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Quantitative *in Vitro* to *in Vivo* Extrapolation (QIVIVE) for Predicting Reduced Anogenital Distance Produced by Anti-Androgenic Pesticides in a Rodent Model for Male Reproductive Disorders

Martin Scholze,[1] Camilla Taxvig,[2*] Andreas Kortenkamp,[1] Julie Boberg,[2] Sofie Christiansen,[2] Terje Svingen,[2] Karin Lauschke,[2] Henrik Frandsen,[3] Sibylle Ermler,[1] Susan Strange Hermann,[3] Mikael Pedersen,[2] Anne Kruse Lykkeberg,[3] Marta Axelstad,[2] and Anne Marie Vinggaard[2]*

[1]Institute of Environment, Health and Societies, Brunel University London, Uxbridge, UK
[2]Division of Diet, Disease Prevention and Toxicology, National Food Institute, Technical University of Denmark, Lyngby, Denmark
[3]Research Group for Analytical Food Chemistry, National Food Institute, Technical University of Denmark, Lyngby, Denmark

Background: Many pesticides can antagonize the androgen receptor (AR) or inhibit androgen synthesis *in vitro* but their potential to cause reproductive toxicity related to disruption of androgen action during fetal life is difficult to predict. Currently no approaches for using *in vitro* data to anticipate such *in vivo* effects exist. Prioritization schemes that limit unnecessary *in vivo* testing are urgently needed.

Objectives: The aim was to develop a quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) approach for predicting *in vivo* anti-androgenicity arising from gestational exposures and manifesting as a shortened anogenital distance (AGD) in male rats.

Methods: We built a physiologically based pharmacokinetic (PBK) model to simulate concentrations of chemicals in the fetus resulting from maternal dosing. The predicted fetal levels were compared with analytically determined concentrations, and these were judged against *in vitro* active concentrations for AR antagonism and androgen synthesis suppression.

Results: We first evaluated our model by using *in vitro* and *in vivo* anti-androgenic data for procymidone, vinclozolin, and linuron. Our PBK model described the measured fetal concentrations of parent compounds and metabolites quite accurately (within a factor of five). We applied the model to nine current-use pesticides, all with *in vitro* evidence for anti-androgenicity but missing *in vivo* data. Seven pesticides (fludioxonil, cyprodinil, dimethomorph, imazalil, quinoxyfen, fenhexamid, *o*-phenylphenol) were predicted to produce a shortened AGD in male pups, whereas two (λ-cyhalothrin, pyrimethanil) were anticipated to be inactive. We tested these expectations for fludioxonil, cyprodinil, and dimethomorph and observed shortened AGD in male pups after gestational exposure. The measured fetal concentrations agreed well with PBK-modeled predictions.

Discussion: Our QIVIVE model newly identified fludioxonil, cyprodinil, and dimethomorph as *in vivo* anti-androgens. With the examples investigated, our approach shows great promise for predicting *in vivo* anti-androgenicity (i.e., AGD shortening) for chemicals with *in vitro* activity and for minimizing unnecessary *in vivo* testing. https://doi.org/10.1289/EHP6774

Introduction

Currently, around 350 pesticides are approved for use in the EU (2009). Even though criteria for assessing the endocrine-disrupting properties of these compounds are now in place within the EU (https://ec.europa.eu/health/endocrine_disruptors/overview_en), most of these substances have not yet been evaluated in terms of adverse effects related to endocrine disruption, including those relevant to human male reproductive health. Many current-use pesticides are capable of antagonizing the androgen receptor (AR) or of inhibiting testosterone synthesis *in vitro* (Bonefeld-Jørgensen et al. 2007; Kelce et al. 1995; Mnif et al. 2011; Orton et al. 2011; Ostby et al. 1999; Vinggaard et al. 2002), but data pertaining to their potential for endocrine disruption *in vivo* are limited or missing altogether. To support such assessments in the future, certain pesticides will need targeted developmental and reproductive toxicity studies, the most demanding type of study in regulatory toxicology in terms of animal numbers and cost, and accounting for no less than 90% of all animals used in regulatory testing (Hartung and Rovida 2009). Until now, no practicable approach existed on how to use *in vitro* data to predict the potential for *in vivo* reproductive and developmental effects related to the disruption of androgen action in fetal life. As a result, prioritization schemes that can limit unnecessary *in vivo* testing are missing altogether (Punt et al. 2011).

Quantitative *in vitro* to *in vivo* extrapolation methods (QIVIVE) have the potential to fill this gap (Fabian et al. 2019). They hold the promise of delivering urgently needed prioritizations for reproductive and developmental toxicity testing. Interest in such approaches has increased considerably since EU and U.S. regulatory authorities regarded the development of alternative animal-free testing strategies as the most important challenge in future chemical risk assessment (EU 2014; Kavlock et al. 2018). The vision is to mobilize mechanism-based toxicological understanding to avoid unnecessary animal testing, and even to replace *in vivo* testing, through the use of new approaches, including *in vitro* data and *in silico* methods (Kavlock et al. 2018). To achieve this goal, a good understanding of the relationship between *in vitro* and *in vivo* endpoints is essential. Most promising are QIVIVE approaches, which relate substance concentrations associated with *in vitro* responses to *in vivo* intake doses and their corresponding concentrations in target tissues. QIVIVE methods are derived from physiologically based kinetic (PBK) models that simulate the kinetic dynamics of a substance within the living organism over time [e.g., absorption, distribution, metabolism, and excretion (ADME)].

There is considerable interest in QIVIVE models for anti-androgenicity because the incidences of male reproductive disorders, such as cryptorchidism, hypospadias, poor semen quality, and

*These authors contributed equally to this work.

Address correspondence to Anne Marie Vinggaard, Division of Diet, Disease Prevention and Toxicology, National Food Institute, Technical University of Denmark, Kemitorvet Building 202, 2800 Kgs. Lyngby, Denmark. Telephone: 45 35887549. Email: annv@food.dtu.dk; or Andreas Kortenkamp, Institute of Environment, Health and Societies, Brunel University–London, Quad North, Kingston Ln., Uxbridge, UB8 3PH, UK. Telephone: 44 1895 266525. Email: andreas.kortenkamp@brunel.ac.uk

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testicular cancer, have risen over the last few decades (Skakkebaek et al. 2016). Human exposure to environmental chemicals, including pesticides, may be among the contributing risk factors, and prenatal or early life exposures are of particular concern (Juul et al. 2014; Swan 2006).

Disruption of androgen action in fetal life by AR antagonists materializes as a syndrome of effects characterized by hypospadias, testes non-descent (cryptorchidism), epididymal lesions, severe prostate lesions, reduced sperm production, and shorter anogenital distance (AGD) (Gray et al. 2004). This syndrome is the manifestation of a disturbance of complex cell signaling processes in addition to a disturbed balance between cell proliferation and apoptosis in androgen-sensitive tissues and arises from the displacement of androgens from the AR by receptor antagonists. Suppression of steroid synthesis can produce a similar constellation of effects (Schwartz et al. 2019). AGD changes are morphometric biomarkers for adverse male reproductive health outcomes originating during fetal life, both in animals (Schwartz et al. 2019) and humans (Thankamony et al. 2016). In rodents and humans, the female AGD is much shorter than the male AGD, and shortened AGDs in males are strongly associated with adverse male reproductive disorders, such as hypospadias, cryptorchidism, and poor sperm quality (Hsieh et al. 2008; Mendiola et al. 2011; Schwartz et al. 2019; Swan 2006). Current test guidelines (TG) from Organisation for Economic Co-operation and Development (OECD) for developmental and reproductive toxicity testing regard the shortening of AGD as an adverse outcome that should be considered for estimating no observed adverse effect levels (NOAELs) (OECD 2013).

We present a QIVIVE approach that uses *in vitro* AR antagonistic and androgen synthesis-suppressing properties of chemicals to predict dose ranges for which shortened AGDs in male fetuses or pups can be expected in rodent studies after gestational exposures to pesticides. We developed a generic PBK model that simulates internal exposure levels in the fetus at critical time windows after repeated maternal dosing. We used data in draft assessment reports (DAR) for pesticide active substances from the European Food Safety Authority (EFSA) to estimate key kinetic parameters and to identify maximal nontoxic dose limits (EFSA 2019). We employed a stepwise proof-of-concept approach in which we

- first evaluated the general applicability of the QIVIVE approach to three well-studied anti-androgenic compounds (procymidone, vinclozolin, and linuron) for which *in vitro* and *in vivo* data clearly show anti-androgenic action *in vitro* and shortened AGD in males;
- applied the QIVIVE approach to nine current-use pesticides, all with *in vitro* evidence for anti-androgenicity but missing *in vivo* confirmation, by simulating dose ranges that are expected to produce a shortened AGD in males; and
- selected three of these pesticides for further *in vivo* studies in which we tested our hypothesis that all these substances produce shortened AGD at low nontoxic doses.

The principles of the QIVIVE approach are illustrated in Figure 1.

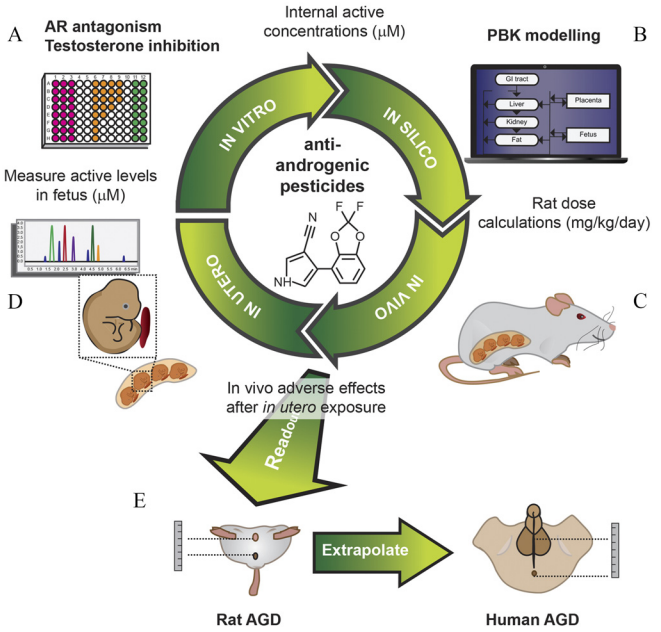

Figure 1. Schematic representation of the strategy used to evaluate the anti-androgenic potential of selected pesticides. (A) *In vitro* assays screen for mechanistic end points such as androgen receptor (AR) antagonism or inhibition of testosterone synthesis. (B) Active *in vitro* concentration ranges determine the target fetal levels for which the corresponding intake doses are simulated by PBK models (reverse dosimetry). These are built on *in vivo* data from existing studies. (C) Rats are exposed prenatally at nontoxic doses that are expected to produce shortened anogenital distance (AGD) in male offspring (*in vivo* study). (D) Internal exposure levels are measured in the fetus and dam and compared with simulations outcomes of B. (E) Exposed offspring are analyzed morphometrically for AGD. Note: PBK, physiologically based pharmacokinetics.

Materials and Methods

Chemicals

The following chemicals were used, all from Sigma-Aldrich unless otherwise stated: procymidone [Chemical Abstracts Service Registry Number (CASRN): 32809-16-8, 99.9% purity], vinclozolin (CASRN: 50471-44-8, 99.6% purity from Sigma-Aldrich for *in vitro* study and 99.5% purity from BOC Sciences for *in vivo* study), linuron (CASRN: 330-55-2, 99.7% purity from Sigma-Aldrich for *in vitro* study and 99.5% purity from Greyhound Chromatography and Allied Chem for *in vivo* study), fludioxonil (CASRN: 131341-86-1, 99.9% purity), cyprodinil (CASRN: 121552-61-2, 98% purity), dimethomorph (CASRN: 110488-70-5, >95% purity), imazalil (CASRN: 35554-44-0, >99% purity), quinoxyphen (CASRN: 124495-18-7, >99% purity), fenhexamid (CASRN: 126833-17-8, >99% purity), *o*-phenylphenol (CASRN: 90-43-7, 99% purity), λ-cyhalothrin (CASRN: 91465-08-6, >99% purity), and pyrimethanil (CASRN: 53112-28-0, >99% purity). Corn oil (C8267-2.5L9) and dimethyl sulfoxide (DMSO) were used as the vehicle for the *in vivo* and *in vitro* studies, respectively, and were purchased from Sigma-Aldrich. The respective positive controls for effects on steroidogenesis, prochloraz (CASRN: 67747-09-5, 98.5% purity), and forskolin (CASRN: 66575-29-9, 98% purity), were purchased from Dr. Ehrenstofer GmbH and Sigma-Aldrich, respectively.

Selection of Test Compounds

To assess the general applicability of the QIVIVE approach we selected three pesticides, vinclozolin, procymidone, and linuron on the basis of their well-known *in vitro* (Orton et al. 2011) and *in vivo* (Gray et al. 2001; Hass et al. 2007) anti-androgenic effects. In addition, QIVIVE simulations were conducted on nine pesticides currently authorized for use on the European market. We selected these substances according to the following criteria: *a*) high relevance in terms of expected exposure to humans (Orton et al. 2011); *b*) AR antagonist properties *in vitro* (Orton et al. 2011); and *c*) lack of data from *in vivo* rodent studies on male reproductive health. The selected pesticides were fludioxonil, cyprodinil, dimethomorph, imazalil, fenhexamid, λ-cyhalothrin, quinoxyfen, pyrimethanil, and *o*-phenylphenol. Of these, three pesticides (fludioxonil, cyprodinil, and dimethomorph) were chosen for follow-up *in vivo* studies on the basis of the outcome of our QIVIVE.

In Vitro *Profiling*

Vinclozolin, procymidone, linuron, and their metabolites were tested for AR antagonistic effects in the AR-EcoScreen assay, an androgen receptor stably transfected transcriptional activation assay described in OECD TG 458 (OECD 2016c). Experiments were run with three technical replicates and repeated three times. The mean value of technical replicates represents one biological replicate. AR-EcoScreen™ cells (JCRB1328; Japanese Collection of Research Bioresources) were grown in CellBIND® surface cell culture flasks (Corning Inc.) in growth medium consisting of Gibco® Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F-12) Nutrient Mixture with L-glutamine and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and without phenol red supplemented with 5% fetal bovine serum (FBS), 1% penicillin-streptomycin, 200 μg/mL Zeocin™ selection reagent, and 100 μg/mL Hygromycin B (all reagents from Invitrogen, Life Technologies). The cells were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide (CO_2) and 95% air. On the day before the experiment, cells were plated in white 96-well plates (Corning Inc.) at a density of 9,000 cells/well in assay medium consisting of Gibco® DMEM/F-12 Nutrient Mixture medium, 1% penicillin-streptomycin, with 5% dextran-coated charcoal-treated FBS (all reagents from Invitrogen, Life Technologies). On the following day, the medium was changed to assay medium containing the known AR agonist R1881 (Perkin Elmer) in 2-fold serial dilutions from 0.008 to 1 nM or the known AR antagonist hydroxyflutamide (CASRN 52806-53-8, Toronto Research Chemicals) in 3-fold serial dilutions from 4,000 to 9,000 nM together with 0.1 nM R1881. The test compounds were added together with 0.1 nM R1881 to the cells in 2-fold serial dilutions ranging from 0.01 to 3.2 μM for vinclozolin, 0.003 to 0.8 μM for procymidone, and 0.3 to 80 μM for linuron. The DMSO vehicle concentrations were kept constant in all wells (0.1%). Following approximately 20 h of exposure to test compounds *firefly* luminescence was measured in a luminometer (LUMIstar® Galaxy, BMG LABTECH) using Dual-Glo® Luciferase Reagent from the Dual-Glo® Luciferase Assay System from Promega. Cell viability was measured by *Renilla* luminescence using Dual-Glo® Stop & Glo® Reagent from the Dual-Glo® Luciferase Assay System.

To capture effects on steroid hormone synthesis (testosterone and androstendione), we tested all 12 pesticides in the H295R assay with the human adrenocortical carcinoma cell line NCI-H295R (American Type Culture Collection no. CRL-2,128, LGC Standards), as previously described (Hecker et al. 2011; Rosenmai et al. 2013). In brief, cells were cultured in DMEM/F-12+glutamine medium with HEPES buffer (Invitrogen) containing 1% insulin-transferrin-sodium selenite plus Premix (VWR) and 2.5% Nu-Serum (BD Bioscience) at 37°C in a 5% CO_2 atmosphere. The day before exposure, cells were seeded 300,000 per well in clear 24-well plates (VWR Corning). Cells were exposed for 48 h to control (Forskolin: 1 μM, 10 μM (Sigma-Aldrich); and Prochloraz: 0.3 μM, 3 μM (VWR-Bie and Berntsen) or test compounds. The vehicle (DMSO) concentration was kept constant in all wells (0.1%). After 48 h, the exposure culture medium was removed and stored at –80°C until the levels of 10 steroid hormones (testosterone, androstenedione, aldosterone, corticosterone, cortisol, dehydroepiandrosterone, 17α-hydroxyprogesterone, progesterone, estrone, and 17β-estradiol) were quantified using the high-performance liquid chromatography–tandem mass spectrometry method described elsewhere (Mortensen and Pedersen 2007). Minor modifications were made to accommodate a smaller sample size and to include more hormones. The liquid chromatography system (Agilent 1100) was equipped with an Atlantis C18 column (2.1 × 150 mm, 3 μm) (Waters Corp.) maintained at 40°C. The sample injection volume was 50 μL. 17β-estradiol and estrone were measured in electrospray ionization (ESI) mode using 65% methanol (MeOH) and 0.01% ammonia for the mobile phase (0.15 mL/min, isocratic flow rate). The remaining steroid hormones were measured in ESI+mode using 65% MeOH and 0.1% acetic acid for the mobile phase (0.2 mL/min, isocratic flow rate). The mass spectrometer was a Quattro Ultima triple quadrupole instrument (Waters Corp.). Calibration standards were run before and after sample analysis at levels of 0.25, 1.25, 2.5, 5.0, and 10.0 ng/mL. The absolute recoveries of the hormones in the cell extracts were estimated to be 70–87%, based on the absolute recoveries of the three internal standards in 90 experiments. Thus, the sensitivity of the method was comparable to the analysis in blood (Mortensen and Pedersen 2007). Each pesticide was tested in 2-fold serial dilutions at concentrations between 0.8 and 50 μM in three independent experiments, and hormone levels were normalized to the solvent controls containing 0.1% DMSO. Cell viability was evaluated by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma) as previously described (Hecker et al. 2011; Rosenmai et al. 2013). Fluorescence was measured (excitation 560 nm, emission 590 nm) using a Wallac Victor2 1420 multilabel counter (PerkinElmer).

PBK Modeling

A generic PBK model was developed with the aim of simulating age-dependent physiological and biochemical changes in rodents associated with ongoing pregnancy after repeated daily oral dosing (gavage) of environmental compounds. The focus of this flow-limited model was on the simulation of internal pesticide concentrations in the blood plasma of the fetus at gestational day (GD) 15 to GD18, a developmental stage considered as most critical for male sexual differentiation in rats (Welsh et al. 2008). The model structure was adopted from (O'Flaherty et al. 1992) with several physiological modifications suggested by (Emond et al. 2006) and included only maternal tissues and kinetic processes that were considered as directly relevant for the estimation of fetal exposure levels (Figure 2): liver and kidney as the major sites of elimination and metabolism, fat tissue to account for potential lipophilicity, blood/plasma for the description of the systemic circulation, and two remaining compartments that include all other well- or poorly perfused organs and tissues lumped together for calculation of the mass balance. Given that all model compounds were nonvolatile, the concentration of chemicals in venous blood was assumed to be equal to its concentration in arterial blood; therefore, a lung compartment was not included. The active uptake into the gastrointestinal (GI) tract and the absorption from the GI tract into the liver were described by first-order kinetics, assuming a 100% oral absorption of the intake dose. The transfer inclusion into the internal blood flow was controlled by a first-order fecal excretion rate. Biliary excretion of the parental compound into the duodenum was modeled as a simple clearance rate from the liver to the intestine (enterohepatic recirculation) with no time delay. The latter was considered as a viable model option a *a*) some pesticides are conjugated with a molecular weight above 325, which is often considered to be the lower bound for the molecular weight for enterohepatic circulation in the rat (LeBlanc 2004); and *b*) to allow more model flexibility. This set of maternal compartments was considered as sufficient to estimate and calibrate kinetic elimination model parameters from *in vivo* kinetic data. The model structure was extended by two placental units (yolk sac and chorioallantoic placenta) and the fetal compartment. For model simplicity, the whole fetus was implemented as a single diffusion-limited compartment with no further division into individual tissues or compartments, where the bidirectional transfer from the placenta units to the embryo/fetus and vice versa was described by first-order diffusion rates (activated on GD6). Elimination

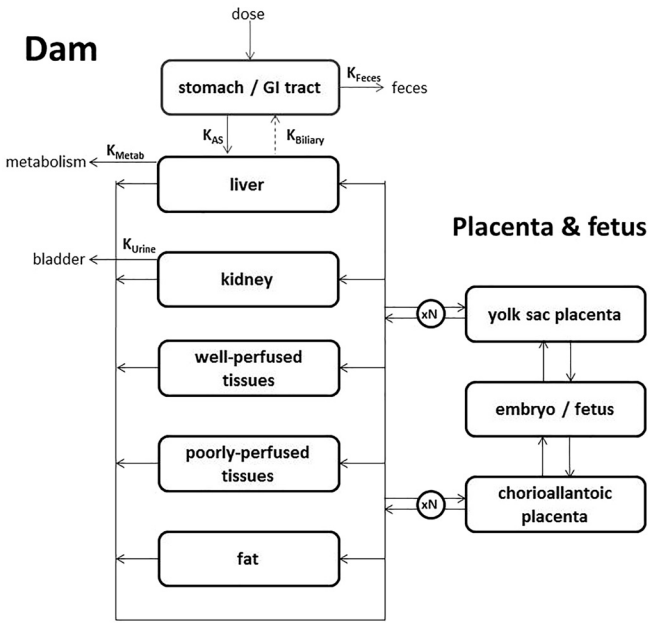

Figure 2. Structure of the PBK model for gestational exposure in rats. This PBK model was adopted from O'Flaherty et al. (1992) with several physiological modifications suggested by Emond et al. (2006). Note: GI, gastrointestinal; K, first-order rate constants; K_{AS}, the portal GI absorption rate into liver; $K_{Biliary}$, the biliary excretion rate; K_{Feces}, the fecal excretion rate from gut; K_{Metab}, the metabolism rate; K_{Urine}, the urine excretion rate; PBK, physiologically based pharmacokinetics; xN, number of concepti.

processes in the fetus were not considered, and transfers between fetal levels and amniotic fluid were not included in the model structure. The fetal plasma concentrations were approximated as the average concentrations over the time interval between GD15 and GD18 and estimated as the area under plasma concentration–time curve (AUC) divided by 72 h (AUCfetus). Similarly, a maternal concentration at GD21 was defined as the average level in the circulating blood system over the time between the last feeding and birth and calculated as AUC divided by 24 h.

An evaluation of the developed PBK model according to the World Health Organization/International Programme (WHO/IPCS) on Chemical Safety guidance on PBK models to be used in risk assessment (WHO/IPCS 2010) is reported in Table S1. All kinetic equations for the rat PBK gestational model are listed below (with the notation of model parameters referring to Table 1 and Table S2).

Maternal Blood Compartment

$$C_{Plasma}(mg/L) = (Q_{Fat} \times CV_{Fat} + Q_{WellP} \times CV_{WellP} + Q_{PoorP} \times CV_{PoorP} + Q_{Kidney} \times CV_{Kidney} + Q_{Liver} \times CV_{Liver} + N_{Concepti} \times (Q_{YPla} \times CV_{YPla} + Q_{CPla} \times CV_{CPla}))/Q_cardiac_p, \quad (1)$$

with Q_X referring to the blood flow rates of compartment X, Q_Cardiac_p to the Cardiac output, and $N_{Concepti}$ to the litter size.

Tissue Compartment (Fat, Well-perfused, Poorly Perfused Tissues)

$$\frac{dA_X}{dt}(mg/h) = R_{plasma} \times Q_X \times (C_{Plasma} - CV_X), \quad (2)$$

$$C_X(mg/L) = A_X/V_X \text{ [concentration in tissue compartment]}, \quad (3)$$

$$CV_X(mg/L) = C_X/Kp_X \text{ [concentration leaving compartment]}, \quad (4)$$

with subscript notations X for Fat, WellP (well-perfused tissue compartment), and PoorP (poorly-perfused tissue compartment).

GI Absorption and Distribution to the Portal System

$$\frac{dA_{Lumen}}{dt}(mg/h) = -\frac{dA_{Fecus}}{dt} - \frac{dA_{Portal}}{dt} + \frac{dA_{Biliary}}{dt} + INTAKE, \quad (5)$$

$$\frac{dA_{Fecus}}{dt}(mg/h) = K_{feces} \times A_{Lumen} \text{ [fecal elimination]}, \quad (6)$$

$$\frac{dA_{Portal}}{dt}(mg/h) = K_{AS} \times A_{Lumen} \text{ [adsorption to the liver]}, \quad (7)$$

$$\frac{dA_{Biliary}}{dt}(mg/h) = K_{Biliary} \times CV_{liver} \times V_{liver} \text{ [enterohepatic circulation]}, \quad (8)$$

with A_{Lumen}, the amount of compound remaining in the gut tract; A_{Fecus}, the amount of compound eliminated in the feces; A_{Portal}, the amount of compound distributed to the liver; $A_{Biliary}$, the amount of compound circulated back to intestine; and INTAKE the rate of compound intake after oral bolus (in milligrams per hour).

Liver Compartment

$$\frac{dA_{liver}}{dt}(mg/h) = R_{PLASMA} \times Q_{Liver} \times (C_{Plasma} - CV_{Liver}) + \frac{dA_{portal}}{dt} - \frac{dA_{Metabolism}}{dt} - \frac{dA_{biliary}}{dt}, \quad (9)$$

$$C_{liver}(mg/L) = A_{liver}/V_{liver} \text{ [concentration in liver]}, \quad (10)$$

$$CV_{liver}(mg/L) = C_{liver}/Kp_{liver} \text{ [concentration leaving liver into blood circulation]}, \quad (11)$$

$$\frac{dA_{metab}}{dt}(mg/h) = K_{Metab} \times CV_{Liver}, \quad (12)$$

with A_{metab} the amount of compound metabolized in the liver.

Kidney Compartment

$$\frac{dA_{kidney}}{dt}(mg/h) = R_{PLASMA} \times Q_{kidney} \times (C_{Plasma} - CV_{kidney}) - \frac{dA_{urine}}{dt}, \quad (13)$$

$$C_{kidney}(mg/L) = A_{kidney}/V_{kidney}\ [\text{Concentration in kidney}], \quad (14)$$

$$CV_{kidney}(mg/L) = C_{kidney}/Kp_{kidney}\ [\text{Concentration leaving kidney}], \quad (15)$$

$$\frac{dA_{urine}}{dt}(mg/h) = K_{urine} \times CV_{kidney}, \quad (16)$$

with A_{urine} the amount of compound excreted into urine.

Yolk-Sac Placenta and Transfer to Fetus

$$\frac{dA_{YPla}}{dt}(mg/h) = R_{PLASMA} \times Q_{YPla} \times (C_{Plasma} - CV_{YPla}) - \frac{dA_{Yolk_to_fetus}}{dt} + \frac{dA_{Fetus_to_Yolk}}{dt}, \quad (17)$$

$$C_{YPla}(mg/L) = A_{YPla}/V_{YPla}\ [\text{Concentration in yolk-sac placenta}], \quad (18)$$

$$CV_{YPla}(mg/L) = C_{YPla}/Kp_{YPla}\ [\text{Concentration leaving yolk-sac placenta}], \quad (19)$$

$$\frac{dA_{Yolk_to_fetus}}{dt}(mg/h) = k_{placenta_to_fetus} \times CV_{YPla}, \quad (20)$$

with $A_{Yolk_to_Fetus}$ the amount of compound transferred from placenta into fetus.

Chorioallontoic Placenta and Transfer to Fetus

$$\frac{dA_{CPla}}{dt}(mg/h) = R_{PLASMA} \times Q_{CPla} \times (C_{Plasma} - CV_{CPla}) - \frac{dA_{Chorio_to_fetus}}{dt} + \frac{dA_{Fetus_to_Chorio}}{dt}, \quad (21)$$

$$C_{CPla}(mg/L) = A_{CPla}/V_{CPla}\ [\text{Concentration in chorioallontoic placenta}], \quad (22)$$

$$CV_{CPla}(mg/L) = C_{CPla}/Kp_{CPla}\ [\text{Concentration leaving chorioallontoic placenta}], \quad (23)$$

$$\frac{dA_{Chorio_to_fetus}}{dt}(mg/h) = k_{placenta_to_fetus} \times CV_{CPla}, \quad (24)$$

with $A_{Chorio_to_Fetus}$ the amount of compound transferred from placenta to fetus.

Fetus and Transfer to Yolk-Sac Placenta

$$\frac{dA_{YFetus}}{dt}(mg/h) = \frac{dA_{Yolk_to_fetus}}{dt} - \frac{dA_{Fetus_to_Yolk}}{dt}, \quad (25)$$

$$C_{YFetus}(mg/kg) = A_{YFetus}/BW_{Fetus}\ [\text{Fetal concentration from yolk-sac placenta}], \quad (26)$$

where BW_{Fetus} is the body weight of the fetus.

$$CV_{YFetus}(mg/kg) = C_{YFetus}/Kp_{Fetus}, \quad (27)$$

$$\frac{dA_{Fetus_to_Yolk}}{dt}(mg/h) = k_{Fetus_to_Placenta} \times CV_{YFetus}, \quad (28)$$

with $A_{Fetus_to_Yol}$ the amount of compound transferred from fetus to placenta compartment.

Fetus and Transfer to Chorioallontoic Placenta

$$\frac{dA_{CFetus}}{dt}(mg/h) = \frac{dA_{Chorio_to_fetus}}{dt} - \frac{dA_{Fetus_to_Chorio}}{dt}, \quad (29)$$

$$C_{CFetus}(mg/kg) = A_{CFetus}/BW_{Fetus}\ [\text{Fetal concentration from chorioallontoic placenta}], \quad (30)$$

$$CV_{CFetus}(mg/kg) = C_{CFetus}/Kp_{Fetus}, \quad (31)$$

$$\frac{dA_{Fetus_to_Chorio}}{dt}(mg/h) = k_{Fetus_to_Placenta} \times CV_{CFetus}, \quad (32)$$

with $A_{Fetus_to_Chorio}$ the amount of compound transferred from fetus to placenta compartment.

$$C_{Fetus}(mg/kg) = C_{YFetus} + C_{CFetus}\ [\text{total concentration in fetus}], \quad (33)$$

For all simulations, Kp_{Fetus} was set to 1, that is, $C_{YFetus} = CV_{YFetus}$ and $C_{CFetus} = CV_{CFetus}$. The PBK model implementation and simulations were carried out with Berkeley Madonna Windows (version 8.3.23) using the Rosenbrock Algorithm for stiff systems. The Berkeley Madonna code is given in "Method S2" in Supplemental Material.

Physiological Parameters

Anatomical and physiological parameters were retrieved from reference-values for laboratory animals and literature (summarized in Table S2). Growth-related body weight (BW) changes in nonpregnant dams were estimated from our data and expressed as function of the GD:

$$BW_{non\text{-}pregnant}(GD)\ [kg] = BW_{GD0} + 0.002429 \times GD, \quad (34)$$

with the body weight at GD0 (BW_{GD0}) set to 0.197 kg. Similarly, we estimated the average BW of the fetus as

$$BW_{fetus}(GD)\ [g] = 5.092 \times 10^{-6} \times EXP\,(0.6413 \times GD). \quad (35)$$

Temporal changes in the blood volume, tissue volume, and blood flow rates of tissues with ongoing pregnancy were chosen according to O'Flaherty et al. (1992) and You et al. (1999), the total BW of the pregnant dam was calculated based on the BW of nonpregnant dams (Equation 34) and corrected by the dynamic changes in the maternal organs due to pregnancy and the BW of the concepti (fetus and placenta) (Table S2). The number of concepti (xN) was set to 10.

Kinetic Parameters

The pesticide-specific kinetic parameters (log K_{ow}, plasma binding) were determined from the literature or *in silico* methods, and tissue-partitioning of pesticides was estimated according to (Poulin and Theil 2002) (Table 1). For the slowly perfused tissue, we used the tissue:plasma partition coefficient (Kp) estimated for muscle, and for the rapidly perfused tissues the Kp estimated for the heart. The Kp for the fetus was set to 1. Absorption and elimination model parameters were estimated directly from kinetic *in vivo* data as reported in EFSA DARs (EFSA 2019) or our own

Table 1. Physicochemical and kinetic model parameters.

Compound (including metabolites)	MW	log K_{ow}[a]	Unbound plasma fraction (%)	Portal GI absorption rate into liver (L/h)	Biliary excretion rate (L/h)	Fecal excretion rate from gut (L/h)	Urine excretion rate from kidney (L/h)	Metabolism rate in liver		Tissue: plasma partition coefficients[b,c]				
								Total[d] (L/h)	Metabolite[e] (L/h)					
				K_{AS}	$K_{Biliary}$	K_{Feces}	K_{Urine}	K_{Metab}		adipose	kidney	liver	Rapidly (heart)	Slowly (muscle)
Procymidone		3.08	3.0[f]	0.3	—	0.04	0.08	0.3	—	2.7	4.4	4.8	3.7	2.7
Vinclozolin	286.11	3.10	59[g]	0.5	—	—	0.05	1.4	—	54.6	6.9	7.5	5.8	4.3
M1[k]	304.13	3.52	22[i]	—	—	—	0.2	—[j]	2.8[h]	10	8.3	9.1	7.0	5.1
M2[l]	260.11	2.95	30[i]	—	—	—	0.2	—[j]	0.9[h]	3.4	6.2	6.7	5.2	3.8
Linuron	249.09	3.20	10.5[g]	0.5	—	0.08	0.05	1.0	—	11.9	5.10	5.50	4.30	3.10
DPMU[m]	235.1	2.70	28[i]	—	—	—	0.05	0.02	0.22	3.8	4.90	5.30	4.20	3.10
DPU[n]	205.04	2.00	52[i]	—	—	—	0.05	—[j]	0.25	0.7	2.00	2.10	1.80	1.40
Fludioxonil	248.19	4.12	0.5[g]	1	—	3	0.01	0.3	—	1.9	5.9	6.4	4.9	3.6
Cyprodinil	225.29	4.00	0.5[g]	1	1.6	1	0.2	0.01	—	1.7	5.8	6.4	4.9	3.5
Dimethomorph	387.87	2.68	9.4[g]	1	—	2	0.01	0.3	—	3.4	3.3	3.6	2.8	2.1
Imazalil	297.18	3.82	4.8[g]	1	—	0.5	0.53	—[j]	—	13.5	5.9	6.5	5.0	3.6
Quinoxyfen	308.14	4.66	5.0[g]	1	1.4	2	0.01	0.3	—	23.0	6.3	6.9	5.3	3.8
Fenhexamide	302.2	3.51	5.7[g]	1	—	3	0.05	0.15	—	7.6	5.5	6.1	4.7	3.4
o-Phenylphenol	170.21	3.09	4.1[g]	1	—	0.1	0.01	0.2	—	3.7	4.5	4.9	3.8	2.8
λ-Cyhalothrin	449.85	6.80	1.0[i]	1	—	4	0.05	0.3	—	24.8	6.4	7.0	5.4	3.9
Pyrimethanil	199.25	2.84	2.0[g]	1	—	0.1	0.01	0.8	—	1.1	3.6	3.9	3.1	2.3

Note: GI, gastrointestinal; K_{AS}, first-order rate constant of the portal gastrointestinal absorption rate into liver; $K_{Biliary}$, first-order rate constant of the biliary excretion rate; K_{Feces}, first-order rate constant of the fecal excretion rate from gut; K_{ow}, partition coefficient; K_{Metab}, first-order rate constant of the metabolism rate; K_{Urine}, first-order rate constant of the urine excretion rate; MW, molecular weight.
[a]Collected from EPI Suite (U.S. EPA 2012), when available, experimental data were preferred over modeled data.
[b]Estimated according to Poulin and Theil (2002).
[c]Tissue: plasma partition coefficient for fetus was set to 1.
[d]Metabolic clearance.
[e]Conversion of parent compound into active metabolite.
[f]WHO/FAO (2007).
[g]Wetmore et al. (2012).
[h]Sierra-Santoyo et al. (2008).
[i]Predicted by pkCSM (Pires et al. 2015).
[j]Separation into urinary and metabolic elimination was not possible.
[k]2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid.
[l]3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide.
[m]1-(3,4-Dichlorophenyl)-3-methoxyurea.
[n]1-(3,4-Dichlorophenyl)urea.

studies (procymidone, vinclozolin, linuron). Data in EFSA DARs are typically reported as residues of the parent compound (and its metabolites), which were measured in adult non-pregnant animals (blood plasma, organs, urine, bile, residual carcass) at several time points following a single oral dose (usually within the first 3 d) and were used to estimate kinetic parameters such as the daily excretion rate, the distribution of the compound as percentage total administered dose per time point of measurement etc. We fitted the PBK model to the reported ADME data in a two-step approach (with preference to data from low doses): at first, we estimated the first-order rates for fecal, urine and metabolic elimination directly from the ADME data without consideration of the PBK model structure (Jónsdóttir et al. 2016); conducted in SAS/STAT® software (version 9.4; SAS Inc.). These estimates guided as input values for the final PBK model calibration, with the full PBK model referring to GD2, that is, placental and fetal compartments are nonactivated at that time. Here we used the entire set of ADME data including exposure measurements in the blood and organs, and the final calibration for the first-order rates (K_{AS}, $K_{Biliary}$, K_{Feces}, K_{Urine}, K_{Metab}) was achieved by visual optimization. The focus was set on describing the reported maternal blood levels as close as possible. Using this approach various sets of parameter values can give equally good fits to the reported data in a way that individual parameters cannot be uniquely identified. To minimize this scenario we fixed the portal GI absorption rate into the liver to 1 L/h unless data evidence or a bad fit suggested a different value. In cases where ADME data from more than one *in vivo* study were reported, the kinetic parameters were estimated for each data set separately, and the final set of model parameters was decided on a case-by-case basis, with preference given to data-rich studies and study designs most similar to that of our animal studies. The simplifying model assumption of this fitting approach is that the elimination rates, which are derived from nonpregnant female rats, can be used as approximation for the kinetic processes during gestation. Furthermore, all kinetic parameters were fixed during the entire gestational period. The placental Kps were assumed to be identical to the poorly perfused Kp.

The blood-to-serum partition ratio (R_{Plasma}) was held fixed to 0.55, which is slightly above the smallest possible value of 0.44 (i.e., 1 – hematocrit) which would be equivalent to the assumption that the compound does not partition into red blood cells. We speculate that the true ratios are well below 1, given that most pesticides in this study are assumed to be highly protein-bound with no high affinity for the red blood cells (erythrocytes). Neither *in vivo* ADME data nor *in silico* tools were available to estimate the bidirectional transplacental diffusion rates between the placenta and fetus. As a pragmatic solution, we considered any value from a worst-case space of 0.005–2 L/h as equally possible. We did not rule out potential differences in the rates between the transplacental transfer from the placenta to the fetus and from the fetus to the placenta, because *a*) these differences have been reported in gestational PBK model although with other compounds (e.g., You et al. 1999); *b*) no elimination processes in

the fetus are implemented in the model structure; and *c*) an imbalance between these two parameters was identified as an important source of variability for the simulated fetal concentrations (File S1: Sensitivity analysis for PBK model). We assumed a maximal difference of $\pm 20\%$ between both clearance rates as equally viable for the simulations. These values were extracted from rodent PBK models that were published with a similar model structure (oral exposure route, transplacental exposure transfer, no elimination process in the fetus) but on different chemicals [e.g., (You et al. 1999) reported rates of 1.6 and 1.9 L/d for *p,p′*-dichlorodiphenyldichloroetheyene (DDE)].

The setup for the PBK model was

$$K_{Placenta_to_Fetus}: 0.005\text{–}2\ L/h,\ K_{Fetus_to_Placenta}: 0.005\text{–}2\ L/h, \tag{36}$$

with a maximally $\pm 20\%$ imbalance between both rates. For a balanced transfer rate of 2 L/h, we simulated two scenarios of maximal imbalance, with *a*) $K_{Placenta_to_Fetus} = 2.4$ L/h and $K_{Fetus_to_Placenta} = 1.6$ L/h assuming a higher transfer rate to the fetus; and *b*) $K_{Placenta_to_Fetus} = 1.6$ L/h and $K_{Fetus_to_Placenta} = 2.4$ L/h assuming a higher transfer rate back to the placenta. A similar setup was used for 0.005 L/h, that is, four simulations were conducted at a given intake dose to establish a range of fetal (and maternal) exposure levels of identical likelihood.

Sensitivity Analysis

A local parameter sensitivity analysis was performed to identify kinetic model parameters that primarily influenced the average fetal concentrations of the parental compound. Each model parameter was increased by 5% in turn, keeping the others constant (Chiu et al. 2007). Normalized sensitivity coefficients were calculated as

$$NCS = \frac{R^C - R}{P^C - P} \times \frac{P}{R}, \tag{37}$$

where R is the initial value of the response variable (AUC_{Fetus}) and R^C the output after a 5% change. P is the initial value of the model parameter of interest (e.g., renal elimination rate) and P^C is the parameter value modified by an increase of 5%. The sensitivity analysis was conducted on model simulations for 50 mg/kg per day linuron (including two main active metabolites), 40 mg/kg per day vinclozolin (including two main active metabolites), and 40 mg/kg per day procymidone, with bidirectional transfer rates between placenta compartments and fetus set to 2 L/h.

QIVIVE

PBK simulations were used to produce an *in vitro–in vivo* profile for the most likely internal exposure concentration in the fetal compartment at a given intake dose and time. We defined a dose window for potential anti-androgenic effects *in vivo* for which the lower and upper limits, respectively, were demarcated by *a*) the dose expected to produce fetal levels equivalent to *in vitro* concentrations associated with at least 20% AR antagonistic activity (i.e., a 20% reduction in AR luciferase activity compared with controls) and/or testosterone inhibition (i.e., reduced testosterone concentration measured in media); and *b*) the dose anticipated to be below a range associated with maternal or prenatal toxicity. This upper dose was derived from data reported in one- or two-generation developmental and reproductive toxicity studies and selected as falling between the highest dose without any observed adverse effects (usually the no observed adverse effect) and the lowest dose at which an adverse effect materialized (the lowest observed adverse effect). All toxicity descriptors that were used to define the anticipated dose range for anti-androgenicity for the nine pesticides can be found in Table S3.

In Vivo *Reproductive/Developmental Toxicity Studies*

In total, four *in vivo* exposure studies were conducted with pesticide exposures to pregnant rats during gestation and lactation to the selected compound (Table 2). First, a selected dose from procymidone and vinclozolin was tested to measure internal body concentrations in the dams and fetuses shortly before birth (GD21) (Study 1). Second, linuron was tested to investigate its internal concentrations, and, because the substance has never been tested before in-house, AGD was assessed in the offspring in order to confirm external outcomes (Ding et al. 2017; McIntyre et al. 2002) (Study 2). Third, fludioxonil, cyprodinil, and dimethomorph were tested to investigate their effects in male offspring and fetuses (Study 3). For one selected dose of each compound, the internal body concentrations in dams and fetuses were determined by cesarean section at GD21. Due to the study outcomes from the third study, we conducted a fourth follow-up study on dimethomorph (Study 4). To demonstrate the applicability of the QIVIVE approach, we have also included the results from previously published *in vivo* studies (Hass et al. 2007) that were carried out in the same laboratory under similar study conditions (Table 2, Studies S1, S3, and S4).

Exposure and Study Design

All conducted *in vivo* studies are listed in Table 2. Study 1, with procymidone and vinclozolin, was performed in Sprague-Dawley rats (NTac:SD strain, SPF; Taconic Europe). For the *in vivo* study with linuron (Study 2), we used Sprague-Dawley rats [CD IGS Rat, Crl:CD(SD); Charles River Laboratories, Sandhofer Weg 7]. Study 3, with fludioxonil, cyprodinil, and dimethomorph, was carried out with time-mated nulliparous, young adult Wistar rats (HanTac:WH, SPF; Taconic Europe) and Study 4, with dimethomorph, on Sprague-Dawley rats [CD IGS Rat, Crl:CD(SD); Charles River Laboratories]. All studies were performed under conditions approved by the Danish Animal Experiments Inspectorate (Council for Animal Experimentation) and protocols were approved by the Technical University of Denmark (DTU) in-house animal welfare committee. The linuron and dimethomorph *in vivo* studies were conducted in the animal facilities at DTU Food, Lyngby, whereas all other animal studies were carried out at the DTU animal facility in Mørkhøj, Denmark. Time-mated dams were delivered on GD3. On GD4, dams were distributed into exposure groups with similar BW distributions. Animals were housed in pairs until GD17 and thereafter individually. The animals were kept under standard conditions in semitransparent polysulfone (PSU) Type III cages (PSU 80-1291HOOSU Type III; Tecniplast) ($15 \times 27 \times 43$ cm) with Aspen wood chip bedding (Tapvei), Enviro Dri nesting material (Brogaarden), and Tapvei Arcade 17 (Aspen wood) shelters (Brogaarden). They were placed in an animal room with controlled environmental conditions: 12-h light: dark cycles with light starting at 2100 hours, temperature $22 \pm 1°C$, humidity $55 \pm 5\%$, and 10 air changes per hour.

Solutions of all test chemicals were prepared in corn oil, and dams were dosed by oral gavage during the morning hours from GD7 to GD21 and again from the day after birth until the pups reached postnatal day (PD) 16 (the day of expected birth was designated PD1). Maternal toxicity has been reported after linuron administration during early gestation (McIntyre et al. 2000); therefore, in Study 2, we exposed dams only from GD13 to GD21 and from PD1 to PD15. Here time-mated rats were divided into four blocks, with each block including a control group and three dose groups (25, 50, or 75 mg/kg per day). Maternal

Table 2. Overview of *in vivo* rat studies.

In vivo study no.	Chemical	Doses (mg/kg BW per day)	Rat strain	Exposure period (dams)	No. of litters/group	AGDI measurement	Internal exposures of dams and fetuses
1	Procymidone	40	SD	GD7–GD21	6 (6 control)	—	GD21
	Vinclozolin	40	SD	GD7–GD21	6 (6 control)	—	GD21
2[a]	Linuron	25, 50, (75[b])	SD	GD13–21[c] and PD1–PD15	9–11 (10 control)	YES	—
	Linuron	25, 50, (75[b])	SD	GD13–21[c]	3 (3 control)	—	GD21
3[a]	Fludioxonil	20, 60, 180	W	GD7–21 and PD1–17	6–10 (8 control)	YES	—
	Cyprodinil	20, 60, 180	W	GD7–21 and PD1–17	6–10 (8 control)	YES	—
	Dimethomorph	6.7, 20, 60	W	GD7–21 and PD1–17	6–10 (8 control)	YES	—
	Fludioxonil	60	W	GD7–21	2 (1 control)	—	GD21
	Cyprodinil	60	W	GD7–21	2 (1 control)	—	GD21
	Dimethomorph	20	W	GD7–21	2 (1 control)	—	GD21
4[a]	Dimethomorph	6.7, 20, 60, 180	SD	GD7–21 and PD1–16	10–11 (11 control)	YES	—
Previous studies (Hass et al. 2007, with study notation from original article):							
S1	Vinclozolin	5, 10, 20, 40, 80, 160	W	GD7–21 and PD1–16	8 (16 control)	YES	—
S3	Procymidone	5, 10, 25, 50, 100, 150	W	GD7–21 and PD1–16	8 (16 control)	YES	—
S4	Vinclozolin	24.5, 95.9	W	GD7–21 and PD1–16	8 (16 control)	YES	—
	Procymidone	14.1, 61.8					

Note: AGD, anogenital distance; BW, body weight; GD, gestational day; OECD TG, Economic Co-operation and Development test guideline; PD, postnatal day; SD, Sprague-Dawley rats; W, Wistar rats.
[a]These studies were modified OECD TG 421 studies (Reproduction/Developmental Toxicity Screening Test) (OECD 2016a).
[b]Maternal toxicity was observed at the highest dose and thus only results from the 25- and 50-mg/kg BW per day doses are included for postnatal end points in this article.
[c]A different exposure period was used in order to reduce maternal toxicity.

toxicity was observed at the highest dose and, for this reason, results about the postnatal end points are only reported for the 25 and 50 mg/kg per day dose groups ($n = 10$–11). For Study 3, 102 time-mated dams were divided into three blocks, with an even allocation to the dose groups and even BW distribution. Dams were dosed by gavage with vehicle control (corn oil) or one of the following doses: fludioxonil 20, 60, and 180 mg/kg per day; cyprodinil 20, 60, and 180 mg/kg per day; and dimethomorph 6.7, 20, and 60 mg/kg per day ($n = 6$–10). In the follow-up study on dimethomorph (Study 4), 60 time-mated dams were dosed by gavage with vehicle control (corn oil) or one of the following doses: 6.7, 20, 60, and 180 mg/kg per day. In all four studies, animals were always exposed during the sensitive fetal masculinization window GD15–GD19.

Pup weights and AGD were measured after birth at PD1 using a stereomicroscope with a micrometer eyepiece and were determined as the distance between the genital papilla and the anus. To discriminate between an anti-androgenic effect and stunted growth, the AGD index (AGDI) was calculated by dividing AGD by the cube root of the BW (as described by Hass et al. 2007). The estimation of AGDI effect doses by regression modeling required a standardized AGDI scale. Therefore, we normalized the AGDI values in relation to the dynamic range spanned by the mean AGDI of the male and the female controls as

$$\text{normalized AGDI} = \frac{\text{mean(AGDI of male controls)} - \text{AGDI(exposed animal)}}{\text{mean(AGDI of male controls)} - \text{mean(AGDI of female controls)}}. \quad (38)$$

Accordingly, a normalized valued of one is equivalent to the mean AGDI of the male controls (signaling no effect). A value of zero on the normalized scale corresponds to the mean AGDI of the female controls (maximal effect, feminization of male). The AGDI of male controls is normally around twice the AGDI of female controls. All percentage changes in AGDI reported in this article refer to this normalized scale; for example, an ED10 stands for the effective dose that caused a 10% AGDI reduction in male pups, equivalent to a value of 0.9 on the normalized AGDI scale (Equation 38). It should be noted that the term reduced AGD always implies that a measure of body size was considered in the assessment (OECD 2016a), and unless stated otherwise, both AGD and AGDI are used synonymously in this article. On PD13–PD14, male and female pups were weighed and examined for the number of areolas/nipples, described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. All measurements were performed blinded with respect to treatment group by the same skilled technician.

Fetal Chemical Exposure Analysis

Cesarean sections were performed at GD21 on additional dams from the following dose groups: *a*) procymidone and vinclozolin (40 mg/kg BW per day both groups, $n = 6$ litters, Study 1); *b*) linuron (25, 50, and 75 mg/kg per day, $n = 3$ litters, Study 2), fludioxonil (60 mg/kg per day, $n = 2$ litters, Study 3); *c*) cyprodinil (60 mg/kg per day, $n = 2$ litters, Study 3); and *d*) dimethomorph (20 mg/kg per day, $n = 2$ litters, Study 3). Dams were decapitated under CO_2/oxygen anesthesia at GD21 and fetuses collected by cesarean section. Prior to decapitation of the fetuses, their BWs and AGDs were recorded. Blood from the dams was collected in heparinized vacutainer tubes, centrifuged at $125 \times g$ for 10 min at 4°C to prepare plasma, and, together with the amniotic fluid, frozen directly. Pooled trunk blood from all female and male fetuses of the same litter (female and male blood separately) was collected into heparin-treated capillary tubes approximately 90 min after dosing and the plasma was prepared and stored at −80°C until analysis for pesticides and selected metabolites by liquid chromatography quadrupole time-of-flight mass spectroscopy (LC-QTOF). Details about the LC-QTOF analyses are provided in "Method S1" in Supplemental Material. Amniotic fluid was

collected from fetuses by pipetting, pooled within each litter, snap frozen in liquid nitrogen, and subsequently stored at −80°C.

Statistics

For the analyses of *in vivo* data, the litter was the statistical unit. When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor. Continuous end points were analyzed by analysis of variance methods (mixed effect modeling), and statistical significance of dose-related effect differences to the controls was assessed using multiple contrast tests, along with the BW as a covariate for AGD and the number of offspring per litter as a covariate for birth weights. The number of nipple/areolas (NR) was assumed to follow a binomial distribution with a response range between 0 and 12, with the latter assumed to reflect the biologically possible maximal number of nipples in rats. Litter effects on NR and overdispersion in the data were accounted for by using generalized estimating equations, as reported by Christiansen et al. (2012). Statistical significance was judged for p-values below the false positive rate $\alpha = 5\%$. Dose–response regression analysis on AGDI and all *in vitro* end points was performed by a best-fit approach (Scholze et al. 2001). All statistical analyses were conducted in SAS (SAS Enterprise Guide 4.3) and GraphPad Prism (version 8; GraphPad Software).

Results

Applicability of the QIVIVE Approach to Three Pesticides with Known Anti-Androgenic Properties

As compounds with a well-documented ability to induce shortened AGD in male rodent offspring, the pesticides procymidone, vinclozolin, and linuron were selected to investigate the general applicability of our QIVIVE approach. First, we tested the three pesticides *in vitro* for AR antagonism in a reporter gene assay and for suppression of androgen synthesis in the H295R steroidogenesis assay. Vinclozolin antagonized the AR but had negligible activity in terms of suppressing steroid synthesis *in vitro*. In contrast, procymidone and linuron both antagonized the AR and inhibited the synthesis of androgens such as testosterone and androstenedione (Figure 3A; Table 3). In both cases, AR antagonism became apparent at approximately one-tenth the concentrations when compared with steroid synthesis inhibition.

Next, we quantified the levels of the parental compounds and selected *in vitro* active metabolites in the blood of fetuses resulting from dosing rat dams daily during GD7–GD21 with 40 mg/kg per day procymidone, 40 mg/kg per day vinclozolin, or three doses of linuron (25, 50, and 75 mg/kg per day), all by gavage. The samples were taken on GD21. At this time point, we also determined the concentrations of all compounds (and their metabolites) in the blood of the dams and amniotic fluid (Table 3; Figure 4). These measurements were used to *a*) inform the PBK model in terms of the metabolites to be included in the simulations; and *b*) to estimate kinetic model parameters (together with literature data or *in silico* methods; Table 1). In the animals exposed to vinclozolin, only the metabolites 2-[[(3,5-dichlorophenyl)-carbamoyl] oxy]-2-methyl-3-butenoic acid (M1) and 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2), but not the parent compound, were detected. The plasma concentration of each measured pesticide and metabolite was similar in male and female fetuses, indicating no gender-related differences (Figure 4).

We determined differences in the AGD between the control and linuron-dosed pups on PND1: male pups from dams dosed at 50 mg/kg per day linuron had significantly smaller AGDs than the control male pups, which corresponded to 0.72 on the normalized AGDI scale (i.e., 28% AGDI reduction) (Figure 3C; Table S7). AGD shortening at higher linuron doses was strongly confounded by maternal toxicity and are not shown here. To provide a complete picture of the QIVIVE, we also show the dose–response data for procymidone and vinclozolin from previously published *in vivo* studies (Hass et al. 2007).

Next, we performed PBK model simulations for a range of doses that included those associated with 10% and 50% reductions on the normalized AGDI scale (ED10 and ED50, respectively; Table S7). This comprised the parent compounds and their active metabolites. The maternal doses, which we used to determine internal tissue concentrations, fell within the modeled range. Figure 3B shows the concentrations in the fetal compartment predicted to result from these doses, together with the range of measured concentrations. Figure 4 complements these data by also showing predicted and observed levels in the blood of dams and amniotic fluid.

Except for the parent compound vinclozolin, the individual measurements were close to the range of the simulated concentrations [<factor 5 (Table 3; Figure 4)]. To a certain degree, the good agreement between simulation and observation was because we had to use the outcome of tissue concentration measurements for the estimation of certain PBK parameters. However, the fetal levels had to be measured at a slightly different time point (GD21) than the simulations (GD15–GD18). Furthermore, the measurements were snapshots of internal exposures for specific time points, whereas the simulations gave values averaged over longer periods.

Local sensitivity analysis was conducted for all kinetic model parameters on AUC_{Fetus} and revealed similar trends in sensitivities across the three parent compounds including their metabolites: Differences between the bidirectional transfer rates between placenta and fetus was by far the most sensitive parameter, followed by the model parameter describing the first-order conversion from the parent compound into the metabolite. Outcomes are described in detail in File S1. Overall, we judged the agreement between simulation and observation as satisfactory and saw no need to modify the PBK model structure.

Strikingly, both the simulated and the measured fetal compartment levels that resulted from doses of procymidone, vinclozolin, and linuron that were associated with reductions in AGDI compared with male controls ($ED10_{AGDI}$ and $ED50_{AGDI}$) overlapped with the concentration ranges associated with *in vitro* AR antagonism and suppression of steroid synthesis [horizontal lines depicting the *in vitro* half-maximal inhibitory concentration (IC_{50}) values in Figure 3B]. For all three compounds, concentrations equivalent to the fetal levels at the $ED10_{AGDI}$ produced almost saturating *in vitro* AR antagonistic effect concentrations.

Overall, our results confirmed our presumption that *in vitro* active concentration ranges, combined with PBK simulations can be used to predict whether active concentrations can be attained in the fetus and whether these are in turn associated with *in vivo* outcomes. This encouraged us to use this approach for predictive assessments of compounds not previously tested *in vivo*.

QIVIVE as a Prediction Tool

To evaluate the robustness of our QIVIVE approach, we selected nine pesticides currently authorized for use in the EU. We previously identified all these compounds as AR antagonists in MDA-kb2 human breast cancer cells (Orton et al. 2011; see Table S3 for a summary of potencies), but information about *in vivo* activity was missing, as were data about their capacity to suppress androgen synthesis. To expand the *in vitro* basis of our QIVIVE, we tested all these pesticides for effects on steroid hormone synthesis in the H295R steroidogenesis assay. Seven of our chosen pesticides (fludioxonil, cyprodinil, dimethomorph, imazalil, quinoxyfen, fenhexamid, and *o*-phenylphenol) inhibited testosterone

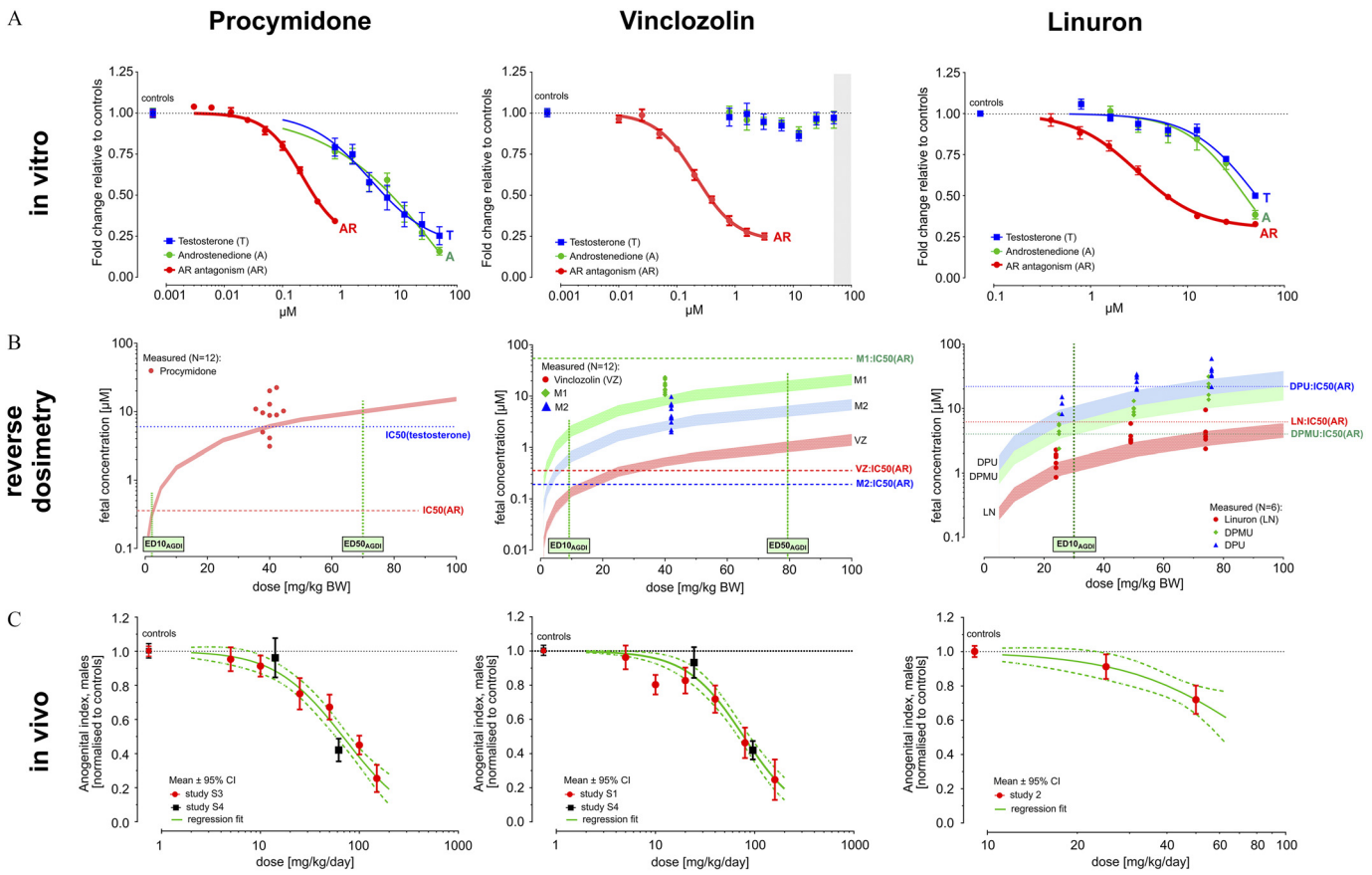

Figure 3. QIVIVE for shortened anogenital distance (AGD) in male offspring following gestational exposure to procymidone, vinclozolin, and linuron. (A) Concentration response data *in vitro* and regression curves for androgen receptor (AR) antagonism (red symbols and lines) as well as testosterone (blue symbols and lines) and androstendione inhibition (green symbols and lines). Data represent mean ± SEM ($n = 9$), the shaded gray area for vinclozolin indicates cytotoxic concentration ranges in the H295R assay. (B) PBK-modeled relationships between fetal plasma concentrations of the parent compounds and their relevant metabolites and doses administered to dams. Symbols (circle, diamond, triangle) show the measured fetal plasma levels in the *in vivo* studies on the three compounds. PBK simulations for GD15–GD18 are shown as shaded areas (red for parent compound, blue and green areas for metabolites). The parent compound vinclozolin could not be detected in blood or amniotic fluid, only the M1 and M2 metabolites were quantified (see Table 3 for full names of metabolites). The shadings reflect least- and worst-case kinetic model assumptions for describing the exposure transport between placenta and fetal compartment (see "Materials and Methods"). Horizontal lines show the concentrations associated with strong *in vitro* activities (IC_{50}), vertical lines show doses ($ED10_{AGDI}$, $ED50_{AGDI}$) that resulted in weak and strong reductions of the AGD index (AGDI), respectively, measured in male rat pups (derived from the data shown in C). (C) Dose–response data and regression curve for AGDI measured in rat male pups shortly after birth following exposure (GD7–GD21) to procymidone, vinclozolin, and linuron. Procymidone and vinclozolin data on AGDI reductions in Wistar rats are from previous studies (Hass et al. 2007, with study notation according to Table 2), and linuron data on AGDI reductions in Sprague-Dawley rats are from Study 2 of Table 2. Absolute AGDI responses in treated males were normalized to relative values between 1 (mean AGDI from male controls, i.e., no effect on male AGDI) and 0 (mean AGDI from female controls, i.e., complete feminization of males) (see Equation 38 in "Materials and Methods"); Data represent mean ± 95% confidence belt ($n = 6$–13 litters), the regression curves are mean ± 95% confidence belt. Note: ED, effective dose; GD, gestational day; IC_{50}, half-maximal inhibitory concentration; PBK, physiologically based pharmacokinetics; SEM, standard error of the mean.

synthesis in addition to its precursor androstendione. Fludioxonil and imazalil showed an activity significantly different from the solvent control cells already at the lowest test concentrations of 1.6 μM and 0.8 μM, respectively (Table S4). Two pesticides, λ-cyhalothrin and pyrimethanil, had no effect on any measured androgen. This potency of cyprodinil, imazalil, and quinoxyfen in suppressing steroid synthesis exceeded that in antagonizing the AR and vice versa for the remaining four compounds (Figure 5, horizontal lines; Figure 6A).

To pinpoint candidates for further *in vivo* investigation, we used the PBK model to simulate fetal concentrations for a wide range of doses (Figure 5). We found no evidence in the literature for potential AR antagonist activity or impacts on steroid hormone synthesis for any of the pesticide metabolites and we therefore limited the simulations to the parent compounds. A compound was considered as a candidate for potential anti-androgenic effects in the male rat *a*) if doses to the dam were predicted to result in fetal compartment levels equivalent to *in vitro* concentrations associated with at least 20% AR antagonist activity and/or testosterone inhibition; and *b*) if these doses were below the lowest observed adverse effect levels (LOAELs) reported from one- or two-generation reproductive toxicity studies and were not associated with maternal and prenatal toxicity. Based on these criteria, we expected seven pesticides to induce shortened male AGD *in vivo*: fludioxonil, cyprodinil, dimethomorph, imazalil, quinoxyfen, fenhexamid, and *o*-phenylphenol. Because AR antagonism was not observed at the fetal levels attained after maternal dosing at levels below the toxic range, we anticipated that λ-cyhalothrin and pyrimethanil would not affect AGD at doses below the maternal toxicity threshold (Figure 5).

To test our predictions, we selected fludioxonil, cyprodinil, and dimethomorph for *in vivo* studies. Fludioxonil antagonized the AR and inhibited testosterone synthesis with similar potency. Cyprodinil was more potent in suppressing testosterone synthesis than in antagonizing the AR, whereas the reverse was true for dimethomorph (Figure 6; Table 3).

Table 3. Measured (GD21) and simulated fetal plasma levels (GD15–GD18) of parent compounds and metabolites after gestational exposure to individual doses of six pesticides, together with AGDI responses in male offspring (PND1 or GD21) and results from *in vitro* profiling for anti-androgenic effects (IC_{50} for androgen receptor (AR) antagonism and testosterone inhibition obtained in NCI-H295R cells).

	In vitro		*In vivo*			*In silico*
Compound	AR antagonism (IC_{50}, μM)	Testosterone inhibition (IC_{50}, μM)	Dose (mg/kg BW per day)	AGDI reduction[a] (%)	Measured concentration in male fetuses[b] (μM)	Simulated concentration in male fetuses[c] (μM)
Procymidone	0.4	6	40	34[d]	7.8	5.9–6.4
Vinclozolin	0.3	NA	40	26[d]	<0.15[e]	0.43–0.64
M1[f]	2.0[g]	NT	—	—	18.0	6.4–49.6
M2[h]	0.2[g]	NT	—	—	3.4	2.1–3.2
Linuron	6	27	25	9	1.6	0.9–1.5
DPMU[i]	4	NA	—	—	5.0	3.4–5.4
DPU[j]	22	5	—	—	11.8	6.0–9.5
DCA[k]	12	NA	—	—	ND	ND
Linuron	6	27	50	28	3.7	1.8–3.0
DPMU[i]	4	NA	—	—	9.6	6.8–10.9
DPU[j]	22	5	—	—	27.4	12.0–19.0
DCA[k]	12	NA	—	—	ND	ND
Linuron	6	27	75	—[l]	4.5	2.9–4.8
DPMU[i]	4	NA	—	—	21.4	10.9–17.3
DPU[j]	22	5	—	—	37.8	19.2–30.4
DCA[k]	12	NA	—	—	ND	ND
Fludioxonil	2.6[m]	10.8	60	13	3.3	3.3–5.4
Cyprodinil	28[m]	8.5	60	16	6.6	11.5–18.3
Dimethomorph	0.9[m]	>50	20	13	1.35	1.0–1.5

Note: —, not applicable; AGDI, anogenital distance index; GD, gestational day; M1, metabolite 1; M2, metabolite 2; NA, not active up to 50 μM; ND, not detected; NT, not tested.
[a]Absolute AGDI responses in treated males were normalized to relative values between 1 (mean AGDI from male controls; i.e., no effect on male AGDI) and 0 (mean AGDI from female controls; i.e., complete feminization of males).
[b]Fetal blood was pooled per litter from all males at GD21 and mean litter value is reported.
[c]Simulations refer to average plasma levels at GD15–GD18.
[d]Estimated from regression model (Hass et al 2007).
[e]Below limit of quantification.
[f]2-[[(3,5-Dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid.
[g]Data derived from Vinggaard et al. (2008).
[h]3′,5′-Dichloro-2-hydroxy-2-methylbut-3-enanilide.
[i]1-(3,4-Dichlorophenyl)-3-methoxyurea.
[j]1-(3,4-Dichlorophenyl)urea.
[k]2,4-Dichloroaniline.
[l]Maternal toxicity.
[m]Orton et al. (2011).

Experimental Assessment of QIVIVE Predicted in Vivo *AGD Effects*

The ability of fludioxonil, dimethomorph, and cyprodinil to induce shortened AGDs after gestational exposure was investigated in a reproductive/developmental toxicity study in the rat. Each pesticide was tested with at least three doses that were selected to fall in the range at which QIVIVE predicted shortened AGDIs in males (Figure 6B, predicted window for *in vivo* activity). At low doses, we anticipated only a weak *in vivo* response (close to the sensitivity limit), whereas at higher doses, we expected a clear response in terms of altered AGDIs, without any interfering maternal toxicity. The high doses were chosen below the threshold for maternal toxicity observed in other studies.

The observed dose–response patterns for AGDI reductions in the newborn male pups were not as clear-cut as we had expected, but all three pesticides did produce statistically significant smaller AGDI toward the center of the predicted dose range (Figure 6C; Table 3). Cyprodinil, at 60 mg/kg per day, produced statistically significant AGDI shortening, but not at 180 mg/kg per day; this dose has been shown to induce maternal liver toxicity (EFSA 2005). Fludioxonil, at 180 mg/kg per day, led to a shortening of AGDI similar in magnitude to those observed at 60 mg/kg per day but with high variations between litters, which may have prevented the values from reaching statistical significance. In the first of the dimethomorph studies, the two lowest doses led to statistically significantly different AGDIs, but the higher dose of 60 mg/kg per day did not (Figure 6C, red circles). This motivated us to repeat in a second study the same doses together with a high dose but with a different rat strain. In this study, statistically significantly shortened AGDIs were seen at doses of 60 and 180 mg/kg per day (Figure 6C, black squares). At 180 mg/kg per day, we observed indications for a mild maternal toxicity (increased postimplantation and perinatal loss, higher postnatal death rate; Table S6). There were no statistically significant effects on nipple retention at PD14 in the studies with fludioxonil, cyprodinil, or dimethomorph (Table S5), but in the second dimethomorph study retained nipples were seen at doses of 7 and 180 mg/kg per day (Table S6).

The predicted levels of fludioxonil, cyprodinil, and dimethomorph in the fetal compartment and the dams' plasma were evaluated by chemical analysis of fetal samples harvested by cesarean section at GD21, and of maternal blood samples drawn on GD21. The measured concentrations in the fetuses and the dams' plasma agreed well with the PBK simulations (Figure 4). The ratios between observation and prediction were in the range of 0.5 to 2, showing good accuracy of the PBK model. However, the maternal plasma levels predicted for dimethomorph were approximately one-tenth that of the analytically determined concentrations.

Discussion

We developed a QIVIVE approach for anti-androgenicity that predicts concentrations of a test compound in the fetal compartment attained after maternal dosing during the male programming window. Comparison of simulated fetal levels with *in vitro* active concentration ranges for AR antagonism and suppression of androgen synthesis enabled us to anticipate whether *in vivo*

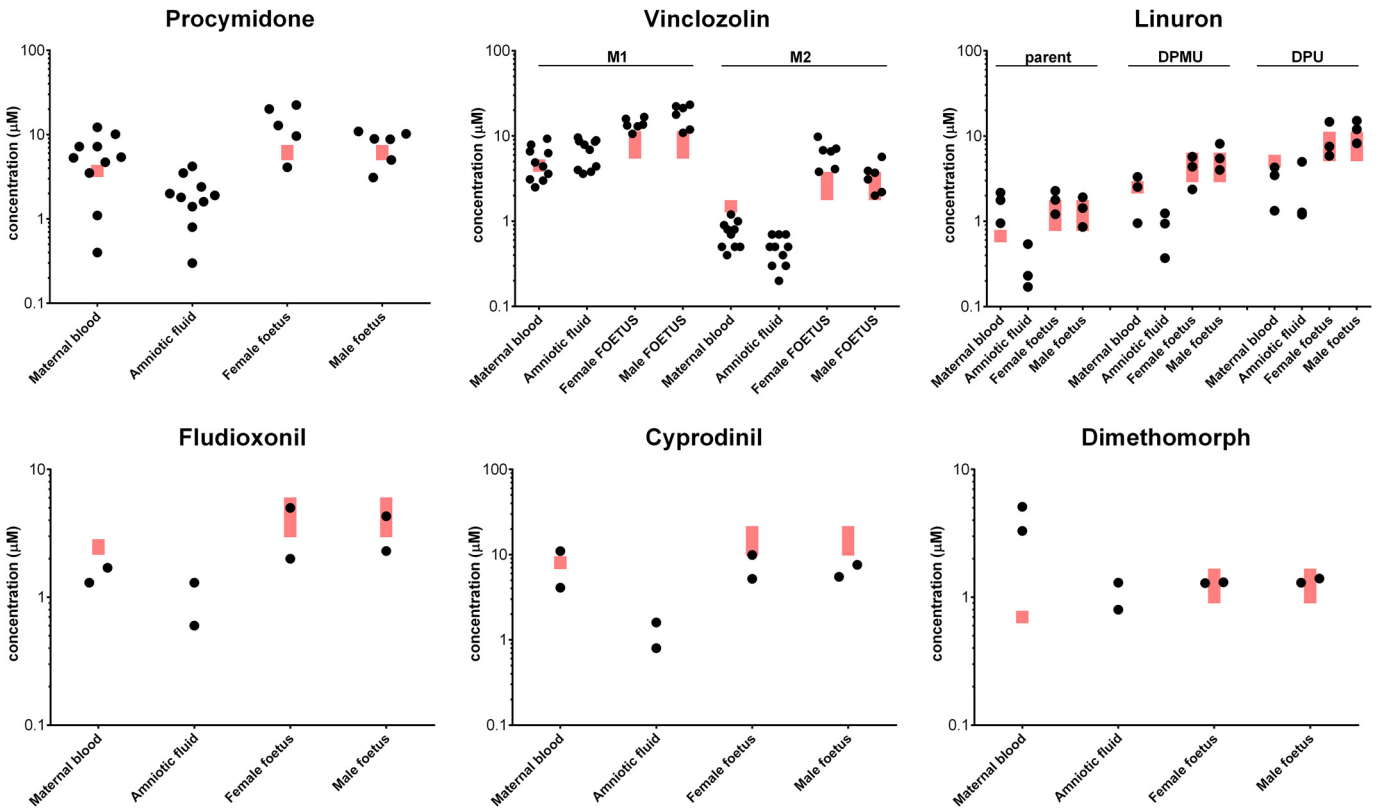

Figure 4. Measured and PBK model-simulated pesticide concentrations in maternal and fetal plasma as well as in amniotic fluid after repeated dosing of procymidone (40 mg/kg BW), vinclozolin (40 mg/kg BW), linuron (25 mg/kg BW), fludioxonil (60 mg/kg BW), cyprodinil (60 mg/kg BW), and dimethomorph (20 mg/kg BW) (GD7–GD21). For vinclozolin, the levels of metabolites M1 and M2 are shown; for linuron, those of dimethylpropyleneurea (DPMU) and 1,3-diphenylurea (DPU) are shown (see Table 3 for full names of metabolites). The ranges of simulated concentrations are shown as red bars and refer to average plasma concentrations on GD21 (dams) or fetal concentrations on GD15–GD18. Measurements are shown as black dots representing one dam (maternal blood), one litter (amniotic fluid), or gender-specific litter means (fetus) and were taken on GD21 approximately 90 min after dosing by oral gavage. Note: BW, body weight; GD, gestational day; PBK, physiologically based pharmacokinetics.

effects on AGD reductions should occur (for simplicity, we use AGD synonymously with AGDI from here on). We demonstrated the applicability of the QIVIVE approach to three well-studied anti-androgenic compounds: procymidone, vinclozolin, and linuron (Gray et al. 2001; Hass et al. 2007). The application of this approach to nine current-use pesticides, all with *in vitro* evidence for anti-androgenicity but missing *in vivo* data, produced seven predicted *in vivo* actives. We confirmed our predictions for three of these seven pesticides (fludioxonil, cyprodinil, and dimethomorph). For two of the nine pesticides, λ-cyhalothrin and pyrimethanil, the simulated fetal concentrations were below the *in vitro* active concentrations at the highest *in vivo* dose tested.

Our method rests on the assumption that the *in vitro* anti-androgenic activity of a chemical (AR antagonism or inhibition of androgen synthesis) will lead to a shortened AGD in male offspring as long as the kinetics of the substance permit that *in vitro* active concentrations can be attained in the fetal compartment after maternal dosing below the toxic range. We further assumed that there were no modulating factors that counteracted the effects by changing endogenous hormone levels. Both kinetic and toxicodynamic considerations were, therefore, decisive in predicting whether an *in vivo* effect was likely to occur.

However, the converse reasoning is not admissible: If QIVIVE suggests that critical fetal concentrations cannot be reached after maternal dosing in the subtoxic range, as was the case with λ-cyhalothrin and pyrimethanil (Figure 5), it is not possible to rule out *in vivo* activity. Other, as yet unknown, modes of action not captured by the *in vitro* assays might also lead to short AGDs (see discussion below). Thus, the accuracy of our QIVIVE is not only conditional on kinetics but also on the selected *in vitro* effect profile. Both these assumptions require critical examination.

Toxicokinetics—Uncertainties of the PBK Model

Our PBK model was designed to be compatible with the data on ADME available from EU pesticide DARs. We used these data to estimate compound-specific first-order elimination rates due to metabolism, renal clearance, and excretion, without the need for predictive *in silico* tools or additional *in vitro* experiments. However, these elimination rates were based on kinetic measurements in nonpregnant animals reported in DARs. It is conceivable that these kinetic parameters are different during gestation, but data to further investigate this possibility were not accessible to us. Our modeling strategy was, therefore, to capture the kinetics of internal exposures during gestation as much as possible by employing physiological parameters as they changed during gestation (e.g., organ weights, blood flow rates, and so on).

Unfortunately, there is no agreed schema by which ADME data for pesticides are reported. There are great variations in terms of the physiological compartments sampled, the timing of sampling, the number of studies conducted and how kinetic measurements are documented (e.g., measurements for individual animals vs. summarizing kinetic descriptors such as AUC). It was, therefore, not possible to pursue a uniform approach when deciding which data to use for deriving PBK model parameters. Rather, this had to be done on a case-by-case basis. As a result,

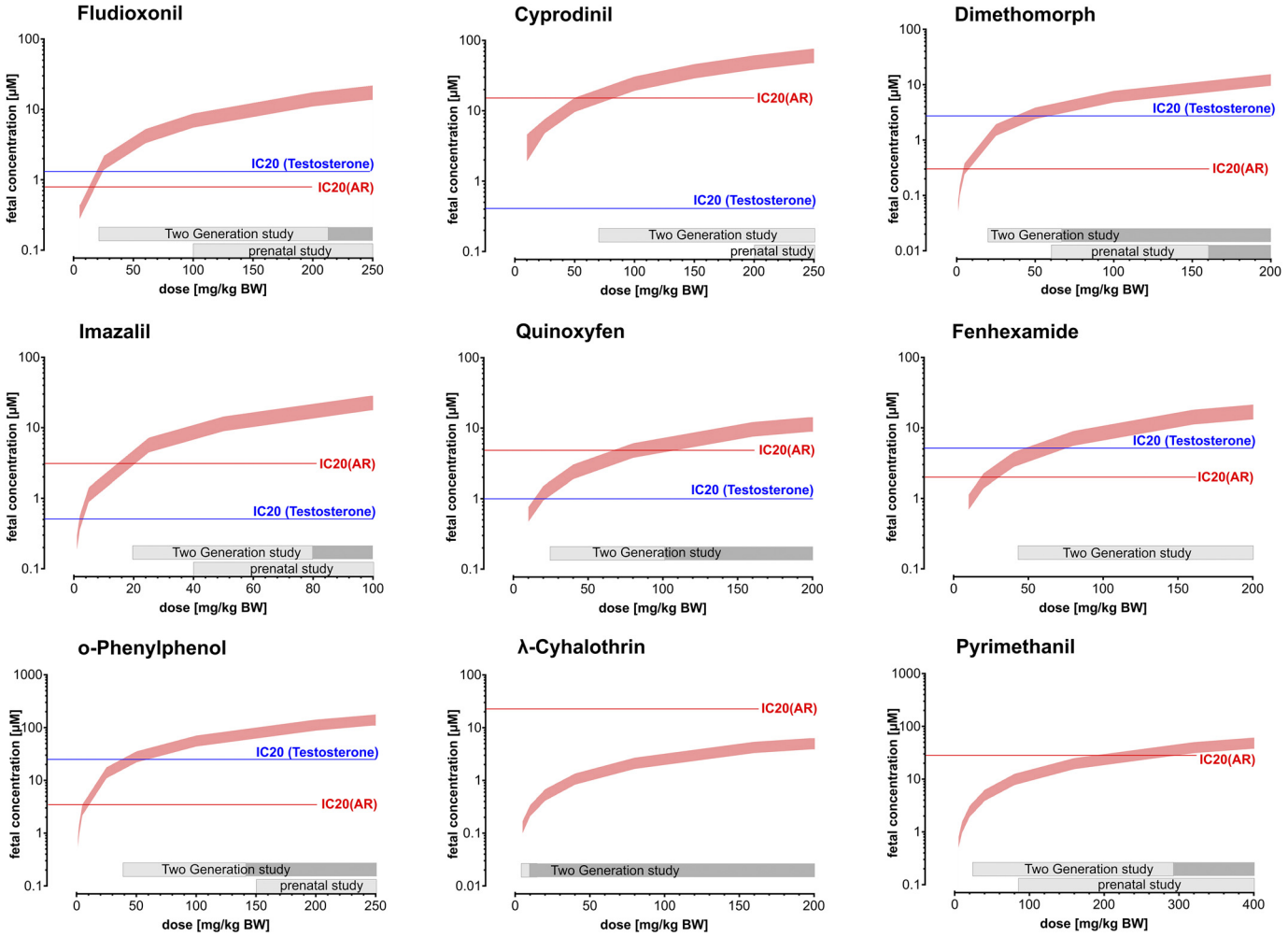

Figure 5. PBK simulations of fetal concentrations of fludioxonil, cyprodinil, dimethomorph, imazalil, quinoxyfen, fenhexamid, *o*-phenylphenol, λ-cyhalothrin, and pyrimethanil, in response to repeated maternal doses between GD7 and GD21, depicted as red shaded areas. The red shadings reflect different kinetic model assumptions for describing the exposure transport between placenta and fetal compartment (see "Materials and Methods"). Horizontal lines indicate the IC_{20} for androgen receptor (AR) antagonism *in vitro* and the IC_{20} for testosterone inhibition *in vitro*. Gray horizontal bars show dose ranges associated with *in vivo* adverse effects reported in two-generation or prenatal toxicity studies in EFSA's Draft Assessment Reports (EFSA 2019), with the light gray bar indicating the range between the no observed adverse effect level (NOAEL) and lowest observed adverse effect level (LOAEL) and the dark gray bar indicating the dose range above the LOAELs (NOAEL and LOAEL for λ-cyhalothrin were chosen from studies on cyhalothrin). Note: EFSA, European Food Safety Authority; GD, gestational day; IC_{20}, inhibitory concentration at 20%; PBK, physiologically based pharmacokinetics.

we occasionally obtained more than one set of model parameters that equally well described the concentrations reported in blood, urine, and feces. Inevitably, therefore, some of the kinetic parameters we selected may not represent the correct elimination rates.

These limitations apply particularly to extensively metabolized substances with complex elimination patterns such as imazalil, where 24 h after dosing at least 25 metabolites were discovered in urine and feces. Imazalil undergoes many different routes of metabolism, including epoxidation, epoxide hydration, oxidative *O*-dealkylation, imidazole oxidation and scission, and oxidative *N*-dealkylation (EFSA 2009). For compounds with such complex and rapid metabolism, analytical determinations of metabolite levels soon after dosing are needed, but in the case of imazalil, such data are not available for time points earlier than 12 h after dosing (Mannens et al. 1993). It is, therefore, possible that imazalil was eliminated much faster than assumed in our PBK simulations. Accordingly, we may have overestimated the concentrations in the fetal compartment, leading to a false positive prediction of *in vivo* activity regarding shortened AGDIs. An ADME *in vivo* study with a focus on establishing metabolite levels within the first 3–6 h after last dosing would be highly desirable to remove the uncertainties in our estimates. These issues are currently being addressed in a follow-up study.

Our PBK model also assumed that the distribution of compounds between compartments, especially the bidirectional transport between placenta and fetus, is by passive diffusion, rather than active transport. Due to a lack of more detailed data, the fetus was modeled as a simple single compartment, without consideration of further distribution processes within the compartment, compartment-specific protein binding, or elimination processes. Because of these simplifications, it was not feasible to characterize the fetal compartment in terms of maximum plasma concentration (C_{max}) or time to maximum plasma concentration (T_{max}). The only reliable estimate of the fetal internal exposure load was the AUC over the period of interest (GD15–GD17). Accordingly, we used AUC per hour as an approximation of average fetal concentrations.

Sensitivity analyses revealed the bidirectional transport of compounds between the placenta and the fetus as the most uncertain and sensitive element of our model simulations. Because of a lack of adequate data from *in silico* or *in vitro* methods, our only option of overcoming this uncertainty was by using a range of

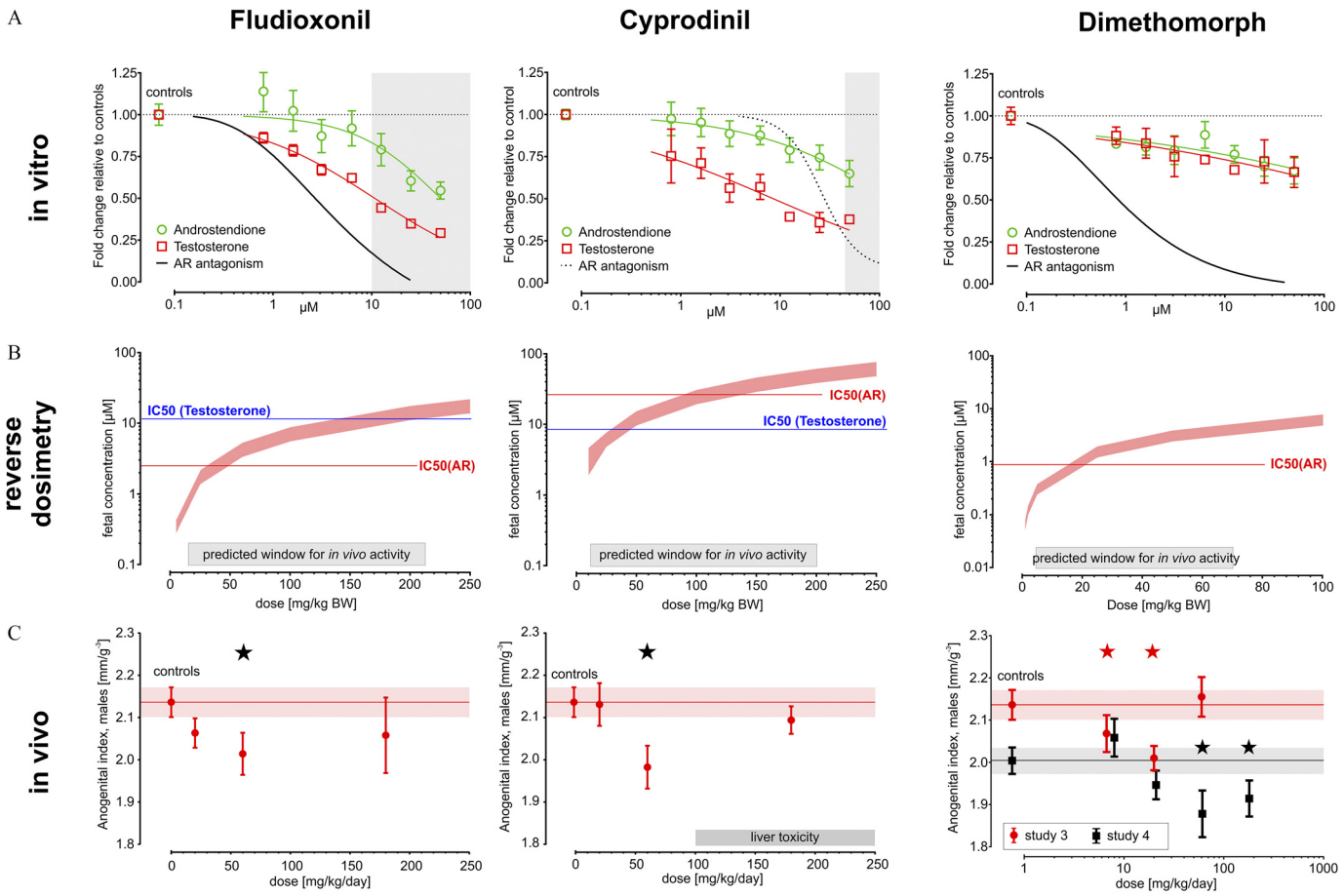

Figure 6. Assessment of the utility of the QIVIVE for anticipating shortened anogenital distance (AGDs) in male offspring following gestational exposure to fludioxonil, cyprodinil, and dimethomorph. (A) Concentration–response curves for androgen receptor (AR) antagonism, testosterone, and androstenedione inhibition *in vitro*. Data represent mean ± SEM ($n = 3$). Gray areas indicate cytotoxic concentration ranges in the H295R assay. (B) Fetal levels of the pesticides predicted by PBK modeling as a function of the *in vivo* dose. The horizontal lines illustrate the *in vitro* activities. The horizontal gray areas (predicted window for *in vivo* activity) depict the dose ranges at which a shorten AGD cannot be ruled out. The red shadings reflect different kinetic model assumptions for describing the exposure transport between placenta and fetal compartment (see "Materials and Methods"). (C) AGD index (AGDI) measured at birth following exposure from GD7 to GD21 to fludioxonil, cyprodinil (20, 60, and 180 mg/kg BW per day) or dimethomorph (6.7, 20, or 60 mg/kg BW per day) (Study 3 on Wistar rats). A follow-up Study 4 was conducted on Sprague-Dawley rats (6.7, 20, 60, or 180 mg/kg BW per day) for dimethomorph. The gray bar for cyprodinil illustrates liver hypertrophy reported *in vivo* in rodent studies (EFSA 2005). Data represents mean ± 95% confidence belt ($n = 6$–10 litters). *, $p < 0.05$ for comparison with the control group (multiple contrast testing in mixed effect ANOVA model). Note: AGD, anogenital distance; ANOVA, analysis of variance; BW, body weight; EFSA, European Food Safety Authority; GD, gestational day; PBK, physiologically based pharmacokinetics; QIVIVE, quantitative *in vitro* to *in vivo* extrapolation; SEM, standard error of the mean.

transplacental rates for the PBK simulations that we considered equally possible, with a maximum relative difference between high and low clearance rates set as ±20%. Whether this range reflects a worst case is debatable, but to our knowledge, no gestational PBK model for rats has been reported that suggests larger parameter differences, at least not for chemicals that have kinetic properties like those of the modeled pesticides.

Despite all these uncertainties, the simulations agreed rather well with the measured values and did not deviate by more than a factor of five. It appears that our relatively simple PBK model structure, combined with the kinetic data contained in DARs, was sufficient to approximate fetal concentrations reasonably well.

Toxicodynamics—from AR Antagonism to Shortening of AGD

All three pesticides selected for testing our QIVIVE predictions induced shortened AGDs, but notable were some unusually shaped dose–response curves. In each case, specific reasons for the observed response patterns may be conjured up. The lack of response at the highest dose of cyprodinil might be due to interference with maternal liver function (EFSA 2005). Furthermore, cyprodinil was an *in vitro* AhR activator and induced expression of CYP1A1 in ovarian granulosa and hepatoma cells (Fang et al. 2013) that may have led to increased hepatic metabolism of steroids at the high dose, thereby preventing the induction of AGD reduction. As discussed above, such phenomena cannot be captured by our PBK modeling. With fludioxonil, there were unusually high variations in AGD at the highest dose, which may have prevented the AGD values from reaching statistical significance. The nonmonotonic dose–response pattern seen with dimethomorph was not replicated in a second study with larger numbers of animals but with a different rat strain. In any case, however, both studies produced evidence of AGD reductions. The example of dimethomorph highlights that our QIVIVE model is not capable of predicting dose–response patterns; rather, it can help in establishing whether compounds induce shortening of AGD in a qualitative sense.

Furthermore, there are differences in the effect strength with which AR antagonists produced AGD reductions, pointing to complex relationships between diminished androgen action in the fetus

and AGD changes. The sex-specific differentiation of the perineum, the area between anus and genitalia, is dependent on the stimulation of the growth of the perineal muscles leviator ani and bulbocavernosus complex, which is mediated by AR activation (Schwartz et al. 2019). Certain AR antagonists such as procymidone, vinclozolin, and flutamide can produce severely shortened AGDs that approach female control values, whereas most other chemicals, including some phthalates, butylparaben, or bisphenol A induce much less marked shortenings (Schwartz et al. 2019). Clearly, fludioxonil, cyprodinil, and dimethomorph fall into the latter category with shortening of AGDI of around 10% compared with male controls (when related to the normalized AGDI scale) (Table 3). The reasons for these differences in effect strength are unknown, but an appealing possibility discussed by Schwartz et al. (2019) is the estrogenicity of some of the anti-androgens.

In any case, our QIVIVE was not designed to predict the effect strength of AGD changes, nor the shapes of dose–response curves. As developed and presented here, the approach is capable of anticipating *in vivo* activity in a qualitative sense, as a binary active–inactive option.

The complex relationships between receptor-mediated and cellular responses and AGD reductions argue for inclusion of as many *in vitro* anti-androgenicity read-outs as possible, at a minimum AR antagonism and suppression of androgen synthesis, as in our study. With certain compounds, there may be merit in additionally including measures of other anti-androgen-related mechanisms of action (e.g., 5α-reductase inhibition or blocking of the membrane AR) as well as *in vitro* estrogenicity.

Prediction of a Wider Anti-Androgenic Effect Spectrum

Chemicals capable of producing severely feminized AGDs typically produce an anti-androgenic effect spectrum also comprising retained nipples, prostate agenesis, penile malformations, and reduced weights of androgen-dependent organs (reviewed by Schwartz et al. 2019). However, with agents that produce milder shortened AGDs, these effects are not consistently observed, indicating that there is no clear relationship between the various manifestations of anti-androgenicity. This is the case with fludioxonil, cyprodinil, and dimethomorph at the doses tested here. Except for dimethomorph, these chemicals did not induce nipple retention at the tested dose levels. Thus, it is difficult to anticipate additional *in vivo* anti-androgenic effects, even when AGD reductions are predicted. However, this is an issue related to our incomplete understanding of the processes leading to the various physical manifestations of androgen insufficiency rather than a weakness of our QIVIVE approach. This calls for further studies of the diversity of patterns/profiles of anti-androgenicity.

False Negative Predictions

The complex processes leading to shortened AGDs in male pups may not be induced only by anti-androgens. Mild analgesics such as paracetamol or acetylsalicylic acid can also induce shortened AGDs, although these substances are not capable of antagonizing the AR or suppressing androgen synthesis (Kristensen et al. 2011; Schwartz et al. 2019). The mechanisms by which shortening of AGD arises after exposure to analgesics remain to be elucidated and compounds that act by such unknown mechanisms will not be detected with our present approach.

False negative predictions can also occur with substances where the levels in the fetal compartment modeled to be attainable after maternal dosing do not reach *in vitro* active AR antagonistic or androgen suppressing effective concentrations. For instance, despite the negative predictions of AGD reductions we made for λ-cyhalothrin and pyrimethanil, we cannot rule out that such substances may lead to incomplete masculinization of male AGDs by mechanisms independent of diminished androgen action. Similarly, inactive parent compounds that are metabolically converted to active AR antagonists, a process currently difficult to capture with *in vitro* assays, may also lead to false negatives.

False Positive Predictions

It appeared that the modeled and measured fetal concentrations were not as active as the *in vitro* data predicted. In some cases, the *in vitro* active concentration ranges of test compounds were lower than the concentration ranges predicted as attainable in the fetal compartment *in vivo*, in other words: All simulated fetal levels were anticipated to be active. This suggests that there were factors at play *in vivo* that led to an apparent diminution of the compounds' activity. False positive predictions might arise if there are bioavailability differences between the *in vitro* and *in vivo* situation. We speculate that differences in the active, free concentrations are a possible explanation, due to differences in protein concentrations between the cell culture media and the blood plasma. Protein binding can be a crucial factor leading to higher *in vitro* effective free concentrations than *in vivo* because smaller proportions of compounds are bound to proteins due to lower *in vitro* protein levels. In principle, the issue could be addressed by simulating the free, unbound fractions of parent compounds and metabolites in the fetus, but this will require the availability of relevant kinetic data for the PBK model, which may pose difficulties. It is at present hard to judge whether the additional data requirements that this entails will lead to significant improvements of the predictive power of our QIVIVE.

False positives could also arise if high doses cause changes in metabolism of compounds to an inactive metabolite. Another possibility for false positive predictions might result from overestimations of internal maternal exposure levels due to a lack of sufficient toxicokinetic data, a possibility that we cannot rule out for imazalil (see the section "Toxicokinetics—Uncertainties of the PBK Model").

To put this issue into perspective, a recent study reported on the performance of the ToxCast/Tox 21 AR *in vitro* model, based on 11 high-throughput assays for predicting a positive anti-androgenic response in the Hershberger rodent model (Kleinstreuer et al. 2018). The AR *in vitro* model had 100% positive predictive value for the *in vivo* response, and chemicals with conclusive *in vitro* results were consistently positive *in vivo*, indicating that few false positives existed.

Testing Strategy

Several OECD test guidelines for reproductive and developmental toxicity require the measurement of AGD in offspring, pups/fetuses [OECD TG 414, 421/422, 443 (OECD 2013, 2016a, 2016b, 2018a, 2018b)], and this end point is vital for the identification of endocrine-disrupting chemicals. AGD reductions can be used for estimating NOAELs or benchmark doses, which form the basis for establishing health-based guidance values (OECD 2013).

Our QIVIVE approach may prove to be a useful tool for the prioritization of chemicals for *in vivo* testing. It is applicable to chemicals assumed to act via a specific pathway, the induction of AGD shortening through androgen insufficiency via AR antagonism or suppression of androgen synthesis. We argue that *in vitro* AR antagonists and androgen synthesis-suppressing chemicals predicted to be active *in vivo* should, by way of a rebuttable hypothesis, be treated as if they were *in vivo* actives, even when appropriate *in vivo* data are not available. This stance should not preclude confirmatory testing if the necessary resources can be made available. However, we suggest that rather than mobilizing resources for confirming predicted *in vivo* positives, chemicals with *in vitro* anti-androgenic

activity, but predicted to be inactive *in vivo*, should be prioritized for *in vivo* testing. This will address the uncertainties associated with factors that lead to false negatives. Such a strategy will be more resource effective and ethical than focusing on predicted *in vivo* actives. To gain an impression of the extent of the testing need, we suggest evaluating all pesticides authorized for use in the EU for *a*) whether ADME data contained in DAR are compatible with our QIVIVE approach; and *b*) whether relevant *in vitro* anti-androgenicity data are available. If both conditions are met, our approach can be used to identify likely *in vivo* active candidates and to make decisions about further testing. Our proposed QIVIVE approach primarily addresses testing prioritizations but should not form the only basis for regulatory decisions at the present time. However, our approach has the potential to be integrated together with other information sources for testing and assessment of chemicals in a regulatory context. Furthermore, our approach may enrich an adverse outcome pathway concept for AR antagonists and contribute data for quantification of the concept.

Conclusion

For three chemicals with *in vitro* activity, we have shown that our QIVIVE approach can successfully predict the fetal levels attained after maternal dosing as well as their *in vivo* anti-androgenicity (shortening of AGD). Our approach is applicable to agents that produce androgen insufficiency by AR antagonism and suppression of androgen synthesis and has great potential for minimizing unnecessary *in vivo* testing.

Our QIVIVE approach is based on PBK models that mimic rat physiology, which will be of utility for *in vivo* test prioritization. However, in the long run, the vision should be to develop the QIVIVE approach based on human PBK models in order to obtain more human-relevant predictions that eliminate the need for species–species extrapolations. If realized, such approaches can have an even greater impact on the reduction of the use of animals for chemical risk assessment.

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