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

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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. \textit{In vitro} toxicity tests provide \textit{in vitro} concentration-response curves for specific target cells, whereas \textit{in vivo} dose-response curves are regularly used for human risk assessment. The present study shows an approach to predict \textit{in vivo} dose-response curves for developmental toxicity by combining \textit{in vitro} toxicity data and \textit{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. \textit{In vitro} toxicity data of these metabolites derived in the embryonic stem cell test were used as input in the PBK model to extrapolate \textit{in vitro} concentration-response curves to predicted \textit{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 \textit{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 \textit{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.

\textbf{Key Words:} developmental toxicity; physiologically based kinetic modeling; glycol ethers; embryonic stem cell test; benchmark dose.

The implementation of the European REACH (\textit{Registration, Evaluation, Authorization and Restriction of Chemicals}) 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 \textit{in vitro} and \textit{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), \textit{in vitro} and \textit{in silico} alternatives for developmental toxicity studies could contribute substantially to the reduction of animal use.

Three alternative methods for \textit{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 (ethyleneglycol monomethyl ether [EGME], ethyleneglycol monooctyl ether [EGOE], ethyleneglycol monobutyl ether [EGBE], and ethyleneglycol 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 BMDLₐ₀ (lower limit of the 95% confidence interval on the benchmark dose at which a benchmark response equivalent to a 10% effect size [BMR₁₀] is reached [BMD₁₀]), 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 phenoxycetic acid (PAA) were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Ethyl acetate and methanol were obtained from Biosolve (Valkenswaard, the Netherlands) and sodium sulfate (Na₂SO₄) 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, 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, 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 (kₐ) 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, EGBE, 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 × 10⁶ hepatocytes/g rat liver (Seglen, 1976) and 99 × 10⁶ 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, EGBE, and EGPE 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 log P and VanderWaals volume (Chang et al., 2000; Soffers et al., 2001), are closest to that of EGBE (Table 3), the biotransformation kinetics of EGPE 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 EGEE (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 an urinary excretion constant (Kₑᵤ) 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 (Groesenneken et al., 1989), EGBE (Groesenneken et al., 1986), and EGPE (Jones and Cocker, 2003; Kezic et al., 2004), respectively, in man, by using a Kₑᵤ 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 Kₑᵤ 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...
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**

<table>
<thead>
<tr>
<th>Physiological parameters</th>
<th>Rat (kg)</th>
<th>Man (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bw</td>
<td>0.25</td>
<td>60.0</td>
</tr>
<tr>
<td>% bw</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>4.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Fat</td>
<td>10.1</td>
<td>27.6</td>
</tr>
<tr>
<td>Rapidly perfused tissue</td>
<td>6.1</td>
<td>3.7</td>
</tr>
<tr>
<td>Slowly perfused tissue</td>
<td>65.0</td>
<td>48.7</td>
</tr>
<tr>
<td>Blood</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>Cardiac output</td>
<td>14.0</td>
<td>19.2</td>
</tr>
</tbody>
</table>

**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 l 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 \(-20^\circ\text{C}\).

**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 μl/min. A gradient was made using a 5mM ammonium formate in methanol/H₂O (20/80, vol/vol) solution (solution A) and a 5mM ammonium formate in methanol/H₂O (90/10, vol/vol) solution (solution B). A linear
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.

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

\[ SC = \frac{C_{#} - C_{0}}{C_{0}} \times \frac{P_{#}}{P} \]

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.

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

### TABLE 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>LogP (^a)</th>
<th>VanderWaals volume (^b) (Å³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGME</td>
<td>-0.61</td>
<td>87.56</td>
</tr>
<tr>
<td>EGEE</td>
<td>-0.22</td>
<td>106.13</td>
</tr>
<tr>
<td>EGBE</td>
<td>0.84</td>
<td>142.79</td>
</tr>
<tr>
<td>EGPE</td>
<td>1.19</td>
<td>152.93</td>
</tr>
</tbody>
</table>

\(^a\)LogP values calculated using ChemBioDraw Ultra 12.0.
\(^b\)VanderWaals volumes calculated using Spartan 04 for Windows version 1.0.3.
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 BMDL10 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 BMDL10 values derived from in vivo data were compared to predicted BMDL10 values, which were acquired by translating the in vitro BMCL10 (lower limit of the 95% confidence interval on the BMC10) 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

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**TABLE 4**

<table>
<thead>
<tr>
<th>Species</th>
<th>Compound</th>
<th>Reference</th>
<th>Exposure route</th>
<th>Exposure</th>
<th>Dose</th>
<th>Data shown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>EGME/MAA</td>
<td>Hays et al. (2000)</td>
<td>Oral</td>
<td>Once</td>
<td>3.3 mmol/kg bw</td>
<td>Fig. 2A</td>
</tr>
<tr>
<td></td>
<td>EGME/MAA</td>
<td>Gargas et al. (2000a)</td>
<td>Inhalation</td>
<td>5 days, 6 h/day</td>
<td>10, 50 ppm</td>
<td>Fig. 2B</td>
</tr>
<tr>
<td></td>
<td>EGEE (EGEEA)/EAA</td>
<td>Gargas et al. (2000b)</td>
<td>Inhalation</td>
<td>5 days, 6 h/day</td>
<td>50, 100 ppm</td>
<td>Fig. 2C</td>
</tr>
<tr>
<td></td>
<td>EGPE/PAA</td>
<td>Ghanayem et al. (1990)</td>
<td>Intravenous</td>
<td>Once</td>
<td>0.26, 0.53, 1.1 mmol/kg bw</td>
<td>Fig. 2D</td>
</tr>
<tr>
<td></td>
<td>Present study</td>
<td></td>
<td>Oral</td>
<td>Once</td>
<td>1.1, 3.3 mmol/kg bw</td>
<td>Fig. 2E</td>
</tr>
<tr>
<td>Man</td>
<td>EGME/MAA</td>
<td>Groeseneken et al. (1989)</td>
<td>Inhalation</td>
<td>4 h</td>
<td>5 ppm</td>
<td>Fig. 3A</td>
</tr>
<tr>
<td></td>
<td>EGEE/EAA</td>
<td>Groeseneken et al. (1986)</td>
<td>Inhalation</td>
<td>4 h</td>
<td>3, 6, 9 ppm</td>
<td>Fig. 3B</td>
</tr>
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<td></td>
<td>EGBE/BAA</td>
<td>Jones and Cocker (2003)</td>
<td>Intravenous</td>
<td>2 h</td>
<td>20 ppm</td>
<td>Fig. 3C</td>
</tr>
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<td></td>
<td>EGBE/BAA</td>
<td>Kezic et al. (2004)</td>
<td>Inhalation</td>
<td>0.5 h</td>
<td>19 ppm</td>
<td>Figs. 3C and 3D</td>
</tr>
<tr>
<td>Compound</td>
<td>Reference</td>
<td>Exposure route</td>
<td>Days of exposure</td>
<td>Dose</td>
<td>Critical end point</td>
<td>Measured BMDL_{10}</td>
</tr>
<tr>
<td>----------</td>
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<tr>
<td>EGME</td>
<td>Toraason et al. (1985)</td>
<td>Oral</td>
<td>GD7–13</td>
<td>0.3, 0.6, 1.1 mmol/kg bw</td>
<td>Cardiac malformations</td>
<td>0.46 mmol/kg bw</td>
</tr>
<tr>
<td>EGME</td>
<td>Doe et al. (1983)</td>
<td>Inhalation</td>
<td>GD6–17 (6 h/day)</td>
<td>100, 300 ppm</td>
<td>Cardiac malformations</td>
<td>0.52 mmol/kg bw</td>
</tr>
<tr>
<td>EGME</td>
<td>Nelson et al. (1984)</td>
<td>Inhalation</td>
<td>GD7–15 (7 h/day)</td>
<td>50, 100, 200 ppm</td>
<td>Skeletal malformations</td>
<td>0.52 mmol/kg bw</td>
</tr>
<tr>
<td>EGEE</td>
<td>Goad and Cranmer (1984)</td>
<td>Oral</td>
<td>GD7–15</td>
<td>2.2 mmol/kg bw</td>
<td>Cardiac malformations</td>
<td>0.14 mmol/kg bw</td>
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<tr>
<td>EGEE</td>
<td>Stenger et al. (1971)</td>
<td>Oral</td>
<td>GD1–21</td>
<td>0.1, 0.3, 0.5, 1.0, 2.1 mmol/kg bw</td>
<td>Fetal deaths</td>
<td>n.d.</td>
</tr>
<tr>
<td>EGEE</td>
<td>Chester et al. (1986)***</td>
<td>Oral</td>
<td>GD1–21</td>
<td>2.3, 3.0, 4.4 mmol/kg bw</td>
<td>Fetal deaths</td>
<td>n.d.</td>
</tr>
<tr>
<td>EGEE</td>
<td>Doe (1984)</td>
<td>Inhalation</td>
<td>GD6–15 (6 h/day)</td>
<td>10, 50, 250 ppm</td>
<td>Skeletal malformations**</td>
<td>0.80 mmol/kg bw</td>
</tr>
<tr>
<td>EGEE</td>
<td>Andrew and Hardin (1984)</td>
<td>Inhalation</td>
<td>GD1–18 (7 h/day)</td>
<td>200, 770 ppm</td>
<td>Cardiac malformations</td>
<td>0.28 mmol/kg bw</td>
</tr>
<tr>
<td>EGBE</td>
<td>Sleet et al. (1989)</td>
<td>Oral</td>
<td>GD9–11</td>
<td>1.3, 2.5 mmol/kg bw</td>
<td>Skeletal malformations</td>
<td>0.80 mmol/kg bw</td>
</tr>
<tr>
<td>EGBE</td>
<td>Tyl et al. (1984)</td>
<td>Inhalation</td>
<td>GD6–15 (6 h/day)</td>
<td>25, 50, 100, 200 ppm</td>
<td>Skeletal malformations</td>
<td>0.28 mmol/kg bw</td>
</tr>
<tr>
<td>EGBE</td>
<td>Nelson et al. (1984)</td>
<td>Inhalation</td>
<td>GD7–15 (7 h/day)</td>
<td>150, 200 ppm</td>
<td>Skeletal malformations</td>
<td>0.28 mmol/kg bw</td>
</tr>
<tr>
<td>EGPE</td>
<td>Unilever Research (1984)</td>
<td>Subcutaneous</td>
<td>GD6–15</td>
<td>0.6, 1.3, 2.5 mmol/kg bw</td>
<td>Fetal deaths</td>
<td>0.14 mmol/kg bw</td>
</tr>
</tbody>
</table>

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.

**Data taken from ECETOC Technical Report 095 (ECETOC, 2005).

***Minor anomalies according to authors.

†Dose at which maternal toxicity including hemolysis was observed.

[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)]
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 EGPE/kg bw.
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...
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 BMDL10 values that could be used for deriving safe exposure limits for human risk assessment. These BMDL10 values derived from in vivo data are presented in Table 5, together with the predicted BMDL10 values, which were acquired by extrapolating the in vitro BMCL10 values to in vivo dose levels using the rat PBK model. When comparing in vivo-derived and in vivo-predicted BMDL10 values for each of the compounds studied, differences in the range of 0.2- to 6-fold were found (Table 5). No BMDL10 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 BMD47 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 (BMC1 to BMC99 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, BMDL10 values for developmental toxicity were predicted for a 1- or 5-day exposure (Table 6). These BMDL10 values were predicted by translating in vitro BMCL10 values to in vivo dose levels using the human PBK model. The results show that upon repeated exposure, predicted BMDL10 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 BMDL10 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 BMC1 up to BMC99 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.
for in vivo developmental toxicity of glycol ethers in rat differed a factor of 0.2–6 from the measured BMDL\textsubscript{10} values determined from reported in vivo developmental toxicity studies. Predicted BMDL\textsubscript{10} values might in the future replace measured BMDL\textsubscript{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\textsubscript{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\textsubscript{10} values were derived for diverse developmental toxicity end points (i.e., resorptions, fetal bw decrease, malformations, and fetal deaths), whereas the predicted BMDL\textsubscript{10} values were derived for one in vitro developmental toxicity end point (i.e.,

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\textsubscript{1} up to BMC\textsubscript{99} from laboratory 1 (straight lines) or laboratory 2 (dashed lines) to in vivo dose levels using the PBK model.
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)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Lab 1</th>
<th>Lab 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGME</td>
<td>110</td>
<td>330</td>
</tr>
<tr>
<td>EGEE</td>
<td>81</td>
<td>370</td>
</tr>
<tr>
<td>EBGE</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>EGPE</td>
<td>1320</td>
<td>4800</td>
</tr>
</tbody>
</table>

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

PREDICTION OF IN VIVO DEVELOPMENTAL TOXICITY

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 1 mM (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 embryotoxicity 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.
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

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 vitro 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\textsubscript{10} values that might be derived from \textit{in vivo} (developmental) toxicity studies, the differences between our predicted BMDL\textsubscript{10} values and the BMDL\textsubscript{10} values derived from \textit{in vivo} studies for glycol ethers are small. The predicted BMDL\textsubscript{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 \textit{in vitro} approach, after further development and evaluation, could contribute to a science-based risk assessment of chemicals, using no or reduced numbers of laboratory animals.

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