconazole, propiconazole and prothioconazole, in incubations with liver and placental microsomes was compared to assess the relative contribution of placental metabolism [62].

This probing of xenobiotic-metabolizing enzyme activities in rat placenta and liver by commonly used assays has shown that most enzyme activities are either lacking in the placenta or are very low compared to the liver (Table 1). In order to confirm this finding for CYP-mediated activities, the disposition of testosterone and four reference antifungal compounds was studied in microsomal incubations fortified with

a NADPH-regenerating system for CYP-mediated metabolism. Testosterone is known to be hydroxylated at various positions of the steroid moiety by multiple isoforms of CYP, and the specific pattern of metabolites depends on the CYP isoforms present [63-65]. The antifungal compounds used in our study have multiple sites for hydroxylation. In order to simplify the analysis, no detailed pattern of metabolites but the recovery of the parent compounds from incubations with active and with heat-inactivated microsomes was determined by HPLC analysis, and the difference was assumed to represent the metabolic turnover.

The results obtained are summarized in Table 2. For testosterone, virtually no turnover was observed with placental microsomes but a complete disappearance of the parent testosterone was noted with hepatic microsomes. For the reference antifungal compounds, turnover with liver microsomes under the conditions applied ranged from 24 to 100%, depending on the individual compound and demonstrating the clear contribution of the hepatic metabolism of these compounds to their systemic clearance. In contrast, the turnover of the azoles with placental microsomes was negligible (Table 2). This finding confirms the notion that the placenta is virtually devoid of CYP activity.

Although our study includes seven major enzymes of xenobiotic metabolism, the placental activities of a few other enzymes still need to be determined, e.g. epoxide hydrolases and sulfotransferases. Moreover, it is likely that enzyme activities change during gestation, and placentas at earlier and later stages should also be studied. For example, glutathione S-transferase (GST) activity was found to be higher in rat placenta on day 16 than on day 20 [66]. Furthermore, placental enzyme activities may be influenced by exposure to other chemicals. For instance, the activity of CYP1A1, which is very low in the placenta of non-smoking women in the first trimester of pregnancy and at full-term, is substantially increased by maternal cigarette smoking [61]. The results of the investigations reported here indicate that, at least for antifungal compounds, it is not necessary to take placental metabolism into consideration for describing clearance because its contribution is negligible in comparison to the liver. However, placental metabolism is still worth investigating, for especially compounds known to be converted to a reactive toxic metabolite, as the placenta is capable of metabolizing several drugs and foreign chemicals having impact on the developmental toxicity outcome. For instance, it was shown that B(a)P is metabolized to its ultimate carcinogenic metabolite, benzo(a)pyrene 7,8-diol-9,10-epoxide, capable of DNA binding by a placental CYP-dependent system [67]. Another example is the observation that human placenta can metabolize retinoids (isotretinoin and tretinoin) to both more and less toxic metabolites [68].

Table 1. Activities of xenobiotic metabolizing enzymes in rat placenta and liver [62].

Enzme	Test substrate	Cell fraction	Placenta	Liver	Unit	LOD/LOQ [a]
Cytochrome P450 1A	**7-*O*-Ethylresorufin**	**Microsomes**	**< LOD/LOQ**	**44.4 ± 3.2**	**pmol min^{-1} mg $protein^{-1}$**	**1.01/2.03**
Cytochrome P450 2B	**7-*O*-Pentylresorufin**	**Microsomes**	**< LOD/LOQ**	**35.7 ± 2.1**	**pmol min^{-1} mg $protein^{-1}$**	**4.75/9.49**
Cytochrome P450 2B/2C/3A	**7-*O*-Benzylresorufin**	**Microsomes**	**< LOD/LOQ**	**114.8 ± 3.0**	**pmol min^{-1} mg $protein^{-1}$**	**1.58/3.17**
NADPH cytochrome c reductase	**Cytochrome c**	**Microsomes**	**29.9 ± 7.7**	**n.m. [b]**	**nmol min^{-1} mg $protein^{-1}$**	**3.77/7.54**
Flavin-containing monooxygenase	**Benzydamine**	**Microsomes**	**0.82 ± 0.64**	**20.8 [c]**	**nmol min^{-1} mg $protein^{-1}$**	**0.02/0.06**
Alcohol dehydrogenase	**Ethanol**	**Cytosol**	**9.15 ± 1.00**	**26.2 ± 4.6**	**nmol min^{-1} mg $protein^{-1}$**	**0.65/1.29**
Aldehyde dehydrogenase	**Propanal**	**Cytosol**	**3.83 ± 0.26**	**9.5 ± 1.0**	**nmol min^{-1} mg $protein^{-1}$**	**0.85/1.70**
Esterase	**Fluorescein diacetate**	**S9 fraction**	**1.27 ± 0.01**	**16.8 [d]**	**nmol min^{-1} mg $protein^{-1}$**	**0.47/0.94**
UDP-glucuronosyltransferase 1	**4-Methylumbelliferone**	**Microsomes**	**< LOD/LOQ**	**334 ± 35 x 10^3**	**FU min^{-1} mg $protein^{-1}$**	**15 x 10^3/30 x 10^3**
UDP-glucuronosyltransferase 2	**4-Hydroxybiphenyl**	**Microsomes**	**< LOD/LOQ**	**81 ± 1 x 10^3**	**FU min^{-1} mg $protein^{-1}$**	**31 x 10^3/62 x 10^3**
Glutathione S-transferase	**1-Chloro-2,4-dinitrobenzene**	**S9 fraction**	**< LOD/LOQ**	**205 ± 18**	**nmol min^{-1} mg $protein^{-1}$**	**35/70**

[a] LOD, limit of detection; LOQ, limit of quantification; < LOD/LOQ, lower than both LOD and LOQ [b].n.m., not measured; [c] individual measurement; [d] duplicates

Table 2. Metabolic turnover of testosterone and four model azole fungicides in incubations of microsomes from rat placenta and liver [62].

		Turnover (%)	
Test compound	***m/z* of $[M+H]^+$ ion/daughter ion** [a]	**Placenta**	**Liver**
Testosterone	n.a. [b]	5.9 ± 3.1	99.7 ± 8.3
Ketoconazole	531.0/489.0	2.8 ± 6.7	24.3 ± 13.6
Tebuconazole	308.1/124.6	n.d. [c]	45.3 ± 3.2
Propiconazole	342.0/158.9	0.2 ± 2.3	99.8 ± 0.1
Prothioconazole	344.0/153.9	n.d.	47.6 ± 1.6

[a] used for quantification in LC-MS/MS; [b] n.a., not applicable; [c] n.d., not detectable (< 0.2%)

Placental toxicity

In the present thesis, intracellular accumulation of test compounds was detected in the BeWo cell model and we discovered that some antifungal compounds showed a high percentage of accumulation in the BeWo cells, amounting to up to 79 % of the total mass added. However, as BeWo cells only represent part of the placental tissue and the placental tissue structure changes over pregnancy, it is difficult to translate this in vitro finding to the exact in vivo situation. If this accumulation in placental cells is also occurring in vivo, then because of the accumulation, high intracellular levels may be reached upon repeated dosing, potentially causing placental toxicity. Given that the placenta is an entry organ to the fetus and vulnerable to the adverse effects of many toxicants, this merits attention. Some toxicants, such as heavy metals and pesticides [43, 69, 70], have been shown to accumulate in the placenta and potentially affect its development or function. Structural or functional damage to the placenta can lead to adverse effects, such as abortion, birth defects and stillbirth [71]. Therefore, it might be of interest to pay attention to possible placental toxicity, for which the in vitro BeWo cell model might be useful for prioritization because as shown in the present study the model is able to provide information on the intracellular accumulation of compounds and detect possible resulting toxicity.

Ad 3) The use and development of PBK models

Another topic that needs to be considered to a further extent in the framework of using alternatives for prediction of in vivo developmental toxicity is the use of PBK model to extrapolate in vitro data into in vivo data that are needed in risk assessment. In the present section, discussion focuses on whether including a placental-fetal unit in a PBK model is needed and how to add such a unit in a PBK model.

The necessity of including a placental-fetal unit in a PBK model

Using the PBK model-facilitated reverse dosimetry approach, we extrapolated the in vitro concentration-response curves into in vivo dose-response curves and derived BMD10 values that correlated well to in vivo data for developmental toxicity of one of the reference antifungal compounds tebuconazole. It should be noted that this PBK model developed for tebuconazole did not include a unit that describes placental-fetal exchange. For tebuconazole, we have demonstrated that a fast placental transfer rate was predicted in the BeWo transport model and that the contribution of placental metabolism is negligible in comparison to the liver. In this case, we assumed that the fetal concentration of tebuconazole is likely equal to its maternal concentration and it was not necessary to include a placental-fetal unit in the PBK model. However, placental transfer should be considered in PBK modelling if no evidence is available to support that the fetal exposure is comparable to internal maternal exposure and placental metabolism should be included in the PBK model if it contributes significantly to the overall metabolism or to the bioactivation of the xenobiotic.

How to include a placental-fetal unit in a PBK model

Various compartmental structures have been used to describe the placental-fetal unit, as previously reviewed by Corley et al. [72]. The placental-fetal unit may include placenta, amniotic fluids, umbilical cord and fetus. The description of the fetus could be further extended to include additional compartments for tissues and organs if fetal tissue exposure is of interest. Fig.1 summarizes the model structures of the placental-fetal unit in the existing pregnancy-PBK (p- PBK) models [72] and they are discussed below in more detail.

For many of the chemicals evaluated to date, simple, blood flow-limited models have generally been used to describe the kinetics in placental-fetal tissue. As shown in Fig.1A, a simple approach on PBK modelling of the placental-fetal unit is to combine the placenta, embryos or fetuses, as well as amniotic fluid within a lumped homogenous compartment based on assumption on their similarities in blood perfusion and chemical partitioning [73-75]. However, this assumption may not be true for all types of chemicals, as tissue/blood partitioning depends on the physico-chemical properties of the chemical and the specific tissue undergoing changes during pregnancy. More importantly, in this simplistic approach, delays in appearance or the concentration of a chemical in embryo or fetal units cannot be explained by simple differences in partitioning and blood perfusion parameters.

Alternatively, it is possible to consider the placental-fetal unit as diffusion-limited. Fig.1B shows the conceptual structure of a PBK model in which all tissues are modeled as well-stirred compartments with blood flow limitations except for the placental-fetal unit, which is modeled as diffusion limited. This model structure has been adopted by most p-PBK models, for example, the models developed by for morphine [76], theophylline [77], methadone [78] and pethidine [79], and for retinoic acid and its metabolites [80]. Moreover, some p-PBK models have modelled the amniotic fluid as a

compartment separated from the embryo/fetus, as shown in Fig. 1C, for example, the models developed for 2-methoxyethanol and 2-methoxyacetic acid and methanol [74, 81, 82]. Such separation would benefit model validation, on the basis of the possibility to get in vivo data from the amniotic fluid sampling.

The most complex approach, as shown in Fig.1D, is to model the fetus using a flow-limited, or diffusion-limited (as well as their combination) compartmental structure of the fetus similar to the maternal part, with mass communication with the maternal PBK model via the placental-barrier [83]. This approach could be used to study the exposure to specific tissues/organs within the fetus. The use of this approach is restricted to modelling the part of the gestation period after the fetal blood circulation and tissues/organs have been established.

As the levels of complexity are added to the p-PBK models, a corresponding increase in the data requirements (and costs) associated with the development of parameters and validation of the models occurs. The simple models provide little insight into the role of embryo or fetal tissue-specific internal doses in developmental toxicity and does not take diffusional transfer into account, which may play an important role in determining fetal concentration. However, the more complex the model applied, the lower the number of chemicals for which data for model verification and validation are available. Therefore, based on the available data, a balanced complexity should be incorporated into PBK models in order to balance the need for reducing uncertainty against the need for refinement of the definition of target tissue doses. Such refinements are dependent on the development of reasonable hypotheses for modes of action and the availability of biological and chemical-specific data [72].

With respect to compounds with low placental transfer rates, e.g. as measured in the BeWo transport model, the incorporation of a placental-fetal unit in a PBK model is needed and for such compounds model B or C are preferable among the 4 models, as these two models take diffusional transfer into account and are less complex than model D. Importantly, parameters for diffusional transfer are needed in model B and model C. However, these parameters are difficult to measure and are generally inferred from in vivo kinetic studies [72]. For most of chemicals, including antifungal compounds, limited in vivo data are available on their diffusional transfer for placenta-embryo/fetus, embryo/fetus-amniotic fluid, or placenta-amniotic fluid. Alternatively, it seems useful to use the ex vivo placental perfusion systems for determining these diffusional transfer parameters, as the ex vivo perfusion systems allows for good control of the physiological environment and can provide an opportunity for studying rate of chemical diffusion across the placenta. However, given that the ex vivo placental perfusion systems are laborious and dependent on the availability of fresh placenta, research is needed to identify the feasibility of using in vitro techniques or in silico methods, such as (Q)SAR models, to determine diffusional transfer parameters for building a placental-fetal unit in a PBK model.

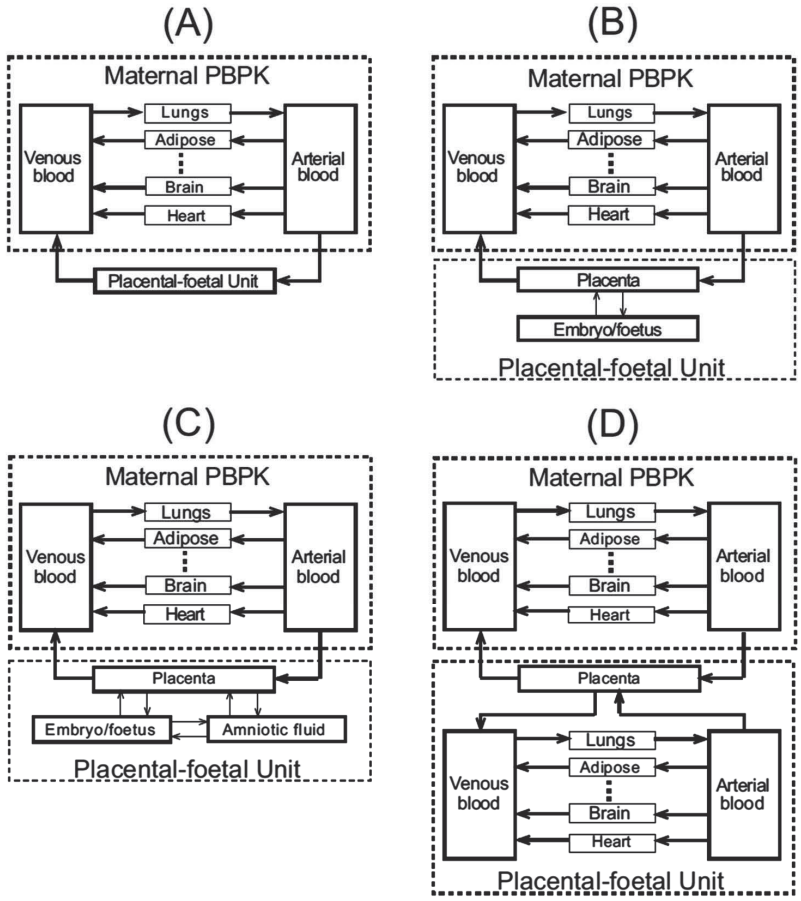


Fig. 1. General model formats utilized in chemical-specific PBK models describing the placental-fetal unit (taken from [72]).

Ad 4) The in vivo data available and their use for validation purposes -- Skeletal malformation or skeletal variation? Litter data or fetus data?

To validate results of the relative developmental toxicity potencies obtained from the in vitro toxicity assays and the predicted dose-response data as determined using the PBK modelling-facilitated reverse dosimetry, in the present thesis the results were compared to available in vivo data from rat studies for developmental toxicity. These in vivo studies are designed to identify chemicals that are potentially hazardous to the developing conceptus, with the ultimate aim to provide appropriate data for the extrapolation to human health hazard and risk assessment. For antifungal compounds studied in the present thesis, in vivo data on skeletal alterations were used in order to validate the results obtained from in vitro and/or in silico results.

Skeletal alterations in development include both malformations and variations. In prenatal developmental toxicity studies, interpretation of skeletal alterations is a difficult area. Part of the difficulty lies in the historic definitions of malformations versus variations and the interpretation of their relevance to human risk assessment [84]. According to the EPA guidelines for developmental toxicity risk assessment: "A malformation is usually defined as a permanent structural change that may adversely affect survival, development, or function. The term teratogenicity is used in these guidelines to refer only to malformations. The term variation is used to indicate a divergence beyond the usual range of structural constitution that may not adversely affect survival or health" [85]. However, this distinction is occasionally blurred, given that there is a continuum ranging from variations to malformations since there exists a continuum of responses from the normal to the extremely deviant [84]. There have been several workshops held in which standardization of the classification of malformations and variations have been discussed [86, 87]. Nevertheless, this area remains highly disputed and depends on species and even strain distribution of abnormalities in the developmental process. For instance, some investigators would classify extra ribs as either variations or malformations depending on the length of the structure [84]. This historic distinction of malformations and variations has often affected the interpretation of the relative importance in predicting risks to humans [85, 88]. In the present thesis, data on both endpoints were chosen to derive BMD10 values for the comparison with the predicted toxicity values, as both endpoints were considered to represent adverse effects on the developing organism. In addition, where it comes to the use of in vivo data, one should be aware that appropriate historical control data can be helpful in the interpretation of malformations and variations, especially those that normally occur at very low incidences, to be able to evaluate whether these adverse findings are relevant indicators of developmental toxicity. When no historical control data are available, the data should be interpreted with caution particularly when a high background incidence in control animals is reported.

In developmental toxicity studies, data can be calculated as proportion of offspring or implants affected per litter (fetus data), but can also be expressed as number and percent of litters with particular endpoints (litter data). Generally, in reproduction toxicity, liter data are considered to be the experimental unit on which (e.g. statistical) evaluations are based. In the evaluation of available in vivo developmental toxicity data to derive BMD10 values for validation purposes of the work presented here, no upfront selection has been made whether litter- or fetus-data are more appropriate to use. The fetal incidence is often a more sensitive indicator of toxic effects than the litter incidence. However, when a high incidence of skeletal alterations is observed in just one litter that is due to dam specific (and not necessarily treatment related) effects, such as genetic background and environmental factors, using fetal incidence as developmental endpoint would be incorrect.. Therefore, it may be inappropriate to only consider one type of incidence data

when analyzing the in vivo data. In the present thesis, data on both litter incidence and fetus incidence were considered for the evaluation in order to derive reference values for validation of values predicted from the in vitro/in silico models.

Ad 5) Future perspectives and conclusions

The present thesis focuses on the use of alternative methods to predict in vivo developmental toxicity. Based on the results obtained in the present thesis, a framework of using in vitro or/and in silico methods for developmental toxicity testing could be proposed for antifungal compounds. As a simpler assay, the ES-D3 cell differentiation assay, when combined with the BeWo cell transport model, can be used in a first tier to predict toxicity potency of chemicals and to detect the compound(s) of high concern, i.e. showing high toxic potency. Then the ex ovo assay of chicken embryos can be applied to test chemical(s) of high concern in a second tier enabling identification of potential structural alterations. In combination, the three in vitro assays could provide a valuable tiered alternative approach for developmental toxicity screening and prioritization. Further on, in vitro concentration-response curves obtained from the two in vitro developmental toxicity assays could be extrapolated into in vivo dose-response curves using the PBK modelling facilitated reverse dosimetry approach. From the predicted in vivo dose-response curve a PoD could be derived which can be used in risk assessment.

The present thesis provides the proof-of-principle that, with the help of PBK-model facilitated reverse dosimetry approach, in vitro concentration response curves can be converted into in vivo dose-response curves for developmental toxicity of antifungal compounds. In order to gain more confidence on the use of this approach as input in risk assessment, further proof-of-principle studies with a wide set of chemicals from diverse nature for developmental toxicity are needed, especially the ones for which human data are available enabling validation of the predictions made for human. So far reverse dosimetry-based predictions have been made for the developmental toxicity of different classes of compounds, including glycol ethers, phenol and retinoic acid [5, 8, 89] and it has been possible to accurately predict a PoD for the risk assessment of these compounds for developmental toxicity, using in vitro and in silico data only. Besides, to increase the impact and applicability of using this PBK-model facilitated reverse dosimetry approach in risk assessment procedures, one should consider to define proofs of principle for other endpoints. Research gaps have been identified in the applicability of in vitro tests for systemic endpoints for regulatory purposes. Therefore, being able to predict PoDs for a broad range of endpoints would allow to assess the level of confidence of using the reverse dosimetry approach in risk assessment. So far, apart from developmental toxicity, this approach has been tested valid in extrapolating in vitro toxicity results to the in vivo situation for other endpoints, including kidney toxicity [90], neurotoxicity [91, 92], acute oral toxicity and repeated dose toxicity [93] and genotoxicity [94] of chemicals of interest.

From a conceptual point of view, it would seem that there is no reason why the value of this approach should be limited to the above mentioned toxicity endpoints. Therefore, it seems reasonable that the applicability of this approach could be extended towards other systemic endpoints. Then the use of alternative methods for risk assessment could even be brought to a new stage, in which based on the PoD values predicted for different endpoints, one could determine what would be the target tissue/organ of a chemical. All in all, this approach is in accordance with the concept recommended by the National Research Council (NRC) for Toxicity Testing in the 21st Century [95], a landmark report stating that scientists must embrace new technologies transitioning from current expensive and lengthy in vivo testing with qualitative and quantitative endpoints to in vitro assays evaluating cellular responses upon chemical exposure, combined with PBK modeling to equate in vitro effect concentrations with tissue doses expected in humans.

Although the PBK modelling-facilitated reverse dosimetry approach has been possible to enhance the applicability of in vitro methods in risk assessment, the application of such an approach will ultimately also depend on the validation of both in vitro assays and PBK models and their acceptation by regulatory authorities [96]. As more and more research activities have been conducted on the PBK modelling-facilitated reverse dosimetry, the possibility of using this approach in risk assessment will increase. When implementing this approach in risk assessment practice, one should consider whether uncertainty factors would be required to account for uncertainties in the accuracy of predicted dose-dependent effects. For the compound studied in the present thesis, differences in predicted BMD10 values and those values derived from reported rat studies were within 5-fold, indicating that an uncertainty factor of 5 may be necessary for uncertainties in the predictions. With earlier reverse dosimetry-based predictions made for the developmental toxicity of glycol ethers, phenol, and retinoic acid [5, 8, 89], the BMD(L)10 values from predicted dose–response data were all within one order of magnitude of the BMD(L)10 values obtained from in vivo data, indicating that so far a factor of 10 seems appropriate for uncertainties in the reverse dosimetry-based predictions. As more examples of the proof-of-principle for other chemicals and other endpoints will be provided, an appropriate overall value for this uncertainty factor may be determined.

In conclusion, the present thesis demonstrates for antifungal compounds, the importance of taking placental transfer of compounds into account when predicting developmental toxicity with in vitro methods. It also shows that in vivo developmental toxicity dose levels can be closely predicted using a PBK modelling-facilitated reverse dosimetry approach. This reverse dosimetry approach could contribute to an increased acceptance and further implementation of in vitro toxicity data in risk assessment procedures. The combined in vitro approaches and the integrated in vitro-in silico approaches appear to be promising for the screening and prioritization of chemicals and to provide reference values, such as BMD10 values, without using animals, therefore contributing to the 3R principle of animal testing.

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CHAPTER 8

Summary

SUMMARY

For the determination of safe human exposure levels of chemicals in toxicological risk assessments, there is large interest in the development of in vitro and/or in silico test systems as alternatives for animal studies. The aim of the present thesis was to evaluate the applicability of an integrated in vitro-in silico approach for prediction of developmental toxicity using a series of antifungal compounds as the model compounds.

In **Chapter 2**, we developed and validated an in vitro placental barrier model using BeWo b30 cells to predict placental transfer. For a set of nine selected model compounds, the relative transport rates obtained in the in vitro model were in very good correlation ($R^2 = 0.95$) with the transfer indices reported for the ex vivo placental transfusion model in the literature. This demonstrated that the BeWo model could be a valuable in vitro model to predict relative placental transfer of compounds.

In **Chapter 3**, the in vitro developmental toxicity of five selected model antifungal compounds was investigated by determining the effect concentrations that inhibit the differentiation of ES-D3 cells into beating cardiomyocytes. When the ES-D3 cell differentiation data were combined with the relative transport rates obtained from the BeWo transport model, the correlation with the in vivo data ($R^2 = 0.95$) was better than using the ES-D3 cell differentiation data alone ($R^2 = 0.57$). This shows that the ES-D3 cell differentiation assay is able to better predict the in vivo developmental toxicity potencies of antifungal compounds when combined with the BeWo transport model, than as a stand-alone assay. To validate this combined in vitro approach to predict in vivo developmental toxicity, we combined ES-D3 cell differentiation data of six novel triazole antifungal compounds with their relative transport rates obtained from the BeWo model and compared the obtained ranking to the developmental toxicity ranking as derived from in vivo data (**Chapter 4**). The data obtained show that the combined in vitro approach provided a correct prediction for the relative in vivo developmental toxicity, whereas the ES-D3 cell differentiation assay as stand-alone did not. We suggest that this combined model, which takes both toxicodynamic and toxicokinetic aspects into account, should be further validated for other chemical classes of developmental toxicants.

In **Chapter 5,** we investigated the applicability of the ex ovo assay of chicken embryos to predict the specific alterations induced by the five antifungal compounds that were used in Chapter 3 and investigated if the combination of the assay with placental transfer information would improve its predictive capacity. The ex ovo assay assesses the developmental toxic potential of compounds based on well-defined morphological endpoints. Each compound produced a characteristic pattern of alterations. When the toxicity data from the ex ovo assay were combined with the relative transport rates from the BeWo model, the correlation with the in vivo data were better than using the toxicity data from the ex ovo assay alone. This revealed that the ex ovo assay of chicken

embryos is able to assess the teratogenic potential of antifungal compounds, and, when combined with the in vitro BeWo transport model, is able to better predict relative in vivo prenatal developmental toxicity potencies.

In **Chapter 6**, we translated in vitro concentration–response data of the antifungal compound tebuconazole, obtained in the ES-D3 cell differentiation assay and the ex ovo assay of chicken embryos, into predicted in vivo dose–response data using physiologically based kinetic modelling-facilitated reverse dosimetry. The results show that the BMD10 values from predicted dose–response data from both assays are in concordance with BMD10 values derived from in vivo data (within 5-fold difference). This revealed that physiologically based kinetic modeling is a promising tool to predict in vivo dose-response curves based on the results of in vitro toxicity assays, and may therefore be used to set a point of departure for deriving safe exposure limits in risk assessment.

In **Chapter 7** we presented a discussion of the data obtained and also future perspectives. It is concluded that it is important to take placental transfer of compounds into account when predicting developmental toxicity with in vitro methods. The results obtained also show that in vivo developmental toxicity dose levels can be closely predicted using a PBK modelling-facilitated reverse dosimetry approach. This reverse dosimetry approach could contribute to an increased acceptance and further implementation of in vitro toxicity data in risk assessment procedures. The combined in vitro approaches and the integrated in vitro-in silico approaches appear to be promising for the screening and prioritization of chemicals and to provide reference values, such as BMD10 values, without using animals, therefore contributing to the 3R principle of animal testing.

APPENDIX

Acknowledgements,
Curriculum Vitae, List of publications,
Overview of completed training activities

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CURRICULUM VITAE

Hequn Li was born on October 2nd, 1988 in Neimongol, China. After receiving her Bachelor degree in Shandong Agricultural University, China in 2009, she came to Wageningen University in the Netherlands for her master study in Food Safety. During her master, Hequn conducted her internship and later became a Junior Research Assistant at Nestlé Research Centre, Lausanne, Switzerland. In 2012, she started her PhD at the Division of Toxicology, Wageningen University, under the supervision of Prof. Ivonne Rietjens and Dr. Jochem Louisse, as well as Prof. Ben van Ravenzwaay from the project sponsor company BASF SE in Germany. During her PhD study, she followed several Postgraduate Education courses in Toxicology, which enables her to register as a European Toxicologist. She was a member of the organizing committee of the international PhD excursion to the United Kingdom in 2013. She was awarded the 2015 Chinese Government Award for Outstanding Self-financed Students Abroad from the China Scholarship Council. From February 2016 onwards, Hequn has been working as an ADME-Biochemist, focusing on PBPK modelling, in Unilever Safety and Environmental Assurance Centre (SEAC) in the United Kingdom.

LIST OF PUBLICATIONS

Li, H., van Ravenzwaay, B., Rietjens, I. M. C. M. and Louisse, J. (2013). Assessment of an in vitro transport model using BeWo b30 cells to predict placental transfer of compounds. *Archives of Toxicology* 87, 1661-1669

Li, H., Rietjens, I.M.C.M., Louisse, J., Blok, M., Wang, X., Snijders, L., van Ravenzwaay, B., (2015). 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* 29, 320-328

Li, H., Flick, B., Rietjens, I.M.C.M., Louisse, J., Schneider, S., van Ravenzwaay, B., (2015). 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*, 1-13

Li, H., Flick, B., Rietjens, I.M.C.M., Louisse, J., van Ravenzwaay, B., (2015). Use of the ex ovo assay of chicken embryos to predict the in vivo developmental toxicity of antifungal compounds. Submitted

Li, H., Rietjens, I.M.C.M., van Ravenzwaay, B., Zhang, M., Louisse, J., (2015). 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. In preparation

Fabian, E., Wang, X., Engel, F., **Li, H.,** Landsiedel, R., van Ravenzwaay, B., (2015). Activities of xenobiotic metabolizing enzymes in rat placenta and liver in vitro, *Toxicology in Vitro,* Available online 2 March 2016

Actis-Goretta, L., Lévèques, A., Rein, M., Teml, A., Schäfer, C., Hofmann, U., **Li, H.,** Schwab, M., Eichelbaum, M. and Williamson, G., (2013). Intestinal absorption, metabolism, and excretion of (–)-epicatechin in healthy humans assessed by using an intestinal perfusion technique. *Am J Clin Nutr* 2013 98: 4 924-933

Sanchez-Bridge, B., Lévèques, A., **Li, H.,** Bertschy, E., Patin, A., Actis-Goretta, L., (2015). Modulation of (–)-Epicatechin Metabolism by Coadministration with Other Polyphenols in Caco-2 Cell Model. *Drug Metabolism and Disposition* 43, 9-16

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

Molecular Toxicology, Postgraduate Education in Toxicology (PET), Amsterdam, 2013
Cell Toxicology, PET, Leiden, 2014
Epidemiology, PET, Utrecht, 2014
Pathobiology, PET, Utrecht, 2013
Laboratory animal science, PET, Utrecht, 2012
Reproductive Toxicology, PET, Utrecht, 2012
Organ Toxicology, PET, Nijmegen, 2013
Mutagenesis & Carcinogenesis, PET, Leiden, 2013

Conferences

BIT's 6th World DNA and Genome Day, Oral Presentation, Nanjing, China, 2015
Society of Toxicology, Oral Presentation, San Diego, USA, 2015
Society of Toxicology, Poster, San Antonio, USA, 2013

General courses

Techniques for Writing and Presenting a Scientific Paper, Wageningen, 2012
Legal and Regulatory Toxicology, PET, Wageningen, 2014
Risk Assessment, PET, Wageningen, 2013
PhD day of the NVT, Soesterberg, 2012
VLAG PhD week, Venlo,

Optional activities

General Toxicology, Wageningen, 2012
Environmental Toxicology, Wageningen, 2013
Attending scientific presentations at Division of Toxicology, 2012-2016
Member of 2013 PhD trip committee, 2013
2013 PhD trip of Tox PhD students to the UK, 2013
Preparing PhD research proposal 2012-2016

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