


Development of a Generic Physiologically Based Kinetic Model to Predict In Vivo Uterotrophic Responses Induced by Estrogenic Chemicals in Rats Based on In Vitro Bioassays

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ABSTRACT

The present study assessed the potential of a generic physiologically based kinetic (PBK) model to convert in vitro data for estrogenicity to predict the in vivo uterotrophic response in rats for diethylstilbestrol (DES), ethinylestradiol (EE2), genistein (GEN), coumestrol (COU), and methoxychlor (MXC). PBK models were developed using a generic approach and in vitro concentration-response data from the MCF-7 proliferation assay and the yeast estrogen screening assay were translated into in vivo dose-response data. Benchmark dose analysis was performed on the predicted data and available in vivo uterotrophic data to evaluate the model predictions. The results reveal that the developed generic PBK model adequately defines the in vivo kinetics of the estrogens. The predicted dose-response data of DES, EE2, GEN, COU, and MXC matched the reported in vivo uterus weight response in a qualitative way, whereas the quantitative comparison was somewhat hampered by the variability in both in vitro and in vivo data. From a safety perspective, the predictions based on the MCF-7 proliferation assay would best guarantee a safe point of departure for further risk assessment although it may be conservative. The current study indicates the feasibility of using a combination of in vitro toxicity data and a generic PBK model to predict the relative in vivo uterotrophic response for estrogenic chemicals.

Key words: generic physiologically based kinetic modeling; reverse dosimetry; quantitative in vitro–in vivo extrapolation; uterotrophic assay.

The rate at which in vitro toxicity data are currently generated is high. The EPA ToxCast project evaluated over 2000 chemicals in more than 700 different high-throughput in vitro screening assays covering a range of endpoints and signaling pathways (Judson *et al.*, 2010). The toxicological profile obtained from in vitro assays plays a major role in hazard identification (Bernauer *et al.*, 2005). In order to be able to judge the impact of the in vitro toxicity data for the in vivo situation, efforts should focus on the development of quantitative in vitro–in vivo extrapolation (QIVIVE) methods (Louisse *et al.*, 2016; Wetmore

et al., 2012). Such methods not only allow the application of in vitro data for preliminary risk assessment, but also are useful in the process of compound development when selecting compounds for which low toxicity is expected in the in vivo situation (Bell *et al.*, 2018).

A method for QIVIVE that has been proven to be of use is physiologically based kinetic (PBK) modeling-based reverse dosimetry. PBK modeling-based reverse dosimetry has been used to predict, for example, the in vivo developmental toxicity, nephrotoxicity, and liver toxicity for diverse compounds using

concentration-response data obtained from relevant *in vitro* assays (Abdullah *et al.*, 2016; Chen *et al.*, 2018; Li *et al.*, 2017; Louisse *et al.*, 2015; Ning *et al.*, 2019; Strikwold *et al.*, 2017). In a previous study, we also showed that the PBK modeling-based reverse dosimetry can be combined with results from the *in vitro* yeast estrogen screening (YES) reporter gene assay to adequately predict the uterus weight response in rats induced by 17 β -estradiol (E2) and bisphenol A (BPA; Zhang *et al.*, 2018). Although considerable research has been devoted to develop the proofs-of-principle of the PBK modeling-based reverse dosimetry for the prediction of diverse toxicological endpoints, it is time and resource consuming to develop a PBK model for each individual compound. Therefore, the development of a generic PBK model that requires only a limited number of parameters to adequately describe the data would be of use. To develop such a generic PBK model, in a first approach a generic PBK model is defined for compounds that are efficiently absorbed upon oral administration by passive diffusion and induce toxic effects as parent compounds although being detoxified by metabolic clearance.

The present study aimed to verify the concept of using a generic PBK model in combination with *in vitro* data for estrogenicity to predict the *in vivo* uterotrophic response for a group of (estrogenic) compounds in rats. Diethylstilbestrol (DES), ethinylestradiol (EE2), genistein (GEN), coumestrol (COU), and methoxychlor (MXC) were selected as the test compounds in the current study, whereas the approach was already shown valid for E2 and BPA previously (Zhang *et al.*, 2018). DES and EE2 have proven to have high estrogenic potency compared with those of COU, GEN, and MXC in both *in vitro* and *in vivo* studies (Andersen *et al.*, 1999; Breinholt and Larsen, 1998; Coldham *et al.*, 1997; Dodge *et al.*, 1996; Fang *et al.*, 2000; Folmar *et al.*, 2002; Gutendorf and Westendorf, 2001; Mueller, 2002). The *in vitro* assay data were obtained from 2 estrogenicity assays, namely, the MCF-7 proliferation assay and the YES assay (Coldham *et al.*, 1997). The *in vitro* concentration-response data were translated into dose-response data, and the predicted data were then compared with the *in vivo* dose-dependent uterus weight response data obtained from reported uterotrophic assay in rats. The uterotrophic assay is the primary *in vivo* assay that measures the uterus weight increase induced by estrogen receptor agonists in either immature juvenile or ovariectomized young adult female rodents. This endpoint is used in hazard and risk assessment to identify and prioritize endocrine active compounds (Caliman and Gavrilescu, 2009; Harvey and Everett, 2006; O'connor *et al.*, 1996; Piersma *et al.*, 2008). Benchmark dose (BMD) analysis of the predicted and available *in vivo* toxicity data was performed to evaluate the model predictions. To this end, BMD values derived from the predicted dose-response data were compared with BMD values obtained from the reported uterus response data.

MATERIALS AND METHODS

Materials

Diethylstilbestrol (DES, CAS 56-53-1), 17 α -ethinylestradiol (EE2, CAS 57-63-6), genistein (GEN, CAS 446-72-0), coumestrol (COU, CAS 479-13-0), methoxychlor (MXC, CAS 72-43-5), reduced nicotinamide adenine dinucleotide phosphate (NADPH), uridine 5'-diphosphoglucuronic acid (UDPGA), adenosine 3'-phosphate 5'-phosphosulfate (PAPS), lithium salt hydrate, acetyl coenzyme A (acetyl CoA) sodium salt, alamethicin, magnesium chloride, sodium phosphate, sodium chloride, and rat serum were

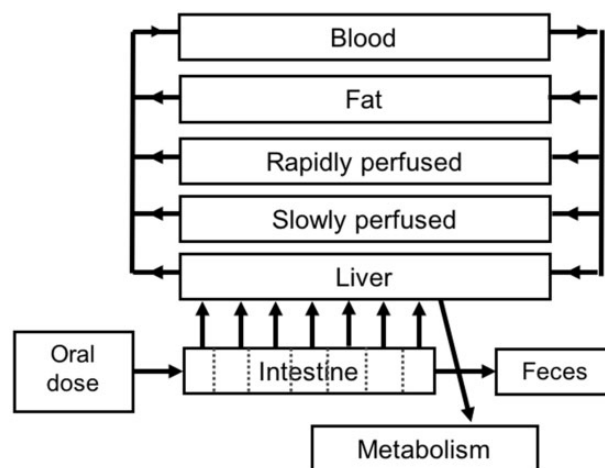


Figure 1. Schematic representation of the generic PBK model. PBK, physiologically based kinetic.

purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Dimethyl sulfoxide (DMSO) was purchased from Acros Organics (Geel, Belgium) and phosphate-buffered saline was purchased from Invitrogen (Breda, the Netherlands). Pooled liver S9 fractions from male and female Sprague-Dawley rats were obtained from Tebu-bio (Heerhugowaard, the Netherlands). Rapid equilibrium dialysis (RED) devices, including RED inserts, RED base plate and sealing tape, were purchased from Fisher (Landsmeer, the Netherlands).

Methods

PBK Modelling-Based Reverse Dosimetry

The PBK modelling-based reverse dosimetry used to predict the uterotrophic growth induced by E2 and BPA (Zhang *et al.*, 2018) was used in the current study, with some minor changes. Five steps were included: (1) development of the PBK models that describe the kinetics of the parent compounds in rats based on a generic conceptual model, (2) PBK model evaluation, (3) quantification of *in vitro* concentration-response data for the selected compounds in *in vitro* estrogenic assays, (4) quantitative extrapolation of the *in vitro* concentration-response data from step iii to *in vivo* dose-response data using the generic PBK model developed in step 1, and (5) evaluation of the predicted dose-dependent estrogenic effects, including BMD analysis on the predicted and available *in vivo* dose-response data obtained from literature.

Development of the PBK models that describe the kinetics of the parent compounds in rats based on a generic conceptual model. In order to use the PBK modelling-based reverse dosimetry for large numbers of compounds, the development of PBK models should be fast and efficient. The PBK models of E2 and BPA developed in our previous study (Zhang *et al.*, 2018) were used as the starting point. The schematic representation of the generic PBK model is displayed in Figure 1. The model consists of 6 compartments including blood, fat, liver, intestines, rapidly perfused tissue, and slowly perfused tissue. Furthermore, to describe the intestinal transition of the parent compound, the intestine compartment was divided in 7 subcompartments. The values for physiological and anatomical parameters were obtained from literature (Brown *et al.*, 1997), and are presented in Supplementary material 1, Table 1. The model equations were coded and

numerically integrated in Berkeley Madonna 8.0.1 (UC Berkeley, CA), using the Rosenbrock's algorithm for stiff systems.

Two administration routes were included in the PBK model, intravenous (IV) and oral administration. The IV exposure was included to enable evaluation of the PBK model predictions using available *in vivo* kinetic data upon IV dosing. The physiological parameter values for the intestine volume, surface area, and transfer rate within the intestines were assumed to be the same for all the subcompartments. The apparent permeability coefficient (P_{app}) value was used to describe the transition rate from each subcompartment to the liver (Louisse *et al.*, 2015; Zhang *et al.*, 2018). The P_{app} values of the parent compounds were determined using the quantitative structure–activity relationship (QSAR) method reported in the literature, using the formula $\text{Log}(P_{app, Caco-2}) = -4.36 - 0.010 \times \text{TPSA}$ (Hou *et al.*, 2004). In this formula, TPSA is the topological polar surface area and the values were computed by PubChem (Kim *et al.*, 2016). The estimated *in vitro* permeability value $P_{app, Caco-2}$ was used to calculate the *in vivo* P_{app} values ($P_{app, in vivo}$) based on the formula $\text{Log}(P_{app, in vivo}) = 0.6836 \times \text{Log}(P_{app, Caco-2}) - 0.5579$ (Sun *et al.*, 2002). The estimated *in vivo* P_{app} values were applied in the PBK models to calculate the intestinal absorption of the parent compounds.

To describe the distribution of the parent compounds to different tissues, the quantitative property relationship (QPPR) approach from DeJongh *et al.* (1997) was used to estimate the partition coefficients. The input parameter of this approach was the octanol-water partition coefficient (P_{ow}). The $\text{Log } P_{ow}$ values of the selected model compounds and the calculated partition coefficients of the compounds are presented in Supplementary material 1, Table 2.

For the reverse dosimetry, it was assumed that the estrogenic effects are induced by the parent compounds and not by their metabolites. This is supported by the fact that metabolites formed were mostly glucuronide conjugates, shown before to be inactive in estrogen bioassays (Islam *et al.*, 2014). This implies that the generic PBK model only needs to include the metabolic clearance of the parent compound. Accordingly, the overall hepatic clearance of the parent compound via both phase I and phase II metabolism was determined by performing the substrate depletion approach, thus accounting for varying types and degrees of metabolism. The underlying conditions for using metabolic clearance instead of Michaelis–Menten kinetics to describe metabolism of the compound in the generic PBK model is that the test concentration of the parent compound remains below the Michaelis constant (K_m) for its metabolic conversion, so that metabolic clearance can be described as a linear process. The test concentration for hepatic clearance was chosen as $3 \mu\text{M}$, assuming that this value is lower than the K_m of the test compounds and the calculation of the clearance values is based on the linear range of the depletion curve. Hepatic clearance of the parent compound was determined, essentially as described before (Zhang *et al.*, 2018), in incubations (total volume $200 \mu\text{l}$) containing $3 \mu\text{M}$ substrate (added from a 100 times concentrated stock solution in DMSO), 0.5 mg/ml rat liver S9 from male or female Sprague-Dawley rats, 3 mM NADPH, 3 mM UDPGA, 0.2 mM PAPS, and 0.5 mM acetyl CoA in 0.1 M K_2HPO_4 (pH 7.4) containing 5 mM MgCl_2 and 0.025 mg/ml alamethicin. The incubation time points were as follows: 0, 1, 2, 3, 4, 5, 7, 8.5, 10, 15, 20, 25, 30, 45, 60, and 90 min. For each incubation time point a corresponding control was included, consisting of an incubation performed in the absence of all the co-factors. All incubations were performed in triplicate in 3 independent studies. The concentration of the parent compound was quantified using

Table 1. The Retention Time and Absorption Wavelength Used for Quantification of the Parent Compounds by UPLC

Compound	Wavelength (nm)	Retention time (min)
DES	230	5.03
EE2	225	4.72
GEN	245	3.83
COU	255	3.76
MXC	240	5.02

ultra-performance liquid chromatography (UPLC) analysis performed as described below. The natural logarithm (\ln) of the ratio of the remaining parent compound concentration in the incubation sample (C_{compound}) and in the control without co-factors (C_{control}) was calculated for each incubation time and the depletion curve of the parent compound ($\ln(C_{\text{compound}}/C_{\text{control}})$) against time was derived. The slope of the linear part of the depletion curve represents the elimination rate (k , 1/min) of the parent compound. By using the following equation, the *in vitro* clearance ($\text{CL}_{\text{int, in vitro}}$) of the parent compound was estimated: $\text{CL}_{\text{int, in vitro}} (\mu\text{l}/\text{min}/\text{mg protein}) = V (\mu\text{l}/\text{mg protein}) \times k (1/\text{min})$ (Obach, 1999; Sjögren *et al.*, 2009). V represents the reciprocal of the protein concentration in the incubation mixture, which is the ratio of the incubation volume (μl) divided by the amount of protein in the mixture (mg). The *in vitro* CL_{int} of the parent compound was scaled to a whole liver by assuming that the S9 protein concentration in rat liver is 87 g/kg liver (Chiu and Ginsberg, 2011).

UPLC ANALYSIS. A UPLC H-Class system (Waters Acquity) equipped with a Waters BEH C18 ($1.7 \mu\text{m}$, $2.1 \times 50 \text{ mm}$) column was used to determine the area under the curve (AUC) of the peaks of the parent compounds. The temperature was set at 40°C for the column and 5°C for the samples. The flow rate was set at $0.6 \text{ ml}/\text{min}$ and the injection volume was $3.5 \mu\text{l}$. A solution of 4 mM ammonium formate was used as mobile phase A and acetonitrile was used as mobile phase B. The initial condition of the eluents was 95% A and this condition was maintained for 1 min. Then the gradient changed to 35% A in 5 min, subsequently returned to the initial conditions in the next minute and was kept for another minute at the initial conditions before the next injection, with a total running time of 8 min. The amount of compound present in each incubation mixture was quantified using a calibration curve made with commercially available reference compounds, using the absorption wavelengths and retention times presented in Table 1.

PBK model evaluation. To evaluate the predictions made by the developed PBK models, the predicted time-dependent blood concentrations of the parent compounds were compared with the available time-dependent blood concentrations in rats reported in the literature.

To investigate the influential parameters of the developed PBK model for the prediction of the maximum blood concentration (C_{max}) of the parent compound, a sensitivity analysis was conducted. To this end, the initial input parameter value was increased by 5% and the sensitivity coefficients (SC) were calculated using the equation $\text{SC} = (C' - C)/(P' - P) \times (P/C)$, in which P and P' represent the initial and modified parameter values, whereas C and C' are the initial and modified model output for C_{max} (Evans and Andersen, 2000; Waters *et al.*, 2008). Each parameter was analyzed individually by changing one parameter at a time keeping the other parameters at their original value,

and the total blood flow fraction was kept as 1. The sensitivity analysis was performed for oral exposure to a single dose of the parent compound, including 4 mg/kg bw DES, 0.007 mg/kg bw EE2, 6.25 mg/kg bw GEN, and 3 mg/kg bw COU, respectively, which were the doses applied in the *in vivo* kinetic studies that were used for the model evaluation (Ako, 2011; Bawarshi-Nassar et al., 1989; Mallis et al., 2003; Teng et al., 2012; Zhou et al., 2008). For MXC, no available *in vivo* kinetic data were found, and the sensitivity analysis was done using a dose of 20 mg/kg bw, which equals one of the test doses in the uterotrophic assay for MXC (Kanno et al., 2003).

Quantification of in vitro effect concentrations of compounds in in vitro estrogenicity assays. MCF-7 proliferation assay data and YES assay data for the current study were obtained from the literature. The MCF-7 proliferation assay measures the increase of cell numbers resulting from the estrogenic activity mediated via the estrogen receptor(s) (Desaulniers et al., 1998; Wang et al., 2012; Zacharewski, 1997). The YES assay is an estrogen receptor gene assay, using recombinant yeast cells containing a reporter gene lacZ that can be activated by the estrogenic compound inducing the formation of β -galactosidase. Then the synthetic β -galactosidase will react with the added substrate chlorophenol red- β -D-galactopyranoside (CPRG) that leads to a measurable color change from yellow to red in the medium as a direct detection for the estrogenic activity of the compound (Kinnberg, 2003; Sonneveld et al., 2006; Zacharewski, 1997). The *in vitro* data were obtained from different studies reported in the literature. Concentration-response curves were expressed in percentage of the maximum response of the compound in each study. The concentration-response curves thus obtained were fitted to a nonlinear regression sigmoidal model (4 parameters) in GraphPad Prism 5 (GraphPad Software Inc., San Diego, California) to derive the EC₅₀ values.

Quantitative extrapolation of in vitro concentration-response data (from step 3) to in vivo dose-response data using the generic PBK model developed in step 1. The *in vitro* concentration-response data obtained were used to predict the dose levels that are required to reach the respective effect concentrations in blood, using PBK modelling-based reverse dosimetry. To this end, it was assumed that it is the unbound fraction (f_{ub}) of the compound that causes the estrogenic effect. This implies that the concentration used in the *in vitro* assay has to be corrected for the difference in protein binding between the *in vitro* assay medium and rat serum prior to applying reverse dosimetry. To this end, RED was performed to determine the f_{ub} of the compounds in the *in vitro* assay medium and in rat serum, according to the protocol described previously (Zhang et al., 2018). The *in vitro* proliferation assay medium used to generate the *in vitro* data contained either 5% fetal bovine serum (FBS; Breinholt and Larsen, 1998; Folmar et al., 2002; Körner et al., 2001; Okubo et al., 2001; Schultis and Metzger, 2004; Wang et al., 2014) or 10% FBS (Dodge et al., 1996; Gutendorf and Westendorf, 2001; Kinjo et al., 2004; Kuruto-Niwa et al., 2007). The f_{ub} values in the *in vitro* proliferation assay medium were measured by RED for medium containing 5% FBS, whereas the f_{ub} values for *in vitro* assay medium containing 10% FBS were assumed to be half of the measured f_{ub} values at 5% FBS, based on the assumption of a linear increase in protein binding with the amount of protein. The YES assay medium does not contain protein; hence, the f_{ub} of compounds in the YES assay medium was set at 1. The determined f_{ub} of the test compounds in the *in vitro* assay medium ($f_{ub, in vitro}$) and in rat

Table 2. In Vitro Hepatic Clearance (CL_{int}) Values of the Parent Compounds Using Male or Female Sprague-Dawley Rat Liver S9

Compound	Male CL _{int} (μl/min/mg protein)	Female CL _{int} (μl/min/mg protein)
EE2	149 ± 9	65 ± 4
GEN	328 ± 6	106 ± 7
COU	650 ± 15	429 ± 12
MXC	448 ± 8	313 ± 5

The *p*-values of the *t*-tests for the CL_{int} between male and female were lower than 0.0001.

serum ($f_{ub, in vivo}$) can be used to estimate the effect (free) concentration *in vitro* ($C_{eff, in vitro}$) and *in vivo* ($C_{eff, in vivo}$) using the following equations: $C_{eff, in vitro} = C_{in vitro} \times f_{ub, in vitro}$ and $C_{eff, in vivo} = C_{in vivo} \times f_{ub, in vivo}$, where $C_{in vitro}$ is the test concentration applied in the *in vitro* assay and $C_{in vivo}$ is the nominal blood concentration in rats. Setting $C_{eff, in vitro}$ equal to $C_{eff, in vivo}$ allows calculation of $C_{in vivo}$ from $C_{in vitro}$ taking protein correction into account using the formula: $C_{in vivo} = (C_{in vitro} \times f_{ub, in vitro}) / f_{ub, in vivo}$. After this correction, the $C_{in vivo}$ was used in the reverse dosimetry to calculate the corresponding dose level. By repeating this analysis for each *in vitro* test concentration, the *in vitro* concentration-response data were translated into *in vivo* dose-response data.

Evaluation of the predicted dose-dependent estrogenic effects. To assess the prediction of the estrogenic effects of the compounds based on the combined *in vitro*-PBK modeling approach, the predicted dose-response data were compared with data reported in the literature for the *in vivo* uterotrophic effects of the compounds in rats. Not all the available references provided the body weight of the rats studied, and therefore, the values for the absolute increase in uterus weight were not normalized to animal body weight. Therefore, the absolute increase in uterus weight was used to define the *in vivo* dose-response curves.

In addition, BMD analysis was performed to compare the points of departure (PODs) that could be derived from the predicted and experimental dose-response curves. PROAST software version 65.5 was used to conduct the BMD analysis, using the exponential model for continuous data. For those *in vitro* data obtained from literature that only provided EC₅₀ values and no corresponding concentration-response curves, the EC₅₀ values were used in the reverse dosimetry to calculate the relevant ED₅₀ values as the POD. In order to present all the data in a consistent manner, for the datasets for which a full dose-response curve could be predicted, the benchmark response (BMR) was defined as 50% increase compared with the control and the BMD₅₀ and corresponding 95% lower and upper bound confidence limits (BMDL₅₀ and BMDU₅₀) were obtained. Furthermore, from an agreement analysis on the BMD values obtained a Bland-Altman plot was created using Graphpad Prism 5 (GraphPad Software Inc., San Diego, California) to analyze the difference in BMD values between the predictions and the *in vivo* uterotrophic data.

RESULTS

Development of the Generic PBK Model

The physiological and anatomy parameter values, obtained from Brown et al. (1997), are presented in Supplementary

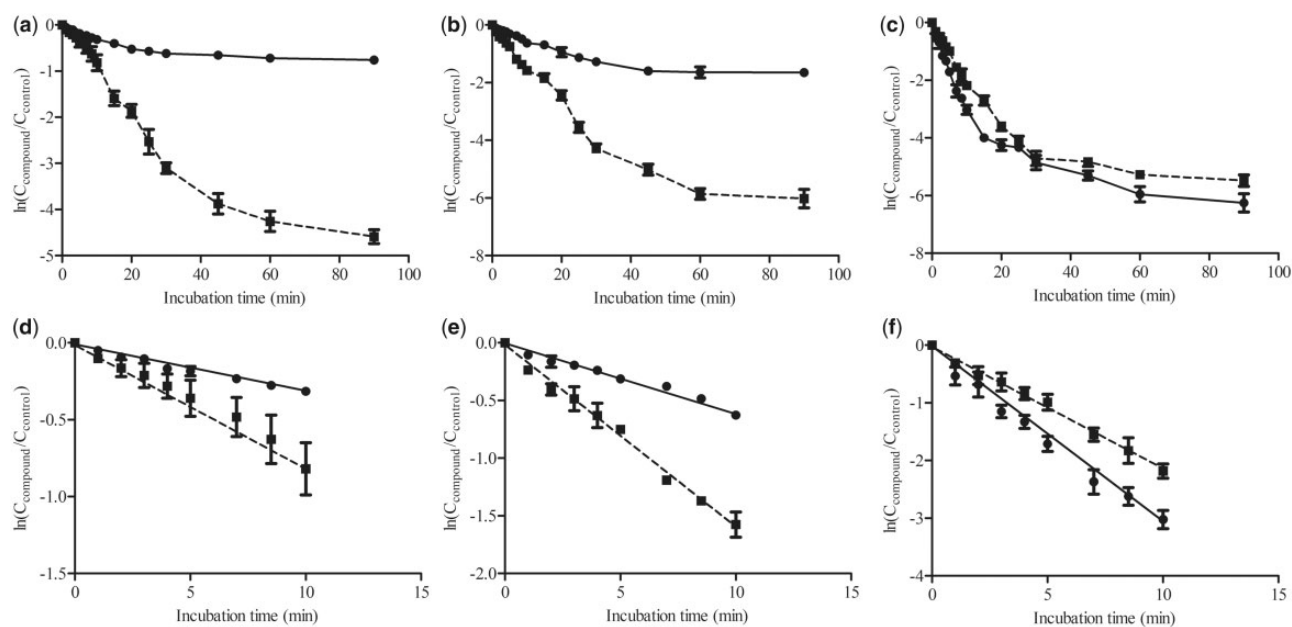


Figure 2. The substrate depletion curves and the linear parts of the curves that use to calculate the clearance values of EE2 (a and d), GEN (b and e), and COU (c and f). Symbols represent the average $\ln(C_{\text{compound}}/C_{\text{control}})$ at different incubation time points. Data derived from incubation with female liver S9 fraction from Sprague-Dawley rats were shown in circles and the depletion curves were shown in straight lines; data obtained from incubation with male liver S9 fraction from Sprague-Dawley rats were shown in squares and the depletion curves were shown in dashed lines. EE2, Ethinylestradiol; GEN, genistein; COU, coumestrol.

Table 3. Characteristics of the in Vivo Kinetic Studies Used to Evaluate the PBK Model Predictions

Compound	Species	Gender	Exposure route	Dose (mg/kg bw)	References
EE2	Sprague-Dawley rats	Male	IV, intraduodenal	0.00175, 0.0035, 0.0070	Bawarshi-Nassar et al. (1989)
GEN	Sprague-Dawley rats	(no information)	IV, oral gavage	IV: 12.5 Oral: 6.25, 12.5, 50	Zhou et al. (2008)
COU	Sprague-Dawley rats	Male	Oral	3	Mallis et al. (2003)

material 1, Table 1. The partition coefficients of all parent compounds calculated using the QPPR method described by DeJongh et al. (1997) are presented in Supplementary material 1, Table 2. The model code of the developed generic PBK model is presented in Supplementary material 2 using the PBK model for EE2 as an example. The in vitro hepatic clearance (CL_{int}), measured in incubations containing male or female rat liver S9 with all co-factors of phase I and phase II reactions in one mixture, is presented in Table 2. The depletion curves were shown in Figure 2. The large difference in clearance of EE2 and GEN by male as compared with female liver S9 was previously also observed for E2 (Zhang et al., 2018). The hepatic clearance of DES and the evaluation of the PBK model predictions were previously reported (Adam et al., 2019).

PBK Model Evaluation

To assess the model predictions, the predicted time-dependent blood concentrations of the selected compounds were compared with in vivo kinetic data in rats reported in the literature. Table 3 presents the characteristics of the in vivo kinetic studies used for the model evaluation.

Figures 3 and 4 present the time-dependent blood concentrations as reported in the literature (symbols) upon, respectively, IV and oral administrations, as compared with the predicted values (lines). The PBK model-predicted blood concentrations were in line with the actual blood concentration of the parent compounds reported in the literature. Figure 3 demonstrates that the

developed generic PBK model adequately predicted the blood concentration of EE2 (Figure 3a) and GEN (Figure 3b) upon IV administration at various dose levels. Moreover, the developed PBK model also predicted the maximum blood concentration of the parent compounds (C_{max}) upon oral administration relatively well (Figure 4). For the oral administration, the difference in C_{max} between the model predictions and the in vivo kinetic data amounted to 3.2- to 3.6-fold for EE2, 1.0- to 1.4-fold for GEN and 2.2-fold for COU. Interestingly, the in vivo blood concentration data upon oral administration of GEN reported by Zhou et al. (2008; Figure 4b) do not show an efficient clearance upon appearance of the parent compound in the systemic circulation. This is in contrast to the in vivo kinetic data reported in the same in vivo study upon IV administration of GEN, which indicate an efficient clearance resulting in an obvious time-dependent decrease in the blood concentration of GEN over time (Figure 3b). Enterohepatic circulation cannot explain this unlogic difference in clearance from the systemic circulation upon IV or oral dosing, because once the compound is fully absorbed and in the systemic circulation clearance would be expected to proceed following similar kinetics.

To identify the most influential parameters of the PBK model on the model prediction of C_{max} upon oral administration, a sensitivity analysis was performed for a different single oral dose level for each compound. Figure 5 displays the parameters for which the absolute value of the SC was higher than 0.1. The results reveal that fraction of liver tissue (VLc), fraction of blood flow to the liver (QLc) and hepatic clearance (CL_{int}) are the most influential parameters. In addition, the parameters related to

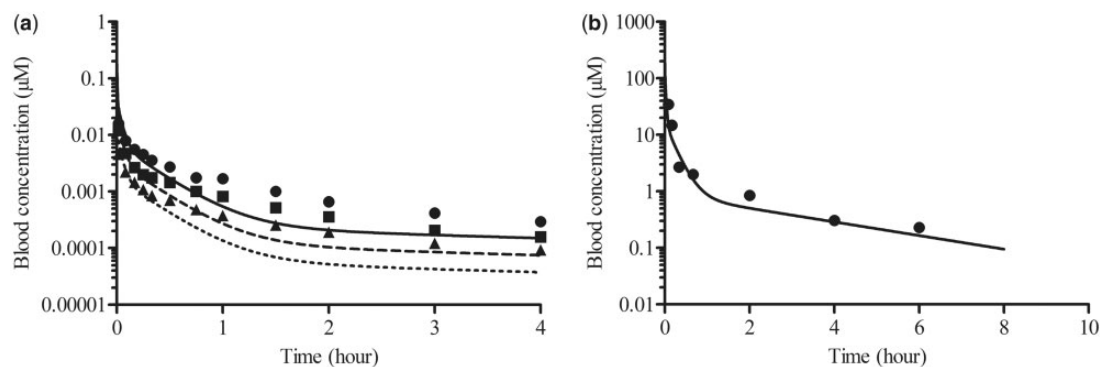


Figure 3. Blood concentrations of the parent compounds in rats after IV administration. Symbols represent the average blood concentrations reported in the literature. Lines represent blood concentrations predicted by the generic PBK model. Compounds and dose levels are as follows: a, EE2, 0.00175 mg/kg bw (triangles, dotted line), 0.0035 mg/kg bw (squares, dashed line) and 0.007 mg/kg bw (circle, straight line) (Bawarshi-Nassar *et al.*, 1989) and b, GEN, 12.5 mg/kg bw (squares, straight line; Zhou *et al.*, 2008). EE2, Ethinylestradiol; GEN, genistein; IV, intravenous; PBK, physiologically based kinetic.

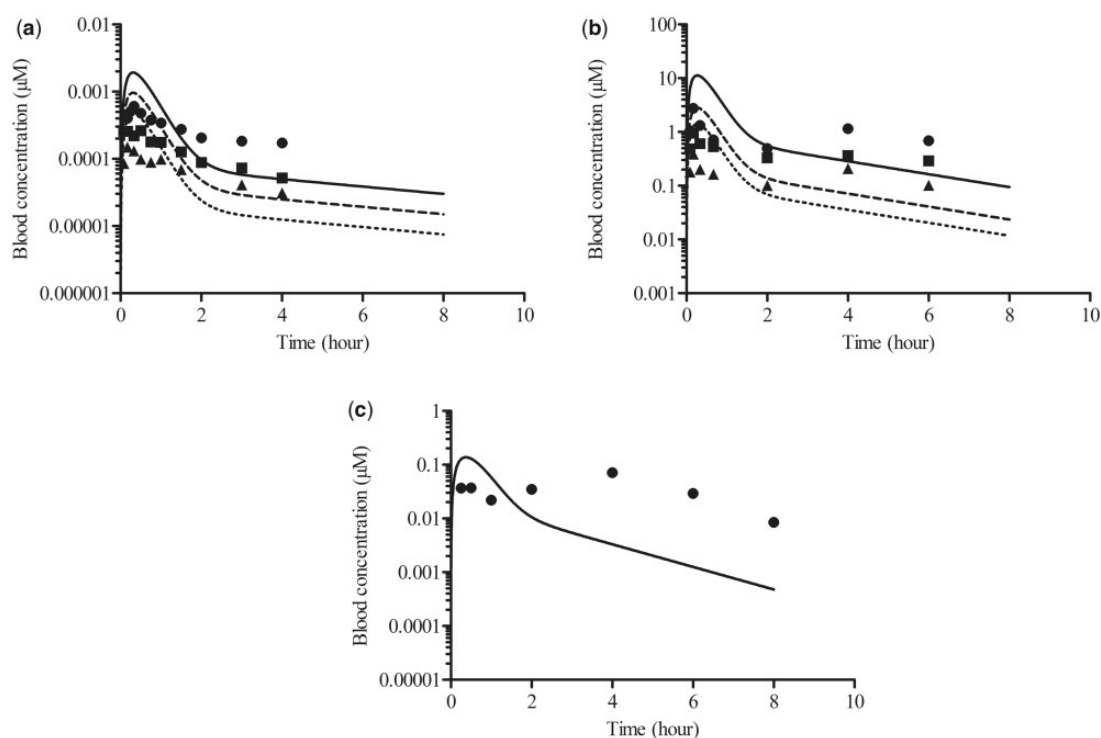


Figure 4. Blood concentrations of the parent compounds in rats after oral administration. Symbols represent the average blood concentrations reported in the literature. Lines represent blood concentrations predicted by the generic PBK model. Compounds and dose levels are as follows: a, EE2, 0.00175 mg/kg bw (triangles, dotted line), 0.0035 mg/kg bw (squares, dashed line) and 0.007 mg/kg bw (circle, straight line) (Bawarshi-Nassar *et al.*, 1989); b, GEN, 6.5 mg/kg bw (triangles, dotted line), 12.5 mg/kg bw (squares, dashed line) and 50 mg/kg bw (circle, straight line) (Zhou *et al.*, 2008), and c, COU, 3 mg/kg bw (circle, straight line) (Mallis *et al.*, 2003). COU, coumestrol; EE2, Ethinylestradiol, GEN, genistein; PBK, physiologically based kinetic

the subcompartments of the intestines are of influence as well, namely intestine volume for each compartment (V_{in}), intestinal surface area for the intestinal compartment (SA_{in}) and the transfer rate from one subcompartment to another (kin).

Quantification of in Vitro Effect Concentrations of Compounds in In Vitro Estrogenicity Assays

Figure 6 displays the in vitro data obtained from the MCF-7 proliferation assay (straight line) and the YES assay (dashed line), presenting each response as a percentage of the maximum response of the compound, for DES (Figure 6a), EE2 (Figure 6b), GEN (Figure 6c), COU (Figure 6d), and MXC (Figure 6e),

respectively. For those in vitro data obtained from literature that only provided EC_{50} values and did not report the underlying concentration-response curves, the available EC_{50} values of the MCF-7 proliferation assay (circles) and YES assay data (triangles) are shown in Figure 6 as well as single data points. The actual EC_{50} values obtained from literature and the values derived from concentration-response curves can be found in Supplementary material 3. The result in Figure 5 reveal that for all the compounds except COU, the MCF-7 proliferation assay is more sensitive compared with the YES assay because the EC_{50} values from the proliferation assay are lower than those from the YES assay. Furthermore, the results also reveal a relatively large variation between the EC_{50} s reported for each compound

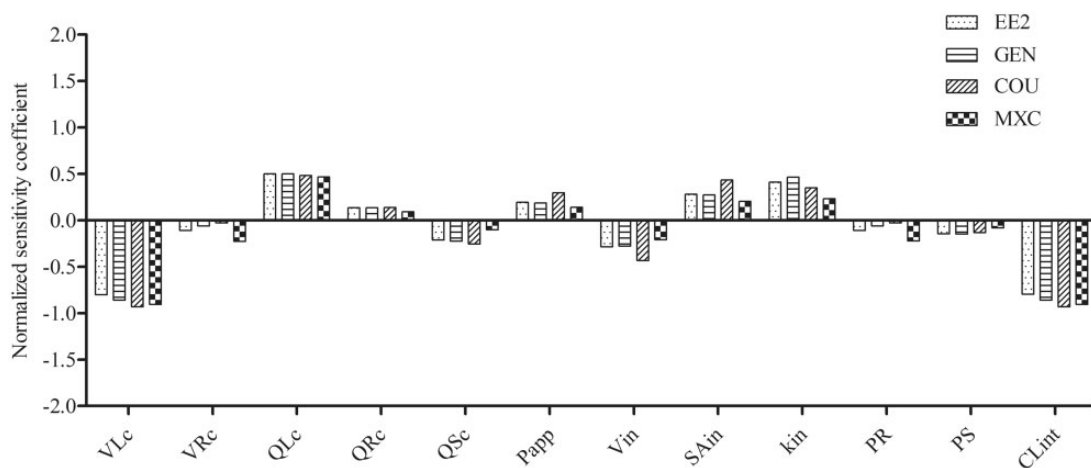


Figure 5. Normalized SCs of PBK model parameters for the predicted C_{max} of parent compounds in blood after oral administration (0.007 mg/kg bw EE2, or 6.25 mg/kg bw GEN, or 3 mg/kg bw COU, or 20 mg/kg bw MXC). All model parameters with normalized SCs with an absolute value higher than 0.1 are shown. SC, sensitivity coefficient; VL_c, fraction of liver tissue; VR_c, fraction of rapidly perfused tissue; VS_c, fraction of slowly perfused tissue; QL_c, fraction of blood flow to liver; QR_c, fraction of blood flow to rapid perfused tissue; QS_c, fraction of blood flow to slowly perfused tissue, $P_{app} = P_{app}$ valued estimated from QSAR method; V_{in}, intestine volume for each compartment; SA_{in}, intestinal surface area for intestinal subcompartment; kin, transfer rate from one subcompartment to another in intestines, PR, rapidly perfused tissue/blood partition coefficient, PS, slowly perfused tissue/blood partition coefficient; CL_{int}, experimental hepatic clearance of parent compound.

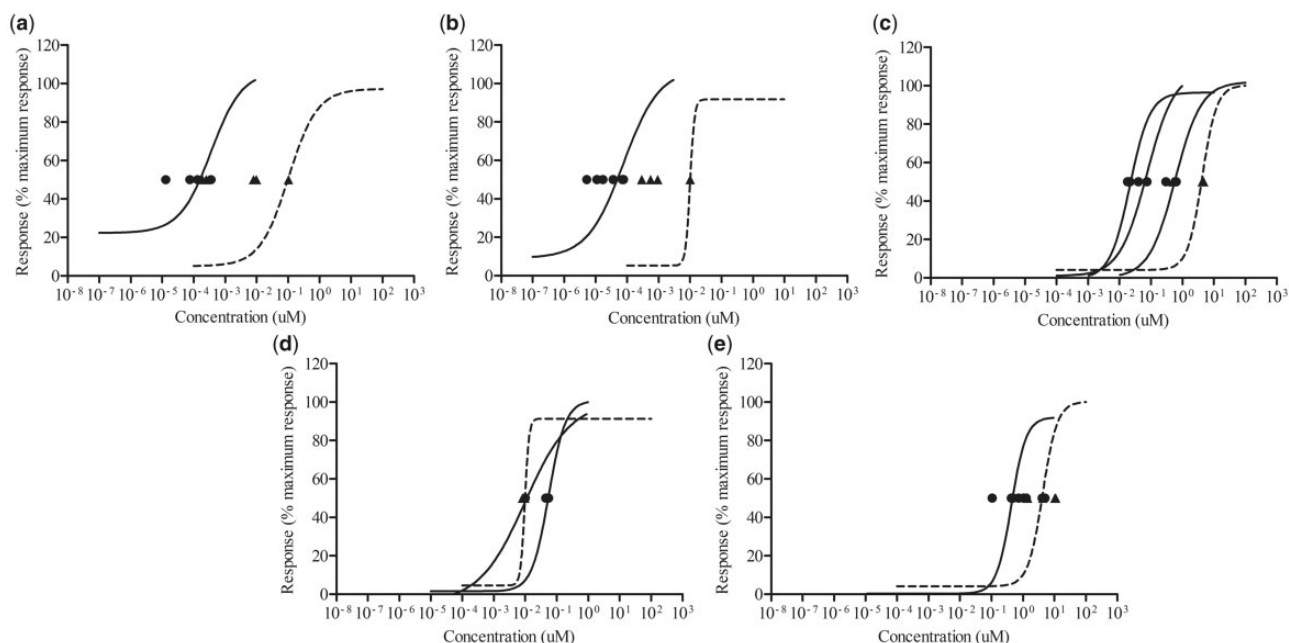


Figure 6. In vitro concentration-response curves (response as percentage of maximum response of the compound) of DES (a), EE2 (b), GEN (c), COU (d), and MXC (e) derived from proliferation assay data (straight line), YES assay data (dashed line), and EC₅₀ values obtained from different in vitro proliferation assays (circles) and YES assay (triangles) reported in the literature (Andersen et al., 1999; Breinholt and Larsen, 1998; Dodge et al., 1996; Folmar et al., 2002; Gutendorf and Westendorf, 2001; Kinjo et al., 2004; Kolle et al., 2010; Körner et al., 2001; Kuruto-Niwa et al., 2007; Okubo et al., 2001; Schultis and Metzger, 2004; Wang et al., 2014).

in the same in vitro assay suggesting substantial variability in the reported in vitro concentration-response data for all model compounds.

Quantitative Extrapolation of In Vitro Concentration-Response Data (from Step 3) to In Vivo Dose-Response Data Using the Generic PBK Model Developed in Step 1

Table 4 presents the f_{ub} values of the model compounds as determined by RED in the in vitro assay medium for MCF-7 proliferation and in rat serum. For the assay mediums that contain 10% FBS, the unbound fraction (f_{ub}) was assumed to be half of

the f_{ub} value for 5% FBS. These values were used to convert the in vitro concentrations to corresponding in vivo blood concentrations, which were subsequently translated into corresponding dose levels using the PBK modeling-based reverse dosimetry. The in vivo dose-response curves thus obtained are presented in Figure 7. Similar to the in vitro concentration-response curves, these predicted dose-response curves show substantial variation depending on the in vitro assay data used to define the in vivo dose-response curves. Predicted curves based on in vitro data obtained from the MCF-7 proliferation assay generally show the effects at lower dose levels than the curves predicted based on YES assay data.

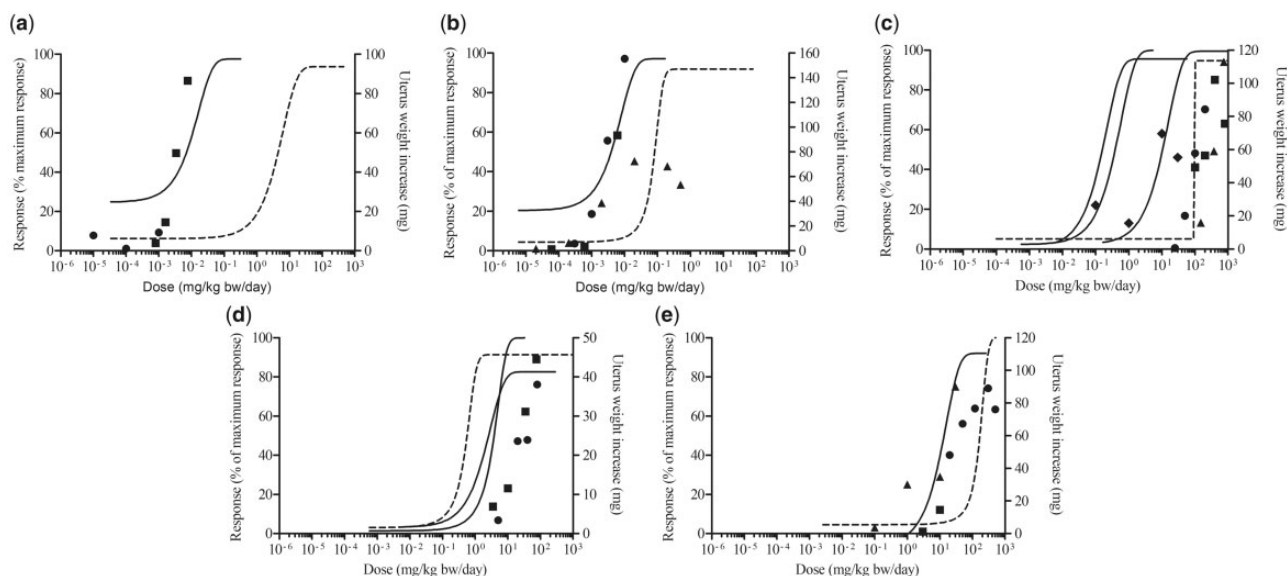


Figure 7. Predicted in vivo dose-response data in rats compared with in vivo data on uterotrophic response upon exposure to DES (a), EE2 (b), GEN (c), COU (d), and MXC (e). The predicted dose-response data were based on in vitro concentration response data obtained in the proliferation assay (straight lines) and on the YES assay (dashed line) (Figure 5). Symbols represent the absolute uterus weight of rats obtained from in vivo uterotrophic assays. For DES (a), in vivo data from Odum et al. (2002) (squares) and from Yamasaki et al. (2000) (circles); for EE2 (b), in vivo data from Yamasaki et al. (2000) (squares), from Kanno et al. (2001) (circles) and from Odum et al. (1997) (triangles); for GEN (c), in vivo data from Patissaul et al. (2002) (squares), from Stroheker et al. (2003) (circles), from Santell et al. (1997) (triangles) and from Dodge et al. (1996) (diamonds); for COU (d), in vivo data from Odum et al. (1997) (squares) and from Baker et al. (1999) (circles) and for MXC (e), in vivo data from Bulger et al. (1978) (squares), from Kanno et al. (2003) (circles) and from Dodge et al. (1996) (triangles).

Evaluation of the Predictions of Dose-Dependent Estrogenic Effects

In addition to the predicted dose-response curves for estrogenicity, Figure 7 also presents the in vivo data reported for the respective compounds in the uterotrophic assay. Table 5 summarizes the information of the in vivo uterotrophic studies in rats that were used to evaluate the predicted in vivo dose-response data. For DES, EE2, and COU, the predictions from the proliferation data match well with the in vivo uterotrophic response data. For GEN, the prediction from the YES assay is in line with the in vivo data. For MXC, the in vivo uterus response data are between the predictions from MCF-7 proliferation and YES assay data. In spite of the variability, the predictions reveal a comparable potency ranking as derived from the in vivo data with the potency in the predicted estrogenicity, with DES and EE2 showing the highest estrogenic potency, GEN and COU being intermediate, and MXC showing the lowest potency. Furthermore, all predictions reveal that the selected model compounds are likely to have estrogenic effects at dose levels below a threshold of 1000 mg/kg bw/day, a dose level above which in vivo testing is not considered of physiological relevance.

To quantitatively compare the predicted and experimental in vivo dose-response data, all dose-response curves were analyzed by BMD modeling to define the BMDL₅₀ to BMDU₅₀ values as the points of comparison. The results are displayed in Figure 8. In this figure, the results for E2 and BPA obtained in our previous study using the same approach (Zhang et al., 2018) were also included for comparison.

Figure 8 facilitates comparison of the potential of the model compounds with DES, E2, and EE2 being the most potent, and BPA being the least potent. For E2, BPA, DES, GEN, and MXC, the BMDL₅₀ to BMDU₅₀ values derived from the predictions based on the YES assay match well with the BMDL₅₀ to BMDU₅₀ ranges obtained from the in vivo studies, whereas for EE2 and COU the

predictions based on the MCF-7 proliferation data are closer to the BMD₅₀ values from the in vivo uterus response data. Besides, the results of the Bland–Altman plot show that the difference between the BMD values from the predictions and the values obtained from in vivo studies were in the range of the 95% limit of agreement (results are shown in Supplementary material 4).

DISCUSSION

The aim of the present study was to assess the potential of using a generic PBK model combined with in vitro toxicity data to predict the in vivo estrogenicity for a group of compounds in rats. In a previous study, QIVIVE using the generic PBK model was shown valid for E2 and BPA. In the present study the approach was challenged using an additional series of model compounds. Five additional compounds, DES, EE2, GEN, COU, and MXC, were selected to investigate whether the in vivo uterus weight increase induced by these compounds in rats can be predicted using the in vitro estrogenic data in combination with the generic PBK model adapted to include the physico-chemical and kinetic parameters of the respective compounds. The in vitro MCF-7 proliferation assay, which measures the increase of cell numbers induced via the estrogen receptor; and the YES assay, in which the yeast cells were engineered with overexpressed human estrogen receptor; were selected as in vitro endpoints for estrogenicity (Coldham et al., 1997). In vitro concentration-response data were derived from literature, and quantitatively translated into predicted dose-response data taking into account the difference in protein binding in the in vitro assay medium and in rat serum as measured in the present study. BMD analysis was performed on the predicted dose-response data and on the available in vivo uterotrophic data in rats. The evaluation of the BMD results indicated that QIVIVE using the generic PBK model in combination with

Table 4. Fraction Unbound (f_{ub}) of the Parent Compounds in the In Vitro Assay Medium for MCF-7 Proliferation Containing 5% FBS and in Rat Serum

Compound	In vitro assay medium (5% FBS)	Recovery rate in vitro assay medium	Rat serum	Recovery rate in rat serum
DES	0.74 ± 0.02	79% ± 0.026	0.48 ± 0.08	77% ± 0.024
EE2	0.72 ± 0.18	86% ± 0.009	0.46 ± 0.03	80% ± 0.015
GEN	0.80 ± 0.04	88% ± 0.030	0.07 ± 0.02	83% ± 0.026
COU	0.69 ± 0.034	79% ± 0.019	0.35 ± 0.07	82% ± 0.015
MXC	0.73 ± 0.29	77% ± 0.021	0.27 ± 0.04	76% ± 0.037

Table 5. Studies Reporting In Vivo Uterotrophic Assay Data That Were Used to Evaluate the Predicted In Vivo Dose-Responses Data Based on the PBK Modelling-Based Reverse Dosimetry of In Vitro Estrogenicity Data

Compound	Species	Exposure route	Dose (mg/kg bw/day)	References
DES	Alpk: APfSD rats	Drinking water	0.0008, 0.0016, 0.0034, 0.0077	Odum et al. (2002)
DES	Sprague-Dawley rats	Oral	0.00001, 0.0001, 0.001	Yamasaki et al. (2000)
EE2	Sprague-Dawley rats	Oral	0.00006, 0.0006, 0.006	Yamasaki et al. (2000)
EE2	Alpk: APfSD rats	Oral gavage	0.00002, 0.0002, 0.002, 0.02, 0.2, 0.5	Odum et al. (1997)
EE2	Sprague-Dawley rats	Oral gavage	0.0001, 0.0003, 0.001, 0.003, 0.001	Kanno et al. (2001)
GEN	Wistar rats	Oral gavage	25, 50, 100, 200	Stroheker et al. (2003)
GEN	Sprague-Dawley rats	Dietary treatment	150, 375, 750	Santell et al. (1997)
GEN	Long Evans rats	Dietary treatment	100, 200, 400, 800	Patisaul et al. (2002)
GEN	Sprague-Dawley rats	Oral gavage	0.1, 1, 10, 30	Dodge et al. (1996)
COU	Alpk: APfSD rats	Oral gavage	3.5, 10, 35, 75	Odum et al. (1997)
COU	Wistar rats	Oral gavage	5, 20, 40, 80	Baker et al. (1999)
MXC	Sprague-Dawley rats	Intraperitoneal injection	3, 10	Bulger et al. (1978)
MXC	Sprague-Dawley rats	Oral gavage	20, 50, 120, 300, 500	Kanno et al. (2003)
MXC	Sprague-Dawley rats	Oral gavage	0.1, 1, 10, 30	Dodge et al. (1996)

reverse dosimetry enables the prediction of the estrogenicity of the selected compounds, with the predictions based on the MCF-7 proliferation assay assuring a safe point of departure for further risk assessment, albeit conservative.

In the present study, the generic PBK model developed in our previous study (Zhang et al., 2018) was used with a minor modification aiming to further enable definition of the PBK model parameters without the need for experimental data. In the current study, the permeability value (P_{app}) of the parent compound, used to describe the transition between the intestines and liver, was quantified using a QSAR approach based on the topological polar surface area of the compound (DeJongh et al., 1997).

One of the assumptions made in the current study was that the estrogenic effects are induced by the parent compounds and not by the metabolites. For MXC, despite the fact that its phase I metabolites mono-demethylated methoxychlor and HPTE have been demonstrated to activate the estrogen receptor in vitro (ATSDR AfTsaDR, 2002; Dehal and Kupfer, 1994; Hu and Kupfer, 2002; Metcalf et al., 1970), due to the rapid phase II metabolism, these cytochrome P450s mediated phase I metabolites are expected to be conjugated and rapidly excreted (Parkinson, 2001). Therefore, the in vivo concentration of these metabolites is unlikely to reach concentrations in the systemic circulation able to induce significant estrogenic effect. This illustrates the importance to consider phase I and phase II metabolism for QIVIVE. In the current study, both phase I and phase II metabolisms were included when measuring the hepatic clearance of the parent compound using all the relevant co-factors and rat liver S9 fraction. The results obtained, showing that also for MXC, the approach did provide reliable predictions, indirectly corroborate that this is a valid approach. In this initial generic approach, it is assumed that there are no effects on liver weight

and/or enzyme inductions upon exposure to the estrogenic compounds. Obviously this would be an interesting further refinement of the approach, but was beyond the aim of the present study.

The time-dependent blood concentrations predicted by the generic PBK model were in line with the in vivo time-dependent blood concentrations upon IV (Figure 3) or oral (Figure 4) administration. Deviation of the overall predicted time-dependent blood concentration from in vivo kinetic data upon oral administration may to some extent be due to the fact that the literature reported in vivo data, for example for COU (Figure 4b), do not show efficient elimination clearance, which may not be fully realistic when taking clearance of the compounds observed upon IV routes from the same literature into account. The maximum blood concentration (C_{max}) which is the parameter used for the QIVIVE was well predicted. Our study reveals that the difference in C_{max} between the prediction and the in vivo kinetic data was 1.0- to 3.6-fold. It was concluded that the developed generic PBK model can adequately predict the C_{max} upon oral administration, and the model can be used to predict the in vivo uterus responses. Considering that the values for most input parameters of the generic PBK model were obtained from available in vivo data or in silico methods, with only 2 parameters that were determined from experiment (CL_{int} and f_{ub}), the adequate prediction of C_{max} demonstrates the efficiency of using the generic PBK model. It is of interest to note that the dose levels tested in the in vivo kinetic studies used to validate the PBK models were lower than the dose levels tested in the uterotrophic studies, especially for GEN and COU. If at these higher dose levels metabolic pathways for clearance would be saturated, the PBK model may under predict the blood concentration of the parent compound, and consequently over predict the estrogenic effects in rats.

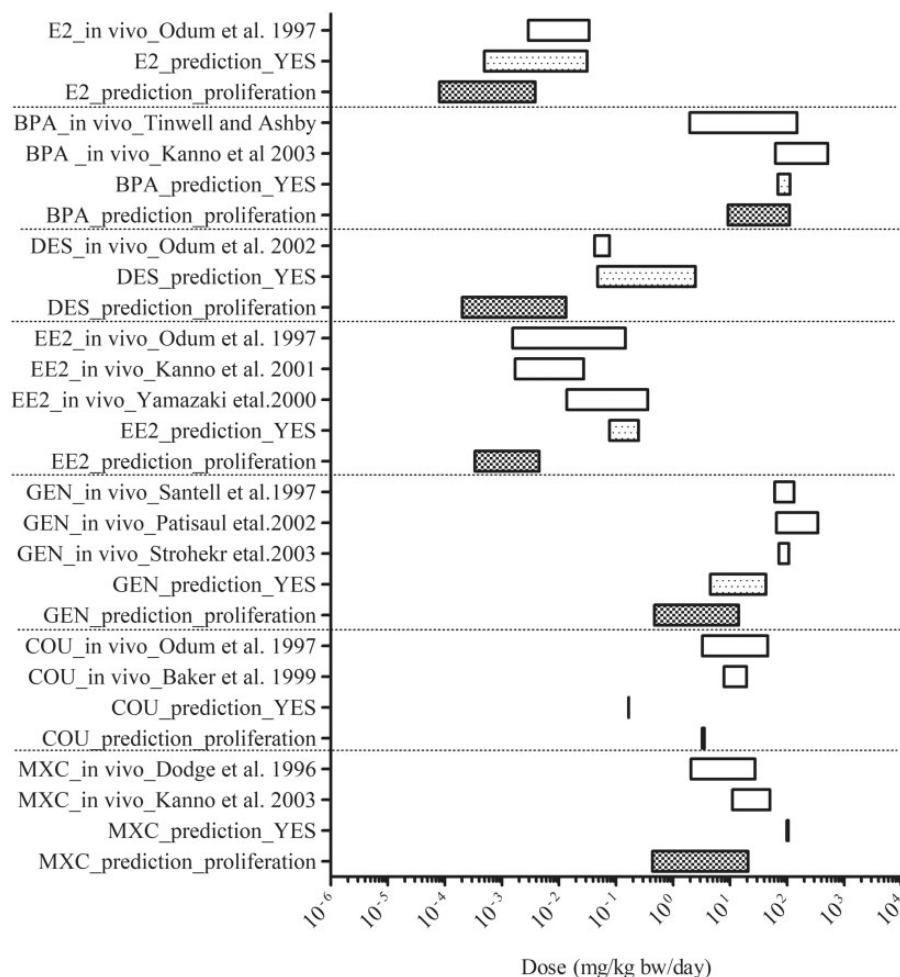


Figure 8. Comparison of the BMD values from predicted dose-response data and in vivo dose-response data of the parent compounds. BMD analyses were performed using the BMR as 50% increase compared with control in PROAST. The results of the in vivo uterotrophic response data are presented as the ranges of BMDL₅₀ to BMDU₅₀ values (empty boxes) obtained from different in vivo studies (Table 5). The range of BMD₅₀ values derived from the predictions based on proliferation assay (dashed boxes) and YES assay data (mosaic boxes) were shown as the box.

The in vitro data of the MCF-7 proliferation assay and the YES assay were derived from available studies reported in the literature (Figure 6). Both in vitro assays are based on cells that mainly contain and detect ER α -mediated estrogenic responses. This makes them adequate cellular models given that the predominant estrogen receptor in rat uterus is ER α , with ER β level in this tissue being extremely low (Byers et al., 1997; Evers et al., 2013). Based on these considerations, it can be expected that the MCF-7 proliferation assay and the YES assay provide adequate in vitro models to mimic the response mediated by the estrogen receptor in rat uterus. Comparison of the results obtained for the selected compounds in the 2 in vitro assays reveals the MCF-7 proliferation assay result in lower EC₅₀ values than the YES assay. This may be due to the high permeability of MCF-7 cell membranes compared with yeast cell membranes (Breinholt and Larsen, 1998; Fang et al., 2000). Furthermore, it is interesting to note the large variation of the EC₅₀ values obtained in the same assay in different studies testing the same compound. This may be related to inter-laboratory differences in the actual assay conditions, a phenomenon observed more often in ring studies aiming to characterize inter-laboratory performance. Taking the MCF-7 proliferation assay as an example, the large variation in the EC₅₀ values shown in Figure 5 may be

due to the difference in assay medium type, the final concentration of the HEPES buffer, the penicillin/streptomycin solution used, the percentage of FBS used in the assay medium, the exposure durations and/or the frequency to renew the medium during the exposure, all representing parameters applied in a different way in the studies reported in the literature (Breinholt and Larsen, 1998; Dodge et al., 1996; Folmar et al., 2002; Gutendorf and Westendorf, 2001; Körner et al., 2001; Okubo et al., 2001; Schultis and Metzger, 2004; Wang et al., 2014).

The in vitro concentration-response data were quantitatively translated into predicted dose-response data using PBK modeling-based reverse dosimetry using C_{max} as the dose metric, given that the C_{max} represents the concentration values that would be able to activate to the estrogen receptor and lead to effects. To facilitate the evaluation of the model prediction, the BMDL₅₀ to BMDU₅₀ values were derived from both the predicted and in vivo dose-response curves using BMD analysis (Figure 7). When more than one in vivo dataset for the uterotrophic assay was available, the BMDL₅₀ to BMDU₅₀ values were consistent for some compounds (EE2, COU, and MEC); but revealed substantial variation such as for GEN, where the BMDL₅₀ to BMDU₅₀ values of 3 available in vivo studies varied up to 2 orders of magnitude. This implies that when evaluating predictions based on QIVIVE,

one should keep in mind that in vivo data may vary from one study to another and that predictions can thus be adequate, even when they deviate from the experimental values as long as this variation falls within the range of variability also observed between different in vivo studies. In order to evaluate QIVIVE based predictions, in vivo data used for the evaluation should preferably come from studies performed according to OECD guidelines and under good laboratory practice (GLP). The in vivo studies from which the uterus weight data used for evaluation of the QIVIVE predictions in the current study were derived were not performed under GLP but were all obtained in studies performed according to the OECD 440 guideline.

This result is in line with what was reported previously for the PBK modeling-based prediction of the developmental toxicity of phenol using in vitro data obtained from the ES-D3 cell differentiation assay of the embryonic stem cell test (EST), where the predictions were within the range of NOAEL values derived from 6 different in vivo studies for which the NOAEL values varied up to 8.3-fold (Strikwold et al., 2013). Comparison of the predicted and experimental BMDL₅₀ to BMDU₅₀ values also demonstrated that the predicted values did not underpredict the in vivo effects, making the approach adequate from a safety evaluation perspective. Despite the variability in the in vitro and in vivo data, the predictions ranked the estrogenic potency of these compounds in the same order as observed in the in vivo uterotrophic assay (Figure 8). The comparison between predicted and in vivo data also revealed that the predictions based on the YES assay qualitatively match well with the in vivo situation for most of the compounds, except for EE2 and COU. This is in line with our previous study of E2 and BPA for which the YES assay qualitatively predicted the uterus response in rats better than proliferation assay (Zhang et al., 2018). The developed generic PBK modeling-based reverse dosimetry approach can be used to classify and prioritize the endocrine active compounds in the process of hazard assessment. Besides, given that for the selected compounds of the present study the predictions of the BMD values based on the MCF-7 proliferation assays were lower than the BMD values obtained from the in vivo uterotrophic studies, the predictions based on the MCF-7 proliferation assays can be used to quantitatively define a point of departure to set safe exposure levels in risk assessment, that may be even conservative.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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