TOXICOLOGICAL SCIENCES **118(2)**, 470–484 (2010)
doi:10.1093/toxsci/kfq270
Advance Access publication September 10, 2010

The Use of *In Vitro* Toxicity Data and Physiologically Based Kinetic Modeling to Predict Dose-Response Curves for *In Vivo* Developmental Toxicity of Glycol Ethers in Rat and Man

Jochem Louisse,*,†,‡,[1] Esther de Jong,§,¶ Johannes J. M. van de Sandt,†,‡ Bas J. Blaauboer,¶ Ruud A. Woutersen,†,‡ Aldert H. Piersma,§,¶ Ivonne M. C. M. Rietjens,*,‡ and Miriam Verwei†,‡

*Division of Toxicology, Wageningen University, 6703 HE Wageningen, The Netherlands; †TNO Quality of Life, 3700 AJ Zeist, The Netherlands; ‡WUR/TNO Centre for Innovative Toxicology, 6700 EA Wageningen, The Netherlands; §National Institute of Public Health and the Environment (RIVM), 3720 BA Bilthoven, The Netherlands; and ¶Institute for Risk Assessment Sciences, Utrecht University, 3508 TD Utrecht, The Netherlands

[1]To whom correspondence should be addressed at Division of Toxicology, Wageningen University, Tuinlaan 5, 6703 HE Wageningen, The Netherlands. Fax: +31-317-484931. E-mail: jochem.louisse@wur.nl.

Received April 1, 2010; accepted August 15, 2010

At present, regulatory assessment of systemic toxicity is almost solely carried out using animal models. The European Commission's REACH legislation stimulates the use of animal-free approaches to obtain information on the toxicity of chemicals. *In vitro* toxicity tests provide *in vitro* concentration-response curves for specific target cells, whereas *in vivo* dose-response curves are regularly used for human risk assessment. The present study shows an approach to predict *in vivo* dose-response curves for developmental toxicity by combining *in vitro* toxicity data and *in silico* kinetic modeling. A physiologically based kinetic (PBK) model was developed, describing the kinetics of four glycol ethers and their embryotoxic alkoxyacetic acid metabolites in rat and man. *In vitro* toxicity data of these metabolites derived in the embryonic stem cell test were used as input in the PBK model to extrapolate *in vitro* concentration-response curves to predicted *in vivo* dose-response curves for developmental toxicity of the parent glycol ethers in rat and man. The predicted dose-response curves for rat were found to be in concordance with the embryotoxic dose levels measured in reported *in vivo* rat studies. Therefore, predicted dose-response curves for rat could be used to set a point of departure for deriving safe exposure limits in human risk assessment. Combining the *in vitro* toxicity data with a human PBK model allows the prediction of dose-response curves for human developmental toxicity. This approach could therefore provide a means to reduce the need for animal testing in human risk assessment practices.

Key Words: **developmental toxicity; physiologically based kinetic modeling; glycol ethers; embryonic stem cell test; benchmark dose.**

The implementation of the European REACH (*R*egistration, *E*valuation, *A*uthorization and Restriction of *Ch*emicals) legislation will lead to the evaluation of the toxicity of a large number of chemicals. The European Commission estimated the number of laboratory animals required for REACH to amount to 3.9 million if the use of alternative methods is not accepted by regulatory authorities. In addition, they indicated that with the expected acceptance scenario for alternative approaches, the total number will amount to 2.6 million (Van der Jagt *et al.*, 2004). Taking into account the offspring produced during the studies, a number of 9 million laboratory animals was estimated (Höfer *et al.*, 2004). Hartung and Rovida (2009) calculated an even higher number of laboratory animals required for REACH, which was contradicted by the European Chemical Agency (ECHA, 2009). Irrespective of the actual number of laboratory animals required for REACH, the development of validated and accepted *in vitro* and *in silico* approaches is urgently needed. Because it is expected that more than 20% of the laboratory animals needed for REACH will be used for developmental toxicity studies (Van der Jagt *et al.*, 2004), *in vitro* and *in silico* alternatives for developmental toxicity studies could contribute substantially to the reduction of animal use.

Three alternative methods for *in vivo* developmental toxicity tests have been scientifically validated so far, that is, the post implantation rat whole-embryo culture test, the rat limb bud micromass test, and the embryonic stem cell test (EST) (Genschow *et al.*, 2002). Only the EST does not require live animals because a mouse embryonic stem cell line is used. De Jong *et al.* (2009) showed that the differentiation assay of the EST, which assesses the effects of compounds on the differentiation of mouse embryonic stem cells into contracting cardiomyocytes, can be used to rank the potency of chemicals within a series of alkoxyacetic acid metabolites formed from glycol ethers. The alkoxyacetic acid metabolites of the glycol ethers, and not the parent glycol ethers themselves, have been identified as the proximate developmental toxicants of these chemicals (Brown *et al.*, 1984; Giavini *et al.*, 1993). The

in vitro potencies of the embryotoxic glycol ether alkoxyacetic acid metabolites, as measured in the EST, were found to correspond with the embryotoxic potency of the corresponding glycol ethers *in vivo* (De Jong *et al*., 2009). However, the EST provides *in vitro* concentration-response curves, whereas for human risk assessment *in vivo* dose-response curves are often required. These *in vitro* data should therefore be extrapolated to *in vivo* data by taking into account *in vivo* kinetics (Verwei *et al*., 2006).

The goal of the present study was to integrate *in vitro* toxicity data and *in silico* kinetic modeling as an approach to predict dose-response curves for developmental toxicity in both rat and man, thereby providing a basis for human risk assessment. To this end, four glycol ethers (ethylene glycol monomethyl ether [EGME], ethylene glycol monoethyl ether [EGEE], ethylene glycol monobutyl ether [EGBE], and ethylene glycol monophenyl ether [EGPE]) were used as model compounds belonging to one chemical class, but showing differences in *in vivo* embryotoxic potencies. To take the *in vivo* kinetics into consideration, the *in vitro* data derived from the EST were used as input in a physiologically based kinetic (PBK) model, extrapolating *in vitro* effect concentrations to predicted *in vivo* embryotoxic dose levels, as described by Verwei *et al*. (2006).

The predicted embryotoxic dose levels are based on the assumption that concentrations of the toxic alkoxyacetic acid metabolites that cause an inhibition of embryonic stem cell differentiation *in vitro* will also cause toxic effects in the developing embryo *in vivo*. By using the EST data as input for the blood concentration in the PBK model, the model allows the calculation of the *in vivo* dose levels of the parent glycol ethers that will lead to these blood concentrations of the toxic alkoxyacetic acid metabolites *in vivo*. By calculating this dose level for each concentration tested in the EST, *in vitro* concentration-response curves for the toxic alkoxyacetic acid metabolites can be converted into predicted *in vivo* dose-response curves for the parent glycol ethers. This dose-response curve could be used to set a point of departure, like a $BMDL_{10}$ (lower limit of the 95% confidence interval on the benchmark dose at which a benchmark response equivalent to a 10% effect size [BMR_{10}] is reached [BMD_{10}]), for deriving safe exposure limits in human risk assessment. Therefore, the combined *in vitro-in silico* approach provides a platform to use *in vitro* toxicity data for risk assessment practices, thereby contributing to the reduction of animal use in chemical risk assessment for embryotoxic end points.

MATERIALS AND METHODS

Chemicals

EGPE, ethylene glycol monopropyl ether, HCl, and phenoxyacetic acid (PAA) were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Ethyl acetate and methanol were obtained from Biosolve (Valkenswaard, the Netherlands) and sodium sulfate (Na_2SO_4) from Merck (Darmstadt, Germany). Ammonium formate was purchased from Fluka (Zwijndrecht, the Netherlands).

In Vitro-In Silico *Approach to Predict Dose-Response Curves for* In Vivo *Developmental Toxicity in Rat and Man*

The *in vitro-in silico* approach to predict embryotoxic dose levels in rats, as proposed by Verwei *et al*. (2006), was used to estimate dose-response curves for the *in vivo* developmental toxicity of the glycol ethers EGME, EGEE, EGBE, and EGPE in both rat and man. This approach consists of the following steps, which are described in more detail in the following sections: (1) development of a PBK model for *in vivo* glycol ether kinetics in rat and man, (2) evaluation of the PBK model, (3) determination of *in vitro* effect concentrations in the EST to be used as input for the PBK model, (4) PBK model-based prediction of dose-response curves for *in vivo* developmental toxicity in rat and man, and (5) evaluation of the potential of the *in vitro-in silico* approach to predict dose-response curves for *in vivo* developmental toxicity in the rat.

Development of a PBK Model for In Vivo *Glycol Ether Kinetics in Rat and Man*

A generic PBK model describing the kinetics of the glycol ethers EGME, EGEE, EGBE, and EGPE and their alkoxyacetic acid metabolites methoxyacetic acid (MAA), ethoxyacetic acid (EAA), butoxyacetic acid (BAA), and PAA, respectively, in rat and man was developed, using the model of EGME and MAA of Gargas *et al*. (2000a) as starting point. A schematic representation of the model is shown in Figure 1.

Physiological parameters for the rat and human model describing *in vivo* kinetics after inhalation exposure were taken from Gargas *et al*. (2000a) (Table 1) and represent the physiological parameters at the beginning of pregnancy. In the present study, the PBK model was adjusted to also describe *in vivo* kinetics after oral exposure. Oral uptake of the glycol ethers was described by an oral absorption constant (k_a) fitted to the *in vivo* plasma concentrations of EGME (Hays *et al*., 2000) and EGPE (present study) in rats that were orally dosed. Partition coefficients of the glycol ethers and their alkoxyacetic acid metabolites for rat and human tissues were estimated using the equation described by Berezhkovskiy (2004) (Table 2). Biotransformation kinetics of EGME, EGEE, and EGBE determined in Fisher 344 rat and human hepatocytes by Green *et al*. (1996) were scaled to whole liver by assuming a number of 128×10^6 hepatocytes/g rat liver (Seglen, 1976) and 99×10^6 hepatocytes/g human liver (Barter *et al*., 2007). No data of the biotransformation kinetics of EGPE to PAA are available in the literature. Because the biotransformation kinetics of EGME, EGEE, and EGBE are alike, it is expected that they are close to those of EGPE. Because the properties that might describe the biotransformation kinetics of EGPE, such as logP and VanderWaals volume (Chang *et al*., 2009; Soffers *et al*., 2001), are closest to that of EGBE (Table 3), the biotransformation kinetics of EGBE were used in the EGPE PBK model. Literature studies describing the kinetics of EGME (Gargas *et al*., 2000a; Hays *et al*., 2000), EGEE acetate (EGEEA) (Gargas *et al*., 2000b), and EGBE (Ghanayem *et al*., 1990) in rats were used to obtain the parameters for urinary excretion of MAA, EAA, and BAA, respectively, in rats by using a urinary excretion constant (K_{ex}) in the model fitted to the *in vivo* plasma concentrations of the alkoxyacetic acid metabolites (Table 2). Because no *in vivo* EGPE kinetic study in rats was found in the literature, an *in vivo* kinetic study with a limited number of rats was carried out to determine the parameters for urinary excretion of PAA in rats. Model parameters for urinary excretion of MAA, EAA, and BAA in man were estimated using *in vivo* kinetic studies of EGME (Groeseneken *et al*., 1989), EGEE (Groeseneken *et al*., 1986), and EGBE (Jones and Cocker, 2003; Kezic *et al*., 2004), respectively, in man, by using a K_{ex} in the model fitted to the *in vivo* urinary excretion rates of the alkoxyacetic acid metabolites (Table 2). Because no study on human EGPE kinetics is described in the literature, the urinary excretion of PAA was determined by fitting the K_{ex} to *in vivo* plasma concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) of man orally exposed to 2,4-D (Sauerhoff *et al*., 1977) (Table 2). The present study shows that PAA's plasma half-life in

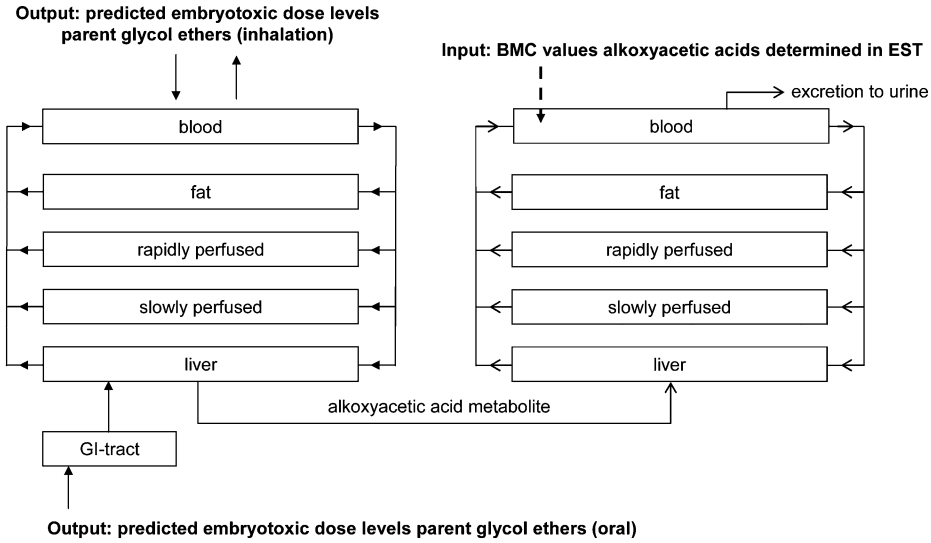

FIG. 1. Schematic representation of the PBK model of glycol ethers and their alkoxyacetic acid metabolites in rat and man. The PBK model is based on the PBK model of EGME and MAA described by Gargas *et al*. (2000a). GI tract, gastrointestinal tract.

the rat is almost equal to 2,4-D's plasma half-life in rat (see the "Discussion" section). Therefore, it was assumed that PAA's plasma half-life in man will be the same as 2,4-D's plasma half-life in man, making it possible to use the *in vivo* kinetic data for 2,4-D in man to estimate the K_{ex}, which is directly related to the plasma half-life, for the human PAA model.

Evaluation of the PBK Model

To evaluate the PBK model describing *in vivo* kinetics of the glycol ethers, *in vivo* kinetic studies for rat and man reported in literature were used (Table 4). The doses, exposure routes, and exposure duration used in the studies were applied in the PBK model simulation, to evaluate the model (Table 4). For the rat EGEE model, an *in vivo* kinetic study with EGEEA was used. No *in vivo* kinetic studies of EGPE were present in the literature. Therefore, *in vivo* EGPE kinetics in rats were determined in the present study, as described in the following. No kinetic data of EGPE in man were available, so for the human EGPE PBK model no evaluation could be carried out.

TABLE 1
Physiological Parameters Used in the PBK Model for Glycol Ethers Taken from Gargas *et al.* (2000a)

Physiological parameters	Rat	Man
bw (kg)	0.25	60.0
% bw		
Liver	4.0	2.4
Fat	10.1	27.6
Rapidly perfused tissue	6.1	3.7
Slowly perfused tissue	65.0	48.7
Blood	5.9	5.9
Cardiac output (l/h/kg bw)	14.0	19.2
% Cardiac output		
Liver: hepatic artery	3.4	3.3
Liver: portal vein	21.6	20.7
Fat	14.2	9.5
Rapidly perfused tissue	45.8	47.5
Slowly perfused tissue	15.0	19.0
Alveolar ventilation (l/h/kg bw)	14.0	15.3

EGPE kinetic study in rat. The experimental protocol of the EGPE kinetic study was approved by the Animal Welfare Committee of the National Institute of Public Health and the Environment (RIVM). Female Sprague-Dawley rats (254.4 ± 5.2 g) at approximately 13 weeks of age were obtained from Harlan Laboratories (Horst, the Netherlands). Rats were maintained in a temperature-, humidity-, and light cycle-controlled facility for 1 week prior to exposure. Feed (SDS, Witham, Essex, UK) and water were provided *ad libitum*. Two groups of 4 rats per group were exposed to either 1.1 mmol/kg body weight (bw) EGPE or 3.3 mmol/kg bw EGPE by oral gavage. Blood samples of 150 µl were taken from the tail vein prior to EGPE exposure and at 5, 15, and 30 min and 1, 2, 4, 6, 8, and 24 h after dosing and were stored in heparin-coated tubes (Sarstedt, Nümbrecht, Germany). Samples were centrifuged at 1300 × g for 10 min. The acquired serum samples were stored at −20°C.

EGPE and PAA analysis of plasma samples using gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry. To determine the EGPE and PAA plasma concentrations, 20 µl plasma was added to 20 µl of a 0.5% (vol/vol) HCl solution. Then, 1 ml ethyl acetate, containing the internal standard ethylene glycol monopropyl ether, was added and samples were vortexed thoroughly. Sodium sulfate was added as a drying agent, after which samples were vortexed and centrifuged. The supernatant was used for EGPE analysis using gas chromatography-mass spectrometry (GC-MS) and PAA analysis using liquid chromatography-mass spectrometry (LC-MS).

EGPE was analyzed with an Agilent HP 5972 GC-MS system (Hewlett-Packard, Palo Alto, CA) equipped with a 60 m × 0.25 mm × 0.5 µm Stabilwax-DA column (Restek; Interscience B.V., Breda, the Netherlands). The oven temperature was initially maintained for 5 min at 60°C after injection and then increased in steps of 15°C/min to 250°C, which was held for 15 min. Samples of 1 µl were injected using a programmed temperature vaporization splitless mode and helium as carrier gas with a constant flow rate of 1.5 ml/min. A positive electron ionization method was used for EGPE and ethylene glycol monopropyl ether (internal standard) detection. The ratio of EGPE ($m/z = 94$) and ethylene glycol monopropyl ether ($m/z = 73$) was used for quantification.

LC-MS analysis was carried out with an HP Agilent 1100 system (Hewlett-Packard) equipped with a Waters Atlantis C18 T3 5-µm column, 150 × 3.0 mm (Waters, Etten-Leur, the Netherlands), coupled to a Waters Quattro Premier XE mass spectrometer (Waters). Aliquots of 5 µl were injected. The flow rate was 0.3 ml/min. A gradient was made using a 5mM ammonium formate in methanol/H_2O (20/80, vol/vol) solution (solution A) and a 5mM ammonium formate in methanol/H_2O (90/10, vol/vol) solution (solution B). A linear

TABLE 2
Compound-Specific Parameters Used in the PBK Model for Glycol Ethers

Partition coefficients[a]	Liver:blood	Fat:blood	Rapidly perfused tissue:blood	Slowly perfused tissue:blood	Blood:air
EGME	0.76	0.37	0.76	0.80	32,800[b]
MAA	0.76	0.13	0.76	0.80	
EGEE	0.77	0.67	0.77	0.80	22,093[b]
EAA	0.76	0.13	0.76	0.79	
EGBE	0.73	3.7	0.73	0.72	7965[b]
BAA	0.64	0.11	0.64	0.67	
EGPE	0.85	8.4	0.85	0.79	5651[c]
PAA	0.64	0.11	0.64	0.67	

	Rat		Man	
Biotransformation kinetics	Apparent K_m (mM)	Apparent V_{max} (nmol/h/10^6 hepatocytes)	Apparent K_m (mM)	Apparent V_{max} (nmol/h/10^6 hepatocytes)
EGME → MAA[d]	6.3	1511	1.7	61.3
EGEE → EAA[d]	6.6	1519	1.2	70.8
EGBE → BAA[d]	0.9	741	0.9	113
EGPE → PAA[e]	0.9	741	0.9	113

Excretion kinetics[f]	Rat, K_{ex} (l/h)	Man, K_{ex} (l/h)
MAA	0.0045	0.18
EAA	0.025	0.30
BAA	0.060	5.0
PAA	0.080	2.0

[a]Estimated using *in silico* method described by Berezhkovskiy (2004).
[b]Determined by Johanson and Dynésius (1988).
[c]Estimated based on linear correlation between LogP values of EGME, EGEE, and EGBE, and the logarithm of their blood:air partition coefficients.
[d]Data from biotransformation studies in hepatocytes (Green *et al*., 1996).
[e]Assumed to be similar as for EGBE (see the ''Materials and Methods'' section).
[f]Fitted to *in vivo* plasma concentrations of alkoxyacetic acid metabolites (MAA: Hays *et al*., 2000; Gargas *et al*., 2000a; EAA: Gargas *et al*., 2000b; BAA: Ghanayem *et al*., 1990; PAA: data from present study) for the rat model and to *in vivo* urinary excretion rates (MAA: Groeseneken *et al*., 1989; EAA: Groeseneken *et al*., 1986; BAA: Jones and Cocker, 2003; Kezic *et al*., 2004) or *in vivo* plasma concentrations (2,4-D: Sauerhoff *et al*., 1977, used for excretion rate of PAA) for the human model.

gradient was applied from 0 to 100% solution B over 5 min, after which solution B was kept at 100% for another 5 min, lowered to 0% in 0.5 min, and equilibrated at these initial conditions for 8.5 min. A positive electrospray ionization mode was used for mass spectrometrical analysis. Sample analysis was carried out by the multiple reaction monitoring scan mode. The *m/z* transition 150.77 → 92.60 was used for PAA quantification.

Data analysis of EGPE kinetic study in rats. The plasma concentrations of EGPE and PAA determined in the EGPE kinetic study were used to calculate EGPE's and PAA's half-lives in plasma after oral dosing of 1.1 or 3.3 mmol EGPE/kg bw, using Kinetica 4.2.

TABLE 3
LogP Values and VanderWaals Volumes of Glycol Ethers

Compound	LogP[a]	VanderWaals volume[b] (Å^3)
EGME	−0.61	87.56
EGEE	−0.22	106.13
EGBE	0.84	142.79
EGPE	1.19	152.93

[a]LogP values calculated using ChemBioDraw Ultra 12.0.
[b]VanderWaals volumes calculated using Spartan 04 for Windows version 1.0.3.

Sensitivity analysis. A sensitivity analysis was carried out to identify the key parameters highly influencing the model output (peak blood concentrations of alkoxyacetic acid metabolites). The effect of a 5% increase in parameter value was evaluated by calculating normalized sensitivity coefficients [SC = $(C' - C)/(P' - P) \times (P/C)$] as was done by Evans and Andersen (2000) (C = initial value of model output, C' = value of model output resulting from 5% increase in parameter value, P = initial parameter value, P' = 5% increased parameter value). This 5% change in parameter value is theoretical and does not necessarily reflect realistic variations. Sensitivity analyses were carried out for the rat and human models using a single oral exposure of 1 mmol/kg bw and an 8-h inhalation exposure of 50 ppm.

Determination of In Vitro *Effect Concentrations in the EST*

The *in vitro* embryotoxic effect data derived in two different labs in our previous study (De Jong *et al*., 2009) were used to calculate benchmark concentrations (BMCs) at which 1-99% (BMC_1 to BMC_{99}) of the embryoid bodies (aggregations of embryonic stem cells) did not differentiate into contracting cardiomyocytes, using Environmental Protection Agency's (EPA) Benchmark Dose Software (BMDS) version 2.0 (Weibull model). These data were used as input in the PBK model to predict dose-response curves for *in vivo*

TABLE 4
***In Vivo* kinetic Data Used to Evaluate the PBK Model**

Species	Compound	Reference	Exposure route	Exposure	Dose	Data shown
Rat	EGME/MAA	Hays *et al.* (2000)	Oral	Once	3.3 mmol/kg bw	Fig. 2A
	EGME/MAA	Gargas *et al.* (2000a)	Inhalation	5 days, 6 h/day	10, 50 ppm	Fig. 2B
	EGEE (EGEEA)/EAA	Gargas *et al.* (2000b)	Inhalation	5 days, 6 h/day	50, 100 ppm	Fig. 2C
	EGBE/BAA	Ghanayem *et al.* (1990)	Intravenous	Once	0.26, 0.53, 1.1 mmol/kg bw	Fig. 2D
	EGPE/PAA	Present study	Oral	Once	1.1, 3.3 mmol/kg bw	Fig. 2E
Man	EGME/MAA	Groeseneken *et al.* (1989)	Inhalation	4 h	5 ppm	Fig. 3A
	EGEE/EAA	Groeseneken *et al.* (1986)	Inhalation	4 h	3, 6, 9 ppm	Fig. 3B
	EGBE/BAA	Jones and Cocker (2003)	Inhalation	2 h	20 ppm	Fig. 3C
	EGBE/BAA	Kezic *et al.* (2004)	Inhalation	0.5 h	19 ppm	Figs. 3C and 3D

developmental toxicity of the glycol ethers in rat and man, based on the *in vitro* EST data of both labs.

PBK Model-Based Prediction of Dose-Response Curves for In Vivo *Developmental Toxicity in Rat and Man*

To predict the dose-response curves for *in vivo* developmental toxicity of the glycol ethers, derived BMCs for the inhibition of embryonic stem cell differentiation by the alkoxyacetic acids were used as input for the peak blood concentrations in the PBK model (Fig. 1). The model was then used to calculate the corresponding dose levels of the parent glycol ethers leading to these concentrations in the blood, resulting in the predicted dose-response curves for *in vivo* developmental toxicity. For rat, dose-response curves were predicted using identical exposure regimens (route of exposure and exposure duration) in the PBK model simulations as applied in the *in vivo* developmental toxicity studies (Table 5). For man, dose-response curves were predicted for single- and repeated-exposure regimens, to investigate the effect of repeated dosing. For repeated dosing, a 5-day exposure regimen was used (8 h/day for inhalation exposure), representing a working week exposure.

Evaluation of the Potential of the In Vitro-In Silico *Approach to Predict Dose-Response Curves for* In Vivo *Developmental Toxicity in Rat*

To evaluate the potential of the *in vitro-in silico* approach to predict dose-response curves for *in vivo* developmental toxicity, the predicted dose-response curves for the rat were compared with data obtained in *in vivo* developmental toxicity studies listed in the ECETOC Technical Report 095 (ECETOC, 2005). Developmental toxicity end points described include resorptions, malformations, fetal deaths, and decrease in fetal bw. The *in vivo* data were used to derive $BMDL_{10}$ values on the basis of dose-response analyses (BMDS version 2.0), which could be used as points of departure in human risk assessment practices (Barlow *et al.*, 2009). These $BMDL_{10}$ values derived from *in vivo* data were compared to predicted $BMDL_{10}$ values, which were acquired by translating the *in vitro* $BMCL_{10}$ (lower limit of the 95% confidence interval on the BMC_{10}) values to dose levels using the rat PBK model. No human glycol ether developmental toxicity data were available. Consequently, predicted dose-response curves for developmental toxicity in man could not be compared with data on developmental toxicity of glycol ethers in man.

RESULTS

Development of a PBK Model for In Vivo *Glycol Ether Kinetics in Rat and Man*

The parameters for oral uptake (k_a) were fitted to *in vivo* kinetic data for EGME and EGPE, as described in the "Materials and Methods" section, and were found to be 4 h^{-1} in both cases. Therefore, in all models, a k_a of 4 h^{-1} was used for oral uptake of the parent glycol ethers. The parameters for urinary excretion of the alkoxyacetic acid metabolites (K_{ex}), obtained by fitting these parameters to *in vivo* kinetic data as described in the "Materials and Methods" section, are shown in Table 2. For the rat model, the K_{ex} value is the lowest for MAA, followed by that for EAA, BAA, and PAA, respectively. For the human model, the K_{ex} value is the lowest for MAA, followed by that for EAA, PAA, and BAA, respectively.

Evaluation of the PBK Model

Figure 2 shows the prediction of the plasma concentrations of EGME/MAA (Figs. 2A and 2B), EGEE/EAA (Fig. 2C), EGBE/BAA (Fig. 2D), and EGPE/PAA (Fig. 2E) in the rat and the measured plasma levels from *in vivo* rat kinetic studies. The plasma concentrations of EGPE and PAA after EGPE exposure were measured in the present study. The results of the PBK simulations show that the predicted plasma concentrations are close to the measured plasma concentrations of the four glycol ethers and their alkoxyacetic metabolites measured in rat. Figures 3A–C show the prediction of the urinary excretion rates of MAA (Fig. 3A), EAA (Fig. 3B), and BAA (Fig. 3C) in man and the measured urinary excretion rates from *in vivo* human kinetic studies. Figure 3D shows the prediction of the EGBE plasma concentrations in man and the measured EGBE plasma concentrations as measured in an *in vivo* human kinetic study. The results show that the model predicted urinary excretion rates and the predicted EGBE plasma levels are within a factor of 10 compared with the measured urinary excretion rates of MAA, EAA, and BAA and the EGBE plasma concentrations measured in man. In general, prediction of *in vivo* kinetics of the glycol ethers by the PBK models was found to be somewhat better in rat than in man (Figs. 2 and 3).

EGPE kinetics in rat. The plasma concentrations of EGPE and PAA in rats exposed to 1.1 and 3.3 mmol EGPE/kg bw are shown in Figure 2E. Maximum EGPE plasma concentrations measured were already reached 5 min after exposure, and were 0.4mM after exposure to 1.1 mmol EGPE/kg bw and 0.5mM after exposure to 3.3 mmol EGPE/kg bw. The maximum PAA plasma concentration of 1.2mM, obtained upon exposure to

TABLE 5

Comparison of $BMDL_{10}$ Values Determined from *In Vivo* Developmental Toxicity Studies and Predicted $BMDL_{10}$ Values for Developmental Toxicity Using the *In Vitro-In Silico* Approach with *in vitro* $BMCL_{10}$ Values as Input (symbols shown correspond with symbols used in Fig. 5)

Compound	Reference	Exposure route	Days of exposure	Dose	Critical end point	Measured $BMDL_{10}$	Predicted $BMDL_{10}$		Figure	Symbol
							Lab 1	Lab 2		
EGME	Toraason *et al.* (1985)	Oral	GD7–13	0.3, 0.6, 1.1 mmol/kg bw	Cardiac malformations	0.46 mmol/kg bw	0.12 mmol/kg bw	0.34 mmol/kg bw	5A	●
					Resorptions	0.52 mmol/kg bw				▲
					Fetal bw decrease	0.14 mmol/kg bw				◆
EGME	Doe *et al.* (1983)	Inhalation	GD6–17 (6 h/day)	100, 300 ppm	Fetal deaths	n.d.			5E	○
EGME	Nelson *et al.* (1984)	Inhalation	GD7–15 (7 h/day)	50, 100, 200 ppm	Skeletal malformations	11 ppm	21 ppm	64 ppm	5E	◆
					Visceral malformations	41 ppm				■
					Resorptions	49 ppm				●
					Fetal bw decrease	37 ppm				▲
EGEE	Goad and Cranmer (1984)[a]	Oral	GD7–15	2.2 mmol/kg bw	Cardiac malformations	n.d.			5B	◆
EGEE	Stenger *et al.* (1971)	Oral	GD1–21	0.1, 0.3, 0.5, 1.0, 2.1 mmol/kg bw	Skeletal malformations	0.80 mmol/kg bw	0.28 mmol/kg bw	1.3 mmol/kg bw	5B	●
EGEE	Chester *et al.* (1986)[a]	Oral	GD1–21	2.3, 3.0, 4.4 mmol/kg bw	Fetal deaths	n.d.			5B	▲
EGEE	Doe (1984)	Inhalation	GD6–15 (6 h/day)	10, 50, 250 ppm	Skeletal malformations[b]	66 ppm	62 ppm	280 ppm	5F	△
					Visceral malformations[b]	n.d.				○
					Fetal bw decrease	n.d.				◇
					Post-implantation loss	n.d.				□
EGEE	Andrew and Hardin (1984)	Inhalation	GD1–18 (7 h/day)	200, 770 ppm	Resorptions	n.d.			5F	●
					Cardiac malformations	n.d.				■
					Reduced ossification[b]	n.d.				◆
					Supernumerary ribs[b]	n.d.				▲
EGBE	Sleet *et al.* (1989)	Oral	GD9–11	1.3, 2.5[c] mmol/kg bw	Resorptions	1.4 mmol/kg bw	2.6 mmol/kg bw	3.1 mmol/kg bw	5C	●
					Skeletal malformations	n.d.				▲
			GD11–13	1.3, 2.5[c], 5.1[c] mmol/kg bw	Resorptions	n.d.				◆
					Skeletal malformations	3.6 mmol/kg bw				■
EGBE	Tyl *et al.* (1984)	Inhalation	GD6–15 (6 h/day)	25, 50, 100, 200[c] ppm	Fetal deaths	n.d.			5G	○
					Total malformations	n.d.				△
EGBE	Nelson *et al.* (1984)	Inhalation	GD7–15 (7 h/day)	150, 200 ppm	Resorptions	n.d.			5G	●
					Fetal bw decrease	n.d.				▲
					Skeletal malformations	n.d.				◆
					Visceral malformations	n.d.				■
EGPE	Unilever Research (1984)	Subcutaneous	GD6–15	0.6, 1.3, 2.5[c] mmol/kg bw	Total malformations	n.d.			5D	●
					Fetal deaths	1.0 mmol/kg bw	2.4 mmol/kg bw	6.2 mmol/kg bw		▲

Note. n.d., $BMDL_{10}$ value could not be determined because of either no effect or unsuitability of data to carry out benchmark dose analysis.

[a]Data taken from ECETOC Technical Report 095 (ECETOC, 2005).

[b]Minor anomalies according to authors.

[c]Dose at which maternal toxicity including hemolysis was observed.

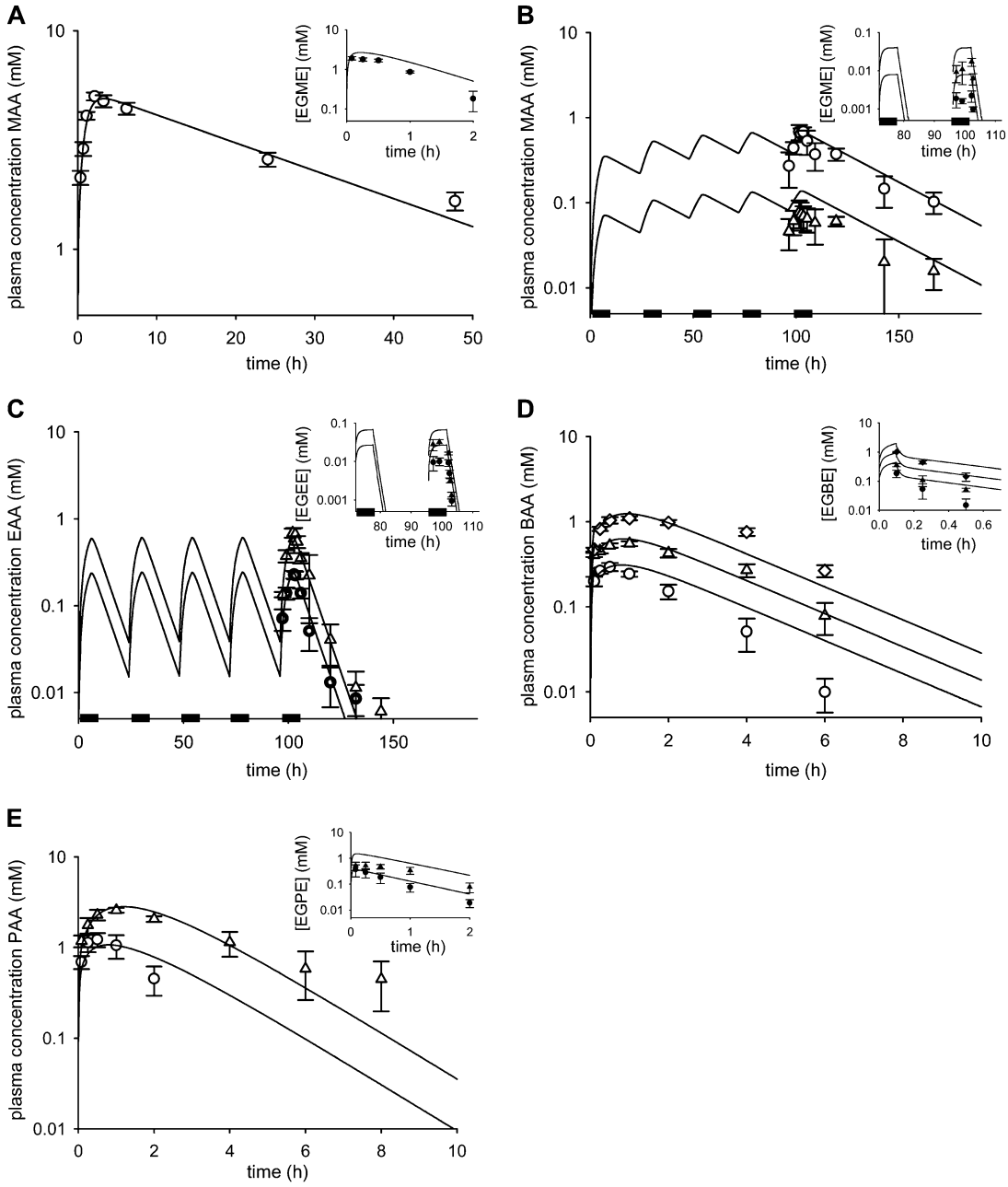

FIG. 2. Measured and predicted plasma concentrations of parent glycol ethers and alkoxyacetic acid metabolites in rats exposed to EGME (A, B), EGEE (C), EGBE (D), and EGPE (E). The main graphs show the plasma alkoxyacetic acid concentrations (open symbols), whereas the insets show the plasma parent glycol ether concentrations (closed symbols). The curves represent the PBK model predictions and the symbols with error bars (= SD) the measured *in vivo* data. (A) Oral exposure to 3.3 mmol EGME/kg bw (Hays *et al*., 2000); (B) 5 days (6 h/day) of inhalation exposure to 10 (●) or 50 (▲) ppm EGME (Gargas *et al*., 2000a). Thick lines on x-axis indicate time interval of glycol ether exposure. Plasma concentrations were determined on days 5, 6, and 7; (C) 5 days (6 h/day) of inhalation exposure to 50 (●) or 100 (▲) ppm EGEE (Gargas *et al*., 2000b). Thick lines on x-axis indicate time interval of glycol ether exposure. Plasma concentrations were determined on days 5, 6, and 7; (D) intravenous exposure to 0.26 (●), 0.53 (▲), or 1.1 (♦) mmol EGBE/kg bw (Ghanayem *et al*., 1990); (E) oral exposure to 1.1 (●) or 3.3 (▲) mmol EGPE/kg bw (present study).

1.1 mmol EGPE/kg bw, was reached 30 min after exposure, whereas the maximal PAA plasma concentration of 2.6mM, obtained upon exposure to 3.3 mmol EGPE/kg bw, was reached 60 min after exposure. EGPE was not detected in the plasma 4 h after exposure to both doses. The half-lives of EGPE in plasma were 0.4 and 0.9 h after exposure to 1.1 and 3.3 mmol EGPE/kg bw, respectively. PAA was not detected 4 h after exposure to 1.1 mmol EGPE/kg bw and 24 h after exposure to 3.3 mmol

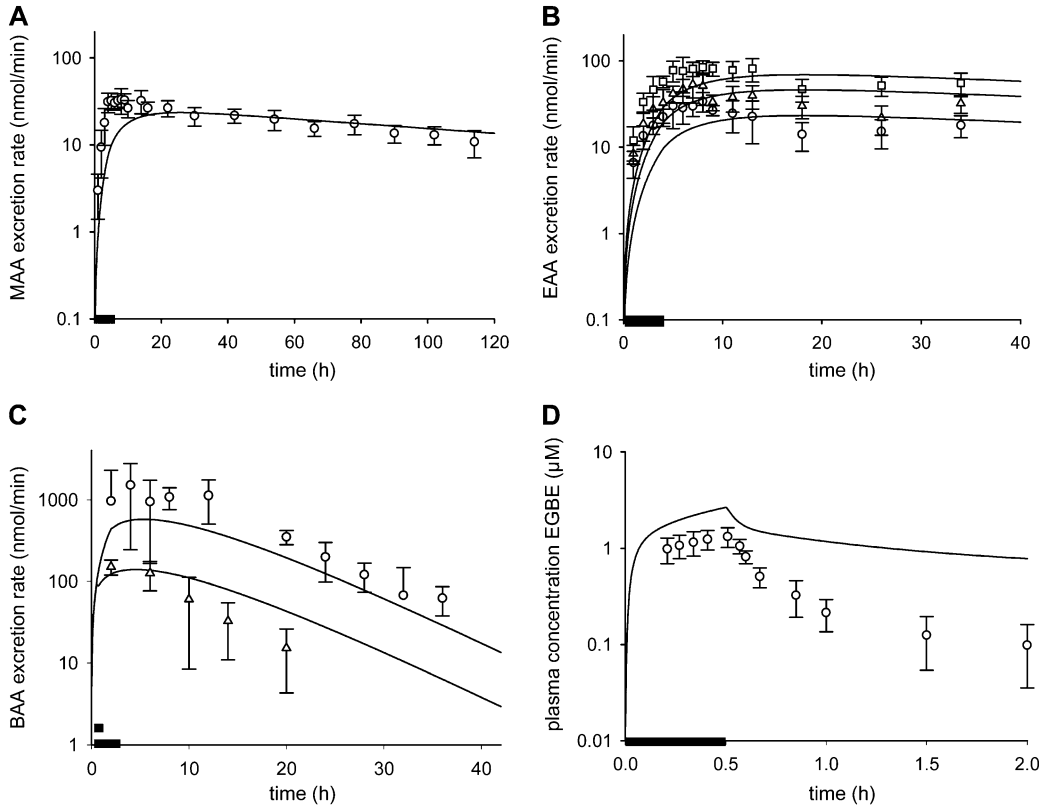

FIG. 3. Measured and predicted urinary excretion rates of MAA (A), EAA (B), and BAA (C) in man after inhalation exposure to EGME, EGEE, and EGBE, respectively, and measured and predicted EGBE plasma concentrations (D) in man after inhalation exposure to EGBE. The curves represent the PBK model predictions and the symbols with error bars (= SD) the measured *in vivo* data. (A) Four-h inhalation exposure to 5 ppm EGME (Groeseneken *et al*., 1989); (B) 4-h inhalation exposure to 3 (○), 6 (Δ), or 9 (□) ppm EGEE (Groeseneken *et al*., 1986); (C) 2-h inhalation exposure to 20 ppm EGBE (Jones and Cocker, 2003; ○) and 0.5-h inhalation exposure to 19 ppm EGBE (Kezic *et al*., 2004; Δ); (D) 0.5-h inhalation exposure to 19 ppm EGBE (Kezic *et al*., 2004). Thick lines on x-axis indicate time interval of glycol ether exposure.

EGPE/kg bw. The half-life of PAA in the plasma was 1.0 h in the rats that were exposed to 1.1 mmol EGPE/kg bw and 2.6 h in the rats that were exposed to 3.3 mmol EGPE/kg bw.

Sensitivity analysis. Figure 4 shows the normalized sensitivity coefficients of the most sensitive model parameters for peak blood concentrations of the alkoxyacetic acids in the rat and human PBK model for EGME/MAA (single exposure). The results of the sensitivity analyses for the PBK models for the other glycol ethers were similar to those of the EGME PBK models (data not shown). The sensitivity analysis revealed that the most sensitive physiological parameters for peak blood concentrations of the alkoxyacetic acids are the alveolar ventilation rate (QP; with inhalation exposure), the blood flow through the portal vein (QPV), and the volume of the slowly perfused tissue compartment (V SP). The most sensitive compound-specific parameters for peak blood concentrations of the alkoxyacetic acids appeared to be the slowly perfused tissue:blood partition coefficient of MAA (PC SP MAA) and the K_{ex}. All SCs for the remaining model parameters are between −0.2 and 0.2.

Comparison of Predicted and Observed Dose-Response Curves for In Vivo *Developmental Toxicity in Rat*

Figure 5 shows the predicted dose-response curves of the glycol ethers EGME, EGEE, EGBE, and EGPE in rat, presenting also the measured developmental toxicity data as derived from literature. The *in vitro* concentration-response curves (at BMC_1 to BMC_{99} values) for the alkoxyacetic acid metabolites in the EST (De Jong *et al*., 2009) extrapolated to *in vivo* dose-response curves are shown in Figure 5A (EGME), Figure 5B (EGEE), and Figure 5C (EGBE) for oral exposure; in Figure 5D (EGPE) for subcutaneous exposure; and in Figure 5E (EGME), Figure 5F (EGEE), Figure 5G (EGBE), and Figure 5H (EGPE) for inhalation exposure. Each figure shows two predicted dose-response curves, derived from the *in vitro* EST data obtained in each of the labs in our previous study (De Jong *et al*., 2009). The individual data points in the graphs (Fig. 5) represent developmental toxicity data from *in vivo* rat studies as reported in the literature (for studies used, see Table 5). For the predicted dose-response curves, the dose regimens used in the corresponding *in vivo* studies were

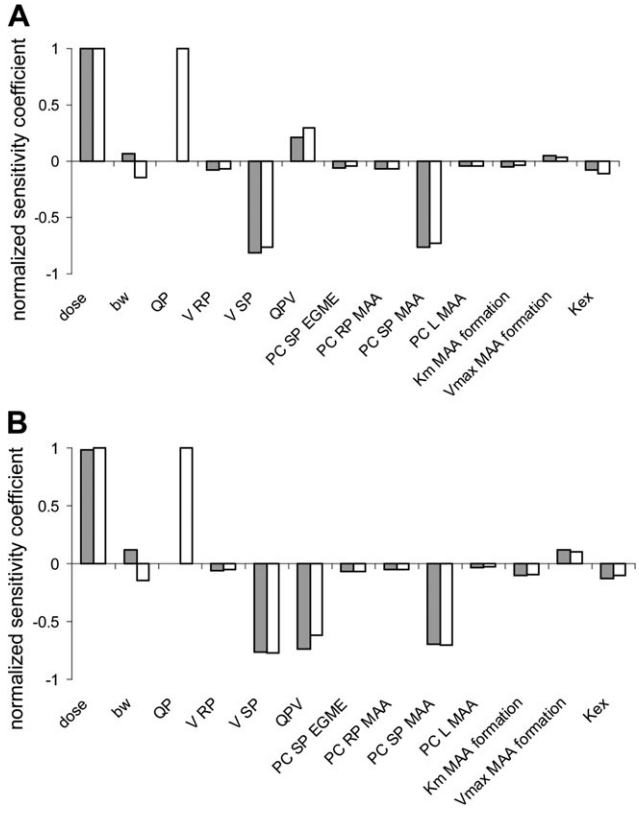

FIG. 4. Sensitivity of the predicted peak MAA plasma concentrations to different model parameters of the rat (A) and human (B) PBK models for EGME and MAA, after oral exposure to 1 mmol EGME/kg bw (gray bars) or inhalation exposure of 8 h to 50 ppm EGME (white bars). All model parameters with normalized sensitivity coefficients smaller than −0.03 and larger than 0.03 are shown. Dose, oral or inhaled dose EGME; bw, body weight; QP, alveolar ventilation rate; V RP, volume richly perfused tissue; V SP, volume slowly perfused tissue; QPV, blood flow through portal vein; PC SP EGME, partition coefficient EGME slowly perfused tissue:blood; PC RP MAA, partition coefficient MAA rapidly perfused tissue:blood; PC SP MAA, partition coefficient MAA slowly perfused tissue:blood; PC L MAA, partition coefficient MAA liver:blood; Km MAA formation, Michaelis-Menten constant for biotransformation of EGME to MAA; Vmax MAA formation, maximum rate of MAA formation; Kex, MAA excretion rate.

applied in the PBK model simulations. In Figures 5E–G, *in vivo* data of inhalation studies are shown in which different exposure regimens have been used, that is, differences in the number of exposure days and differences in exposure duration per day (either 6 or 7 h/day). Predicted dose-response curves in these figures are shown for the studies using a 7 h/day exposure regimen (Figs. 5E–H). When applying a 6 h/day exposure regimen in the PBK model simulations, predicted dose-response curves are slightly shifted to the right (data not shown).

The developmental toxicity data from reported *in vivo* rat studies were used to derive $BMDL_{10}$ values that could be used for deriving safe exposure limits for human risk assessment. These $BMDL_{10}$ values derived from *in vivo* data are presented in Table 5, together with the predicted $BMDL_{10}$ values, which were acquired by extrapolating the *in vitro* $BMCL_{10}$ values to *in vivo* dose levels using the rat PBK model. When comparing *in vivo*-derived and *in vivo*-predicted $BMDL_{10}$ values for each of the compounds studied, differences in the range of 0.2- to 6-fold were found (Table 5). No $BMDL_{10}$ value for developmental toxicity could be derived for the EGBE inhalation exposure study in rats. However, when comparing the individual data points of the *in vivo* studies and the predicted dose-response curve (Fig. 5G), a somewhat larger discrepancy between the predicted dose-response curve and the reported embryotoxic dose levels was found. In rats exposed to 200 ppm EGBE, 47% of the fetuses died (Tyl *et al.*, 1984) (Fig. 5G), whereas the predicted BMD_{47} value for developmental toxicity amounted to 1300 (predicted dose-response curve 1) and 1900 (predicted dose-response curve 2) ppm EGBE, resulting in approximately a 7- to 10-fold difference in predicted versus reported embryotoxic dose levels.

Prediction of In Vivo *Dose-Response Curves for Developmental Toxicity in Man*

The predicted dose-response curves for *in vivo* developmental toxicity of the glycol ethers EGME, EGEE, and EGPE in man are shown in Figure 6 for a single-exposure (black lines) and for a repeated-exposure regimen (gray lines). For repeated exposure, a 5-day exposure regimen was chosen, representing daily exposure during a working week (8 h/day for inhalation exposure). No dose-response curves could be predicted for EGBE because the maximum BAA plasma concentration reached using the human PBK model was lower than the BAA concentrations that affect ES-D3 cell differentiation. The *in vitro* concentration-response curves (BMC_1 to BMC_{99} values) for the alkoxyacetic acid metabolites in the EST (De Jong *et al.*, 2009) extrapolated to *in vivo* dose-response curves for man are shown in Figures 6A–C for oral exposure and in Figures 6D–F for inhalation exposure. For inhalation exposure, which is a more relevant exposure route for glycol ethers than oral exposure, $BMDL_{10}$ values for developmental toxicity were predicted for a 1- or 5-day exposure (Table 6). These $BMDL_{10}$ values were predicted by translating *in vitro* $BMCL_{10}$ values to *in vivo* dose levels using the human PBK model. The results show that upon repeated exposure, predicted $BMDL_{10}$ values decrease compared with single exposure.

DISCUSSION

The aim of the present study was to investigate the feasibility of using a combination of *in vitro* toxicity data and PBK modeling to predict *in vivo* dose-response curves for developmental toxicity, thereby providing a platform to use *in vitro* toxicity data not only qualitatively (presence or absence of effect) but also quantitatively (prediction of dose-response curves). This study shows that our predicted $BMDL_{10}$ values

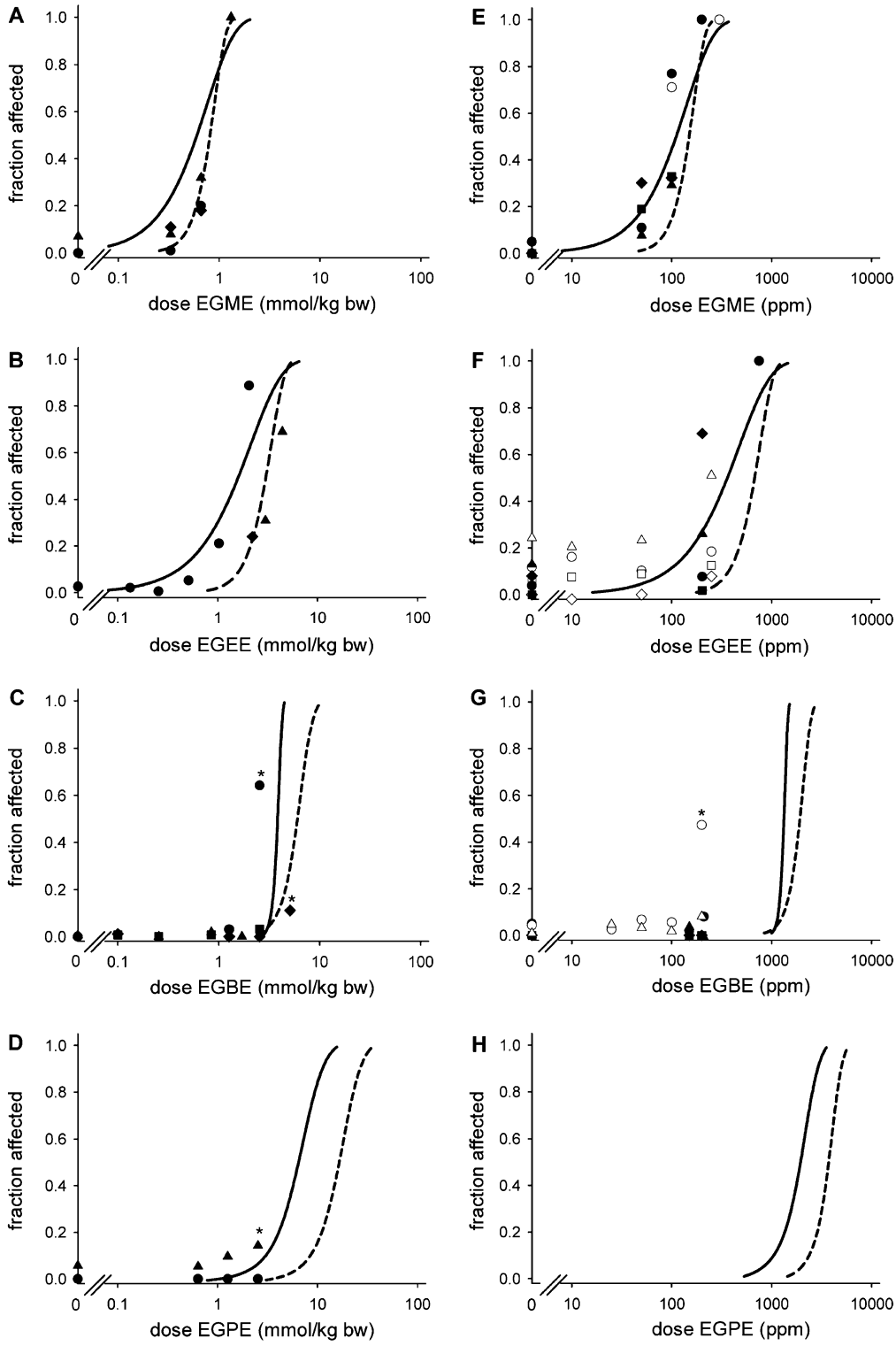

FIG. 5. Predicted dose-response curves for developmental toxicity of glycol ethers EGME (A and E), EGEE (B and F), EGBE, (C and G), and EGPE (D and H) after repeated oral (A–C), subcutaneous (D), or inhalation (E–H) exposure in the rat. The individual data points represent measured developmental toxicity data are taken from *in vivo* developmental toxicity studies as reported in the literature. The embryotoxic end points represented by the symbols are shown in Table 4. For all end points, except for fetal bw decrease, the fraction of affected embryos or fetuses was calculated. For the end point fetal bw decrease, the average decrease in fetal bw of fetuses in the exposed groups was calculated as fraction of the average bw of fetuses of the control group. The curves represent the predicted dose-response curves for developmental toxicity and are acquired by extrapolating *in vitro* effect concentrations ranging from BMC_1 up to BMC_{99} from laboratory 1 (straight line) or laboratory 2 (dashed line) to *in vivo* dose levels using the PBK model. ''*'' indicates a dose at which maternal toxicity including hemolysis was observed.

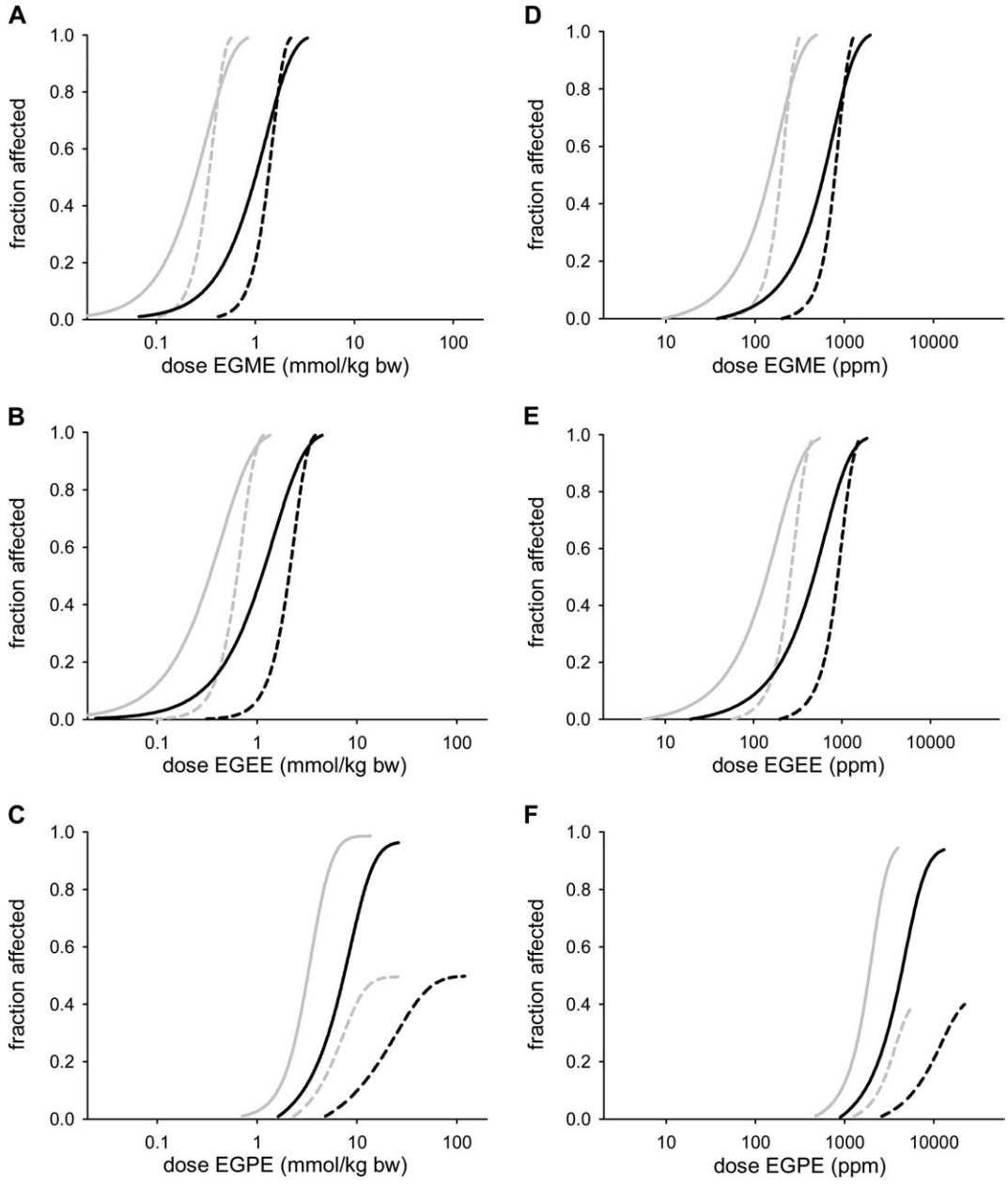

FIG. 6. Predicted dose-response curves for developmental toxicity of glycol ethers EGME (A and D), EGEE (B and E), and EGPE, (C and F) after oral (A–C) or inhalation (D–F) exposure in man. The curves represent the predicted dose-response curves for developmental toxicity for a 1-day (black lines) or a 5-day (gray lines) exposure and are acquired by translating *in vitro* effect concentrations ranging from BMC_1 up to BMC_{99} from laboratory 1 (straight lines) or laboratory 2 (dashed lines) to *in vivo* dose levels using the PBK model.

for *in vivo* developmental toxicity of glycol ethers in rat differed a factor of 0.2–6 from the measured $BMDL_{10}$ values determined from reported *in vivo* developmental toxicity studies. Predicted $BMDL_{10}$ values might in the future replace measured $BMDL_{10}$ values determined in *in vivo* developmental toxicity studies and could be used as points of departure in risk assessment to derive safe exposure levels for man. This could be achieved by applying uncertainty factors for interspecies variation, as also currently used in risk assessment based on animal toxicity studies. In addition, one might even use the human PBK models to obtain dose-response curves and $BMDL_{10}$ values for man, thereby eliminating the need for an uncertainty factor for interspecies differences in kinetics.

From the *in vivo* developmental toxicity studies, $BMDL_{10}$ values were derived for diverse developmental toxicity end points (i.e., resorptions, fetal bw decrease, malformations, and fetal deaths), whereas the predicted $BMDL_{10}$ values were derived for one *in vitro* developmental toxicity end point (i.e.,

TABLE 6
Predicted $BMDL_{10}$ Values for Developmental Toxicity in Man Exposed for 1 or 5 Days to Glycol Ethers via Inhalation (8 h/day) Using the *In Vitro-In Silico* Approach (*in vitro* toxicity data were taken from two different laboratories [De Jong *et al.*, 2009] leading to different predicted $BMDL_{10}$ values per compound)

	Lab 1		Lab 2	
Compound	1-day exposure (ppm)	5-day exposure (ppm)	1-day exposure (ppm)	5-day exposure (ppm)
EGME	110	28	330	83
EGEE	81	24	370	110
EGBE	n.d.	n.d.	n.d.	n.d.
EGPE	1320	715	4800	2000

Note. n.d., $BMDL_{10}$ could not be determined because $BMCL_{10}$ concentration of BAA could not be reached using the human PBK model.

inhibition of embryonic stem cell differentiation into contracting cardiomyocytes). Although the EST determines the concentrations of a compound that inhibit cardiomyocyte differentiation, the present study was not intended to specifically predict dose levels affecting cardiac development during embryonic development. The inhibition of cardiac differentiation was rather used to represent a sensitive *in vitro* developmental toxicity end point (Genschow *et al.*, 2004), which in the ideal situation could represent the most sensitive *in vivo* developmental toxicity end point. However, one might argue that other developmental toxicity end points such as developmental neurotoxicity might be less well predicted with the use of the *in vitro* effect concentrations that inhibit cardiomyocyte differentiation. Therefore, for predicting $BMDL_{10}$ values for other developmental toxicity end points such as developmental neurotoxicity, an *in vitro* assay focusing on this specific end point (Breier *et al.*, 2010) might improve the approach to an even further extent. The type of observed glycol ether-induced malformations will probably depend on the developmental stage at which the embryo is exposed to the glycol ethers (and their alkoxyacetic acid metabolites). Horton *et al.* (1985) showed, for example, that exposure of mice to EGME on gestational day 7 (GD7) resulted in exencephaly, whereas paw anomalies dominated when mice were exposed later in pregnancy (GD11). Because the present study did not intend to predict the developmental toxicity for a specific teratogenic end point, no critical window of glycol ether exposure was defined for the predictions.

The predicted dose-response curves for developmental toxicity were acquired by using the peak plasma concentrations of the alkoxyacetic acid metabolites as input in the PBK models. Sweeney *et al.* (2001) evaluated for different EGME developmental toxicity studies in rat and mice the relation between the MAA peak plasma concentration and the percentage of malformed fetuses, and the relation between the average daily area under the blood concentration-time curve (AUC) of MAA and the percentage of malformed fetuses. No preferred dose metric (MAA peak plasma concentration or average daily AUC of MAA) was obtained. For the extrapolation of *in vitro* data to *in vivo* data in the present study, alkoxyacetic acid peak concentrations were selected as the preferred dose metric to be used as input values for the PBK models.

In the *in vivo* developmental toxicity studies with EGBE and EGPE, developmental toxicity was only observed at dose levels that also caused maternal toxicity, including hemolysis (Sleet *et al.*, 1989; Tyl *et al.*, 1984; Unilever Research, 1984). *In vitro* studies showed that hemolytic effects of BAA are already found at 1mM (lowest concentration tested; Ghanayem *et al.*, 1989), indicating that hemolysis occurs at lower BAA concentrations than the concentrations inhibiting embryonic stem cell differentiation (De Jong *et al.*, 2009). Because maternal toxicity was not taken into account in the present study, in which we aimed to predict developmental toxicity, this may explain why our predicted embryotoxic dose levels are higher than the embryotoxic dose levels measured in *in vivo* studies, due to embryotoxicty as a secondary effect of maternal toxicity.

De Jong *et al.* (2009) showed that the ranking of the potencies of the glycol ether alkoxyacetic acid metabolites in the differentiation assay of the EST is the same as that of the potencies of their parent glycol ethers *in vivo*. However, differences in *in vitro* potencies were small (at maximum threefold) compared with differences in *in vivo* potencies for developmental toxicity (at maximum 24-fold) (De Jong *et al.*, 2009). The present study shows that the predicted differences in potencies of the glycol ethers improve, when combining the *in vitro* toxicity data with PBK models simulating *in vivo* kinetics (Table 7).

The physiological parameters used in the rat and human PBK models are representative for the beginning of pregnancy (Gargas *et al.*, 2000a). During pregnancy, some physiological parameters will change, for example, the size of some tissue compartments. It is not expected that this would have a large influence on the model outcome of the present models, because the sensitivity analysis of the model revealed that the influence of these parameters, including richly perfused tissue (fetus) (Gargas *et al.*, 2000a), is low (Fig. 4). Although the approach used assumes that *in vitro* embryotoxic concentrations have to reach the embryo to result in developmental toxicity, it was not necessary to include a specific fetal compartment in the model, because fetal MAA and EAA concentrations were found to be identical to maternal plasma concentrations in rats exposed to EGME (Gargas *et al.*, 2000a) and EGEEA (Gargas *et al.*, 2000b), respectively. It was assumed that this is also true for BAA and PAA. We aimed to build our PBK model based on parameters derived from *in silico* (partition coefficients) or *in vitro* (metabolism) methods. The parameters for EGPE metabolism were assumed to be equal

TABLE 7
Measured and Predicted $BMDL_{10}$ Values for Glycol Ether-Induced Malformations in Rat and Relative Potency of These Glycol Ethers in Inducing Malformations as Measured *In Vivo* and Predicted *In Vivo* (*in vitro* toxicity data were taken from two different laboratories [De Jong et al., 2009] leading to different predicted $BMDL_{10}$ values per compound)

	Measured $BMDL_{10}$ *in vivo* for malformations (mmol/kg bw)	Predicted $BMDL_{10}$ *in vivo* (mmol/kg bw)	
Compound		Lab 1	Lab 2
EGME (MAA)	0.46	0.12	0.34
EGEE (EAA)	0.80	0.28	1.3
EGBE (BAA)	3.6	2.6	3.1
EGPE (PAA)	>2.5	2.4	6.2
Relative potency			
EGME (MAA)/EGME (MAA)	1.0	1.0	1.0
EGEE (EAA)/EGME (MAA)	1.7	2.3	3.8
EGBE (BAA)/EGME (MAA)	7.8	22	9.1
EGPE (PAA)/EGME (MAA)	>5.4	20	18

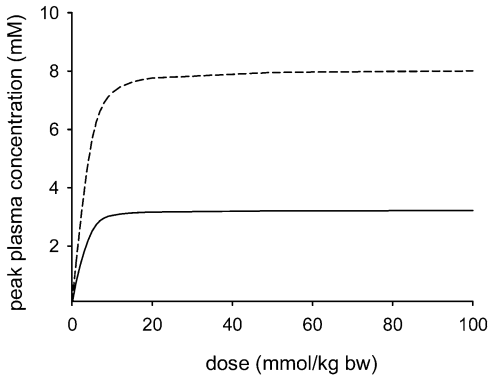

FIG. 7. Predicted peak plasma concentrations of BAA (solid line) and PAA (dashed line) in man upon five subsequent daily exposures to EGBE and EGPE respectively, at doses ranging from 0 to 100 mmol/kg bw.

to those of EGBE based on comparable LogP values and VanderWaals volumes (Table 3), which are important descriptors in Quantitative Structure-Activity Relationships (QSARs) for metabolism (Chang *et al.*, 2009; Soffers *et al.*, 2001). Given the outcomes of the sensitivity analysis, which revealed that metabolic parameters were of limited influence, and considering the model outcomes of the EGPE model (Fig. 2F), it seems to be justified to use the kinetic parameters for EGBE metabolism in the EGPE model. The parameters for the oral uptake (k_a) of the glycol ethers and the parameters for urinary excretion of the alkoxyacetic acid metabolites (K_{ex}) were acquired using *in vivo* kinetic data. The sensitivity analysis showed that the k_a and the K_{ex} were not sensitive parameters for the prediction of peak plasma levels of the alkoxyacetic acids (SC $k_a = 0$, SC $K_{ex} = -0.1$). Although the K_{ex} is not a sensitive parameter, differences in peak plasma concentrations of MAA, EAA, BAA, and PAA reached are to a large extent the result of the large differences in K_{ex} values (at maximum 18-fold for rat and 11-fold for man), which are directly related to the plasma half-lives of the alkoxyacetic acid metabolites. The present study shows that PAA has a shorter plasma half-life in rats (1.0–2.6 h) than BAA (1.5–3.2 h; Ghanayem *et al.*, 1990), EAA (7.6–10 h; Aasmoe and Aarbakke, 1997; Aasmoe *et al.*, 1999), and MAA (14–19 h; Aasmoe and Aarbakke, 1997; Aasmoe *et al.*, 1999). This indicates that the embryotoxic potencies of the glycol ethers EGME, EGEE, EGBE, and EGPE in rats are related to the plasma half-lives of their alkoxyacetic acid metabolites, as we suggested before (De Jong *et al.*, 2009). The plasma half-life of PAA, as measured in rats in the present study, was comparable with that of 2,4-D in rats (1.1–2.1 h; Timchalk, 2004). Because no human data on the excretion kinetics of PAA are available in the literature, the excretion data of 2,4-D in man were therefore assumed to be suitable to derive the parameter for PAA excretion for the human PBK model. However, because the K_{ex} seems to play an important role in determining differences in *in vivo* embryotoxic potencies of the glycol ethers, an inaccurate estimation of this parameter value would result in incorrectly predicted embryotoxic dose levels.

No dose-response curves for developmental toxicity of EGBE could be predicted for man. Due to the high excretion rate of BAA in the human model, a steady-state level of BAA is reached (Fig. 7), which is lower than the BAA concentrations that decrease the ES-D3 cell differentiation *in vitro* by 10%. The predicted dose-response curve for developmental toxicity of EGPE for man, using the *in vitro* EST data of laboratory 2, does not reach a 100% of affected fetuses (Figs. 6C and 6F). This is due to the steady-state level that is reached for PAA (Fig. 7), which equals the BMC_{50} value of PAA derived from the EST data of laboratory 2.

The extrapolation of *in vitro* toxicity data with human PBK models may predict safe dose levels for man, without the need for interspecies extrapolation for differences in kinetics. With the use of probabilistic PBK models, in which distributions rather than single points are used for parameter values, one might even take the variability of *in vivo* kinetics in the human population into account. This would enable a better prediction of dose-response curves for *in vivo* developmental toxicity for man and eliminate the need not only for an uncertainty factor for interspecies differences in kinetics, but also for an uncertainty factor for intraspecies differences in kinetics. However, predicted embryotoxic dose levels for man cannot be verified with *in vivo* human data. Consequently, possible interspecies differences in dynamics might be overlooked. Therefore, one might want to keep the uncertainty factor for these differences in dynamics.

Altogether, the results of the present study show that our predicted dose-response curves for glycol ether-induced developmental toxicity in rats, based on EST data in combination with PBK modeling, are in good concordance with glycol ether

developmental toxicity dose levels reported in literature. Considering the experimental differences in $BMDL_{10}$ values that might be derived from *in vivo* (developmental) toxicity studies, the differences between our predicted $BMDL_{10}$ values and the $BMDL_{10}$ values derived from *in vivo* studies for glycol ethers are small. The predicted $BMDL_{10}$ values could be used as points of departure in risk assessment practices, thereby contributing to the reduction of animal use in the risk assessment of these chemicals. It is concluded that the combined *in vitro-in silico* approach, after further development and evaluation, could contribute to a science-based risk assessment of chemicals, using no or reduced numbers of laboratory animals.

FUNDING

The Netherlands Organization for Health Research and Development (ZonMw; Project No. 114000088).

ACKNOWLEDGMENTS

The authors thank Ans Soffers for helping with the calculations of LogP values and VanderWaals volumes. The authors declare that there are no conflicts of interest.

REFERENCES

Aasmoe, L., and Aarbakke, J. (1997). Gender difference in the elimination of 2-methoxyethanol, methoxyacetic acid and ethoxyacetic acid in rat. *Xenobiotica* **27,** 1237–1244.

Aasmoe, L., Mathiesen, M., and Sager, G. (1999). Elimination of methoxyacetic acid and ethoxyacetic acid in rat. *Xenobiotica* **29,** 417–424.

Andrew, F. D., and Hardin, B. D. (1984). Developmental effects after inhalation exposure of gravid rabbits and rats to ethylene glycol monoethyl ether. *Environ. Health Perspect.* **57,** 13–23.

Barlow, S., Chesson, A., Collins, J. D., Flynn, A., Hardy, A., Jany, K., Knaap, A., Kuiper, H., Larsen, J., Lovell, D., *et al.* (2009). Guidance of the scientific committee on a request from EFSA on the use of the benchmark dose approach in risk assessment. *EFSA J.* **1150,** 1–72.

Barter, Z. E., Bayliss, M. K., Beaune, P. H., Boobis, A. R., Carlile, D. J., Edwards, R. J., Houston, J. B., Lake, B. G., Lipscomb, J. C., Pelkonen, O. R., *et al.* (2007). Scaling factors for the extrapolation of *in vivo* metabolic drug clearance from *in vitro* data: reaching a consensus on values of human microsomal protein and hepatocellularity per gram of liver. *Curr. Drug Metab.* **8,** 33–45.

Berezhkovskiy, L. M. (2004). Volume of distribution at steady state for a linear pharmacokinetic system with peripheral elimination. *J. Pharm. Sci.* **93,** 1628–1640.

Breier, J. M., Gassmann, K., Kayser, R., Stegeman, H., de Groot, D., Fritsche, E., and Shafer, T. J. (2010). Neural progenitor cells as models for high-throughput screens of developmental neurotoxicity: state of the science. *Neurotoxicol. Teratol.* **32,** 4–15.

Brown, N. A., Holt, D., and Webb, M. (1984). The teratogenicity of methoxyacetic acid in the rat. *Toxicol. Lett.* **22,** 93–100.

Chang, C., Duignan, D. B., Johnson, K. D., Lee, P. H., Cowan, G. S., Gifford, E. M., Stankovic, C. J., Lepsy, C. S., and Stoner, C. L. (2009). The development and validation of a computational model to predict rat liver microsomal clearance. *J. Pharm. Sci.* **98,** 2857–2867.

Chester, A., Hull, J., and Andrew, F. (1986). Lack of teratogenic effect after ethylene glycol monoethyl ether (EGEE) in rats via drinking water. *Teratology* **33,** C57 (Abstract P24).

De Jong, E., Louisse, J., Verwei, M., Blaauboer, B. J., van de Sandt, J. J. M., Woutersen, R. A., Rietjens, I. M. C. M., and Piersma, A. H. (2009). Relative developmental toxicity of glycol ether alkoxy acid metabolites in the embryonic stem cell test as compared to the *in vivo* potency of their parent compounds. *Toxicol. Sci.* **110,** 117–124.

Doe, J. E. (1984). Ethylene glycol monoethyl ether and ethylene glycol monoethyl ether acetate teratology studies. *Environ. Health Perspect.* **57,** 33–41.

Doe, J. E., Samuels, D. M., Tinston, D. J., and de Silva Wickramaratne, G. A. (1983). Comparative aspects of the reproductive toxicology by inhalation in rats of ethylene glycol monomethyl ether and propylene glycol monomethyl ether. *Toxicol. Appl. Pharmacol.* **69,** 43–47.

ECETOC. (2005). The toxicology of glycol ethers and its relevance to man. *Technical Report* 095, 4th ed. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium.

ECHA. (2009). New study on the number of test animals for REACH. *Press Release ECHA/PR/09/11* (28 August 2009).

Evans, M. V., and Andersen, M. E. (2000). Sensitivity analysis of a physiological model for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): assessing the impact of specific model parameters on sequestration in liver and fat in the rat. *Toxicol. Sci.* **54,** 71–80.

Gargas, M. L., Tyler, T. R., Sweeney, L. M., Corley, R. A., Weitz, K. K., Mast, T. J., Paustenbach, D. J., and Hays, S. M. (2000a). A toxicokinetic study of inhaled ethylene glycol monomethyl ether (2-ME) and validation of a physiologically based pharmacokinetic model for the pregnant rat and human. *Toxicol. Appl. Pharmacol.* **165,** 53–62.

Gargas, M. L., Tyler, T. R., Sweeney, L. M., Corley, R. A., Weitz, K. K., Mast, T. J., Paustenbach, D. J., and Hays, S. M. (2000b). A toxicokinetic study of inhaled ethylene glycol ethyl ether acetate and validation of a physiologically based pharmacokinetic model for rat and human. *Toxicol. Appl. Pharmacol.* **165,** 63–73.

Genschow, E., Spielmann, H., Scholz, G., Pohl, I., Seiler, A., Clemann, N., Bremer, S., and Becker, K. (2004). Validation of the embryonic stem cell test in the international ECVAM validation study on three *in vitro* embryotoxicity tests. *Altern. Lab. Anim.* **32,** 209–244.

Genschow, E., Spielmann, H., Scholz, G., Seiler, A., Brown, N., Piersma, A., Brady, M., Clemann, N., Huuskonen, H., Paillard, F., *et al.* (2002). The ECVAM international validation study on *in vitro* embryotoxicity tests: results of the definitive phase and evaluation of prediction models. European Centre for the Validation of Alternative Methods. *Atern. Lab. Anim.* **30,** 151–176.

Ghanayem, B. I., Burka, L. T., and Matthews, H. B. (1989). Structure-activity relationships for the *in vitro* hematotoxicity of N-alkoxyacetic acids, the toxic metabolites of glycol ethers. *Chem. Biol. Interact.* **70,** 339–352.

Ghanayem, B. I., Sanders, J. M., Clark, A. M., Bailer, J., and Matthews, H. B. (1990). Effects of dose, age, inhibition of metabolism and elimination on the toxicokinetics of 2-butoxyethanol and its metabolites. *J. Pharmacol. Exp. Ther.* **253,** 136–143.

Giavini, E., Broccia, M. L., Menegola, E., and Prati, M. (1993). Comparative *in vitro* study of the embryotoxic effects of three glycol ethers and their metabolites, the alkoxyacids. *Toxicol. In Vitro* **7,** 777–784.

Goad, P. T., and Cranmer, J. M. (1984). Gestation period sensitivity of ethylene glycol monoethyl ether in rats. *Toxicologist* **4,** 87. (Abstract 345).

Green, C. E., Gordon, G. R., Cohen, P. M., Nolen, H. W., Peters, J. H., and Tyson, C. A. (1996). *In vitro* metabolism of glycol ethers by human and rat hepatocytes. *Occup. Hyg.* **2,** 67–75.

Groeseneken, D., Veulemans, H., and Masschelein, R. (1986). Urinary excretion of ethoxyacetic acid after experimental human exposure to ethylene glycol monoethyl ether. *Br. J. Ind. Med.* **43,** 615–619.

Groeseneken, D., Veulemans, H., Masschelein, R., and Van Vlem, E. (1989). Experimental human exposure to ethylene glycol monomethyl ether. *Int. Arch. Occup. Environ. Health* **61,** 243–247.

Hartung, T., and Rovida, C. (2009). Chemical regulators have overreached. *Nature* **460,** 1080–1081.

Hays, S. M., Elswick, B. A., Blumenthal, G. M., Welsch, F., Conolly, R. B., and Gargas, M. L. (2000). Development of a physiologically based pharmacokinetic model of 2-methoxyethanol and 2-methoxyacetic acid disposition in pregnant rats. *Toxicol. Appl. Pharmacol.* **163,** 67–74.

Höfer, T., Gerner, I., Gundert-Remy, U., Liebsch, M., Schulte, A., Spielmann, H., Vogel, R., and Wettig, K. (2004). Animal testing and alternative approaches for the human health risk assessment under the proposed new European chemicals regulation. *Arch. Toxicol.* **78,** 549–564.

Horton, V. L., Sleet, R. B., John-Greene, J. A., and Welsch, F. (1985). Developmental phase-specific and dose-related teratogenic effects of ethylene glycol monomethyl ether in CD-1 mice. *Toxicol. Appl. Pharmacol.* **80,** 108–118.

Johanson, G., and Dynésius, B. (1988). Liquid/air partition coefficients of six commonly used glycol ethers. *Br. J. Ind. Med.* **45,** 561–564.

Jones, K., and Cocker, J. (2003). A human exposure study to investigate biological monitoring methods for 2-butoxyethanol. *Biomarkers* **8,** 360–370.

Kezic, S., Meuling, W. J. A., and Jakasa, I. (2004). Free and total urinary 2-butoxyacetic acid following dermal and inhalation exposure to 2-butoxyethanol in human volunteers. *Int. Arch. Occup. Environ. Health* **77,** 580–586.

Nelson, B. K., Setzer, J. V., Brightwell, W. S., Mathinos, P. R., Kuczuk, M. H., Weaver, T. E., and Goad, P. T. (1984). Comparative inhalation teratogenicity of four glycol ether solvents and an amino derivative in rats. *Environ. Health Perspect.* **57,** 261–271.

Sauerhoff, M. W., Braun, W. H., Blau, G. E., and Gehring, P. J. (1977). The fate of 2,4-dichlorophenoxyacetic acid (2,4-D) following oral administration to man. *Toxicology* **8,** 3–11.

Seglen, P. O. (1976). Preparation of isolated rat liver cells. *Methods Cell Biol.* **13,** 29–83.

Sleet, R. B., Price, C. J., Marr, M. C., Morrissey, R. E., and Schwetz, B. A. (1989). Teratologic evaluation of ethylene glycol monobutyl ether (CAS no.111-76-2) administered to Fisher-344 rats on either gestational days 9 through 11 or days 11 through 13. *NTP Report* 89-058. National Toxicology Program and National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Soffers, A. E. M. F., Boersma, M. G., Vaes, W. H. J., Vervoort, J., Tyrakowska, B., Hermens, J. L. M., and Rietjens, I. M. C. M. (2001). Computer-modeling-based QSARs for analyzing experimental data on biotransformation and toxicity. *Toxicol. In Vitro* **15,** 539–551.

Stenger, E. G., Aeppli, L., Müller, D., Peheim, E., and Thomann, P. (1971). Toxicology of ethyleneglycol-monoethyl ether. *Arzneimittelforschung* **21,** 880–885.

Sweeney, L. M., Tyler, T. R., Kirman, C. R., Corley, R. A., Reitz, R. H., Paustenbach, D. J., Holson, J. F., Whorton, M. D., Thompson, K. M., and Gargas, M. L. (2001). Proposed occupational exposure limits for select ethylene glycol ethers using PBPK models and Monte Carlo simulations. *Toxicol. Sci.* **62,** 124–139.

Timchalk, C. (2004). Comparative inter-species pharmacokinetics of phenoxyacetic acid herbicides and related organic acids; evidence that the dog is not a relevant species for evaluation of human health risk. *Toxicology* **200,** 1–19.

Toraason, M., Stringer, B., Stober, P., and Hardin, B. D. (1985). Electrocardiographic study of rat fetuses exposed to ethylene glycol monomethyl ether (EGME). *Teratology* **32,** 33–39.

Tyl, R. W., Millicovsky, G., Dodd, D. E., Pritts, I. M., France, K. A., and Fisher, L. C. (1984). Teratologic evaluation of ethylene glycol monobutyl ether in Fischer 344 rats and New Zealand white rabbits following inhalation exposure. *Environ. Health Perspect.* **57,** 47–68.

Unilever Research. (1984). Teratogenicity of phenoxyethanol by subcutaneous injection in Colworth Wistar rats. *Research Report* PES 841023. Unilever Research, UK.

Van der Jagt, K., Munn, S., Tørsløv, J., and de Bruijn, J. (2004). Alternative approaches can reduce the use of test animals under REACH. *Report EUR* 21405.

Verwei, M., van Burgsteden, J. A., Krul, C. A. M., van de Sandt, J. J. M., and Freidig, A. P. (2006). Prediction of *in vivo* embryotoxic effect levels with a combination of *in vitro* studies and PBPK modelling. *Toxicol. Lett.* **165,** 79–87.