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Relative Developmental Toxicity of Glycol Ether Alkoxy Acid Metabolites in the Embryonic Stem Cell Test as compared with the *In Vivo* Potency of their Parent Compounds

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The embryonic stem cell test (EST) has been proposed as an *in vitro* assay that might reduce animal experimentation in regulatory developmental toxicology. So far, evaluation of the EST was not performed using compounds within distinct chemical classes. Evaluation within a distinct class of chemically related compounds can define the usefulness of the assay for the chemical class tested. The aim of the present study was to evaluate the relative sensitivity of the EST for a selected series of homologous compounds and to compare the data to the relative developmental toxicity of the compounds *in vivo*. To this end a series of proximate developmentally toxic glycol ether alkoxy acid metabolites was tested in the EST. All glycol ether alkoxy acid metabolites tested showed a concentration-dependent inhibition of cardiomyocyte differentiation at noncytotoxic concentrations, with methoxyacetic acid as the most potent compound followed by ethoxyacetic acid, butoxyacetic acid, and phenoxyacetic acid, respectively. The potency ranking of the compounds in the EST corresponds with the available *in vivo* data. The relative differences between the potencies of the compounds appeared more pronounced in the *in vivo* studies than in the EST. A possible explanation for this discrepancy could be the difference in the kinetics of the compounds *in vivo* as compared with their *in vitro* kinetics. This study illustrates that the EST can be used to set priorities for developmental toxicity testing within classes of related compounds.

Key Words: **embryonic stem cells; glycol ethers; alternatives to animal testing; developmental toxicology.**

In the context of the European REACH (Registration, Evaluation, and Authorization of CHemicals) legislation the evaluation of the developmental toxicity of a large number of chemicals is foreseen. Unfortunately, the current regulatory animal tests for the evaluation of developmental toxicity of chemicals require a high number of laboratory animals, are time-consuming and expensive (Van der Jagt *et al.*, 2004). Consequently, there is a great need to develop alternative approaches and methods (Schaafsma *et al.*, 2009). *In vitro* alternatives could accelerate the process of chemical safety testing compared with classical *in vivo* testing, also reducing costs and animal use.

Currently three *in vitro* developmental toxicity tests have been formally validated, namely the whole embryo culture, the limb bud micromass and the embryonic stem cell test (EST) (Genschow *et al.*, 2004). Of these three tests only the last does not require pregnant animals to obtain embryonic tissue. Embryonic stem (ES) cell lines have been derived from the inner cell mass of blastocysts and have the advantage of being able to differentiate into all cell types of the three primary germ layers (Martin, 1981). The EST uses this ability to study the effect of compounds on the differentiation of ES cells into beating cardiomyocytes as an indication of developmental toxicity. The assay has been validated under the supervision of the European Centre for the Validation of Alternative Methods (ECVAM) in four different laboratories using 20 different chemicals resulting in an overall accuracy of 78% (Genschow *et al.*, 2004). However, it remains to be answered whether a validation study using 20 chemicals of diverse nature is sufficient to make a definitive statement on the validity of the test system. In addition the added value of the use of 3T3 cytotoxicity in the prediction model has been questioned (Spielmann *et al.*, 2006). The applicability domain of the EST in terms of the chemical classes for which the test makes reliable predictions is still not sufficiently defined to allow regulatory implementation and may vary with different classes of compounds tested (Hartung *et al.*, 2004; Spielmann *et al.*, 2006). The category approach assumes that if the *in vitro* ranking of the class of compounds corresponds with the *in vivo* ranking, the test system will give reliable results for new

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compounds within the chemical class. Therefore, evaluation of the EST within a distinct class of chemically related compounds can define the usefulness of the assay for the chemical class under study. The aim of the present study was to evaluate the performance of the EST for a selected series of homologous compounds. To this end a series of glycol ethers and their glycol ether alkoxy acid metabolites (Fig. 1) was tested in the EST in two independent laboratories. Results were compared among compounds as well as between laboratories, and with available data from *in vivo* animal studies. This class of compounds was selected based on the availability of sufficient *in vivo* data in order to allow comparison with the EST data. Also, these compounds showed differences in embryotoxic potencies among the chemicals within the class, which facilitates *in vitro-in vivo* comparison. Glycol ethers are widely used as solvents in cosmetics, printing inks, plastics, household and industrial cleaning products and textile dyes. Some of them have been shown to cause developmental toxicity through several routes of exposure in mice, rats and rabbits (Feuston *et al*., 1990; Hanley *et al*., 1984; Nagano *et al*., 1981; Wier *et al*., 1987). Frequently occurring malformations include spina bifida occulta, fused, small or missing digits and exencephaly. As the glycol ether alkoxy acid metabolites have been identified as the proximate developmental toxicants in animal studies and in whole embryo culture (Brown *et al*., 1984; Giavini *et al*., 1993), a series of glycol ether alkoxy acid metabolites was selected for the present study together with two parent compounds, namely ethylene glycol methyl ether (EGME) and ethylene glycol ethyl ether (EGEE) (Fig. 1). The specificity of the effects of the glycol ether metabolites in the EST was further studied by comparison with the effects of two structurally similar nonglycol ether organic acids.

To determine the applicability of the EST for glycol ethers, the differentiation inhibition dose-response data obtained for several glycol ether alkoxy acid metabolites were compared with *in vivo* developmental toxicity data of their parent compounds found in literature, using the benchmark dose (BMD) approach. In the BMD approach a dose-response curve is fitted to determine the BMD for *in vivo* data and the benchmark concentration (BMC) for *in vitro* data corresponding to the dose or concentration at which a certain benchmark response (BMR) is reached (Crump 1984). The method has several principal advantages above the classical No Observed

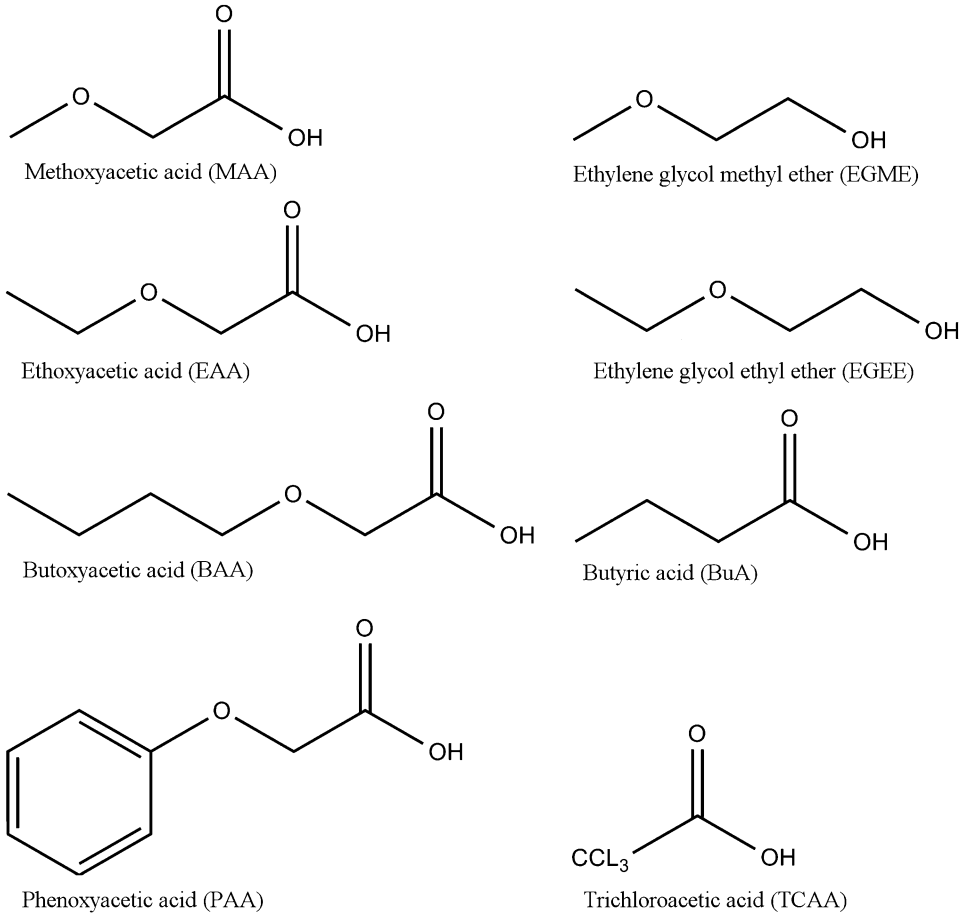

FIG. 1. Chemical structure of the glycol ethers, glycol ether alkoxy acid metabolites, and two structural analogs included in the present study.

Adverse Effect Level (NOAEL) approach as discussed elsewhere (Slob, 2002). One of the uncertainties in the BMD approach, as with any type of dose-response modeling, lies in the model chosen to mimic the "true" dose-response curve. The choice of the model fitted could influence the BMC values calculated and thereby influence the ranking of the compounds within the EST. Therefore, multiple models were used in the analysis to address this uncertainty. Another issue is defining the BMR level used to determine the BMC values. When dose-response curves are not of a similar shape, different response levels could lead to a difference in the ranking of the compounds based on the BMCs derived *in vitro*. This study investigated these uncertainties by deriving BMC values for multiple BMR values and with multiple types of dose-response models based on EST data for the glycol ether alkoxy acid metabolites as the selected chemical class. In addition, the effect of interlaboratory variation on compound ranking was addressed by testing the same compound set in two independent laboratories. Finally, the resultant ranking was compared with *in vivo* data to assess the predictive value of the EST for the developmental toxicity of glycol ethers.

MATERIALS AND METHODS

Cell Line and Culture Conditions

The mouse embryonic stem cell line D3 (ATCC, Rockville, MD) was grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Breda, The Netherlands) at 37°C and 5% CO_2 and routinely passaged three times per week. Medium was supplemented with 20% fetal calf serum (Hyclone, Thermo-Fisher Scientific, Etten-Leur, The Netherlands), 2mM glutamine (Invitrogen), 50 U/ml penicillin (Invitrogen), 50 μg/ml streptomycin (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1mM β-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, The Netherlands), and 1000 U/ml murine leukemia inhibiting factor (mLIF) (Chemicon, Amsterdam, The Netherlands).

Chemicals Tested

All chemicals were obtained from Sigma-Aldrich unless stated otherwise. A series of glycol ether alkoxy acid metabolites, namely methoxyacetic acid (MAA, cat. no. 194557), ethoxyacetic acid (EAA, cat. no. 137111), butoxyacetic acid (BAA, Tokyo Chemical Industries, Zwijndrecht, Belgium, cat. no. B1467), and phenoxyacetic acid (cat. no. 77740), was selected (Fig. 1). Other chemicals tested included the structural analogs trichloroacetic acid (TCAA, cat. no. 91228) and butyric acid (BuA, cat. no. B103500), and the parent compounds EGME (cat. no. 800858) and EGEE (cat no. 128082). All chemicals were dissolved and diluted in DMSO (0.1% vol/vol final concentration in culture medium) with the exception of MAA, EAA, EGME, and EGEE which were dissolved and diluted directly in DMEM. 5-Fluorouracil, dissolved in phosphate buffered saline (PBS, Invitrogen), was used as a positive control in the EST (Genschow *et al*. 2004).

Cytotoxicity Assay

To determine cytotoxicity of the compounds, ES-D3 cells were seeded in 96-well plates at 500 cells per well in routine culture medium and allowed to attach for 2 h before chemical exposure at equal concentrations as used in the differentiation assay. The cells were subsequently cultured for 5 days at 37°C and 5% CO_2, with a medium renewal containing the corresponding compound for exposure on day 3. On day 5 and approximately 80% cell confluence, CellTiter-blue (Promega, Leiden, The Netherlands) was added to each well and incubated for 2 h. After the incubation period the fluorescence was read using a FLUOstar spectrofluorometer (FLUOstar Optima, BMG Labtech, de Meeren, The Netherlands) at 544 (excitation) and 590 nm (emission). Three independent experiments were done for each compound.

Differentiation into Beating Cardiomyocytes

The EST for cardiac differentiation was carried out as previously described using culture medium in the absence of mLIF (Genschow *et al*., 2004). Briefly, on day 0 of the assay 20 μl of an ES cell suspension (3.75×10^4 cells/ml) was placed onto the inner side of the lid of a 10-cm Petri dish (Greiner) containing 5 ml of PBS and incubated at 37°C and 5% CO_2 for three days. On day 3, the resulting embryoid bodies (EBs) were transferred to bacteriological Petri dishes (Greiner). On day 5, the EBs were transferred to 24-well plates (TPP, Trasadingen, Switzerland). On day 10, the number of wells containing beating EBs was determined by light microscopy and compared with the number of wells containing beating EBs in the solvent control plate. Cells were exposed to the test compound in concentrations up to 1 mg/ml from day 0 onwards with a medium renewal containing the corresponding compound for exposure on day 3 and day 5. Solvent controls were included in each experiment. Tests were accepted for further analysis if the solvent control plate contained at least 21 beating EBs out of 24 EBs incubated. Three independent experiments were done for each of the compounds.

BMC and BMD Derivation

In vitro *data.* The results from the EST were analyzed using the BMD approach, meaning that a concentration-response curve was fitted to determine the concentration associated with a predefined BMR. For each of the compounds a 50% change in the number of beating EBs was selected as the BMR to calculate the benchmark concentrations for the differentiation (BMC_d50). BMD values at a BMR of 25 and 10% were also determined to study the effect of the choice of the response level on the ranking of the compounds within the chemical class. For the viability concentration-response curves, a 50% change in cell viability was selected as the BMR to calculate the benchmark concentrations for the viability (BMC_v50). For the continuous data from the viability assay a model was selected according to the procedure previously described by Slob (2002) in which a family of concentration-response models are fitted together. The goodness of fit was determined by the loglikelihoods of each model. The final model selected was the model with the lowest number of parameters which gave the best significant fit. For the quantal data from the differentiation assay several models with a statistically equal goodness of fit for the data were used to determine the effect of the model choice on the results. Models fitted included logistic, probit, weibull, and gamma. The BMC calculations were done using PROAST software (Slob, 2002).

In vivo *data.* For each of the compounds a literature search was performed for *in vivo* embryotoxicity and developmental toxicity of the parent compounds and their glycol ether alkoxy acid metabolites. For the glycol ether alkoxy acid metabolites, which are considered as the proximate embryotoxicants (Brown *et al*., 1984; Giavini *et al*., 1993), no relevant *in vivo* studies were available. Search terms included the compound name together with combinations of the search terms teratogen, teratogenic, teratogenicity, development, developmental toxicity, malformation, and embryotoxicity. Only studies containing multiple exposure times, multiple doses, and an oral exposure route were included in the data analysis for each of the glycol ethers. Fetal body weight and incidence of malformations were selected as the *in vivo* endpoints. The BMDs were calculated using dose-response modeling as done with the *in vitro* dataset for continuous data. When required, dose-response modeling using constrained fit parameters was applied. The BMR for the BMD for each compound was defined as a 10% decrease in fetal weight and a 10% additional incidence of malformations. These effect levels were selected because they could be estimated within each of the selected studies and could be distinguished from the background variation. This approach has previously been used in (Piersma *et al*., 2008). The BMD calculations were done using PROAST software (Slob, 2002).

RESULTS

Cytotoxicity Assay

Viability assays were performed to evaluate the cytotoxic effects of the compounds on the ES-D3 cell line (Fig. 2). The concentration-response curves obtained were used to calculate the BMC_v50 values corresponding to a 50% decline in cell viability (Table 1). Of the glycol ether alkoxy acid metabolites MAA was the most potent, reducing the cell viability of ES cells with a BMC_v50 of 6.7mM. The nonglycol ethers TCAA and BuA resulted in lower BMC_v50 values of 4.9mM and 0.9mM, respectively, reflecting higher cytotoxic properties of these compounds than of the glycol ether alkoxy acid metabolites tested. The parent compounds EGME and EGEE did not cause any reduction in cell viability (data not shown).

Differentiation into Beating Cardiomyocytes

To study the *in vitro* developmental toxicity potency of the chemicals the effect of each of the compounds on the differentiation of ES cells into beating cardiomyocytes was evaluated in two independent laboratories (Fig. 2). The Weibull model gave the best fit for the dataset obtained, based on the loglikelihood of the fitted curves and was used to determine the BMC_d50 values for both labs corresponding to a 50% decline

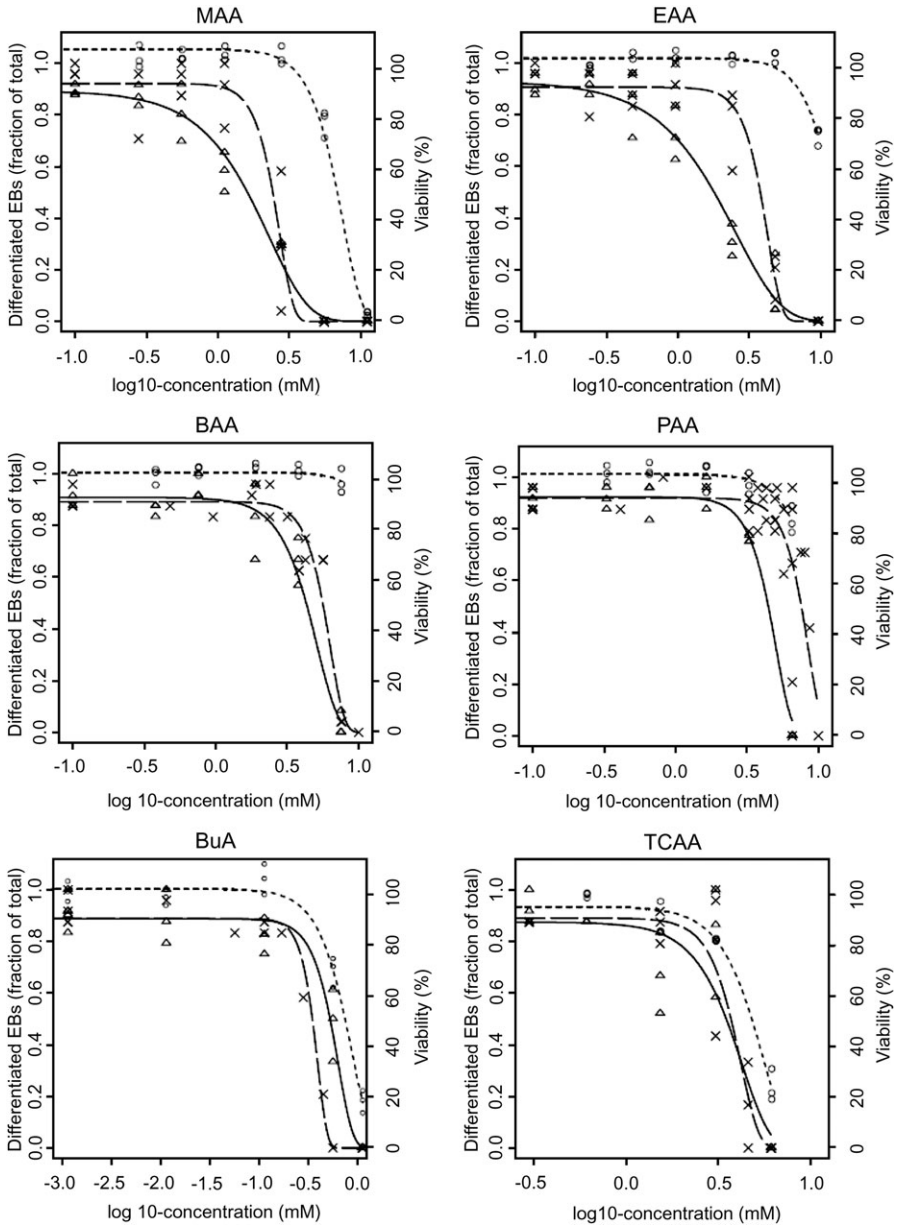

FIG. 2. Concentration-dependent effects of glycol ether alkoxy acid metabolites and two structural analogs on the viability in lab 1 (---) of murine ES cells and on differentiation into beating EBs in lab 1 (——) and lab 2 (— —).

TABLE 1
Viability (BMC_v50) and Viability at BMC_d50 Value of ES Cells after Exposure to Four Glycol Ether Alkoxy Acid Metabolites and Two Structural Analogs

	BMC_v50 (mM), Lab 1	Cell viability at BMC_d50 (%), Lab 1[a]
MAA	6.7 (6.3–7.0)	105
EAA	11.9 (11.1–13.1)	103
BAA	ND[b]	102
PAA	9.7 (8.6–11.8)	99
BuA	0.9 (0.8–0.9)	78
TCAA	4.9 (4.8–5.1)	70

Note. The 90% confidence interval is giving between brackets.
[a]Determined by extrapolation to fitted viability curve.
[b]ND = not detected.

in the fraction of beating EBs in comparison to the solvent control (Table 2).

The results show that all the glycol ether alkoxy acid metabolites exert a concentration-dependent inhibition on the differentiation of ES cells into beating cardiomyocytes. Furthermore, the BMC_d50 values occurred at concentrations that did not cause cytotoxicity indicating that the differentiation of the ES cells into beating cardiomyocytes is already affected at noncytotoxic concentrations of these compounds (Table 1). The difference between the BMC_v50 and the BMC_d50 ranged from a 2.1-fold difference for PAA to a 4-fold difference for EAA. Of the four glycol ether alkoxy acid metabolites tested MAA was the most potent compound followed by EAA, BAA and PAA, respectively (Table 2). In both laboratories, BuA had a strong effect on the differentiation of ES cells into beating cardiomyocytes with a BMC_d50 value four times lower than the most potent glycol ether alkoxy acid metabolite, MAA. However it should be noted that both for BuA and for TCAA the reduction in beating EBs only occurred at concentrations where cell viability was also reduced. The BMC_v50 values for BuA and TCAA were only 1.3-fold and 1.5-fold higher than the BMC_d50s. At the BMC_d50 values cell viability was reduced to 78 and 70%, respectively (Table 1). Unlike the glycol ether alkoxy acid metabolites, both parent compounds tested (EGME and EGEE) did not inhibit differentiation (data not shown), corroborating that the metabolites are the proximate developmental toxicants *in vivo* after exposure to the parent compounds. Therefore, the potency of the glycol ether alkoxy acid metabolites in the EST was compared with *in vivo* toxicity data of the glycol ethers.

The interlaboratory reproducibility of the results was determined by comparing the BMC_d50 values obtained at the two labs (Fig. 3). Interlaboratory variation is represented by the R^2, where the R^2 can range between 0 (no correlation) and 1 (equal values). The R^2 of 0.89 indicates a good correlation between the two laboratories whereas the BMC_d50 values were on average about 1.3-fold higher in Lab 2 than in Lab 1.

Choice of BMR and Dose-Response Model

For the *in vitro* data set a 50% decline in differentiated EBs was arbitrarily selected as the BMR for the BMC_d derivation to compare the results of the different compounds. The ranking of the different compounds might differ with the magnitude of the BMR chosen. This possibility was investigated by also calculating the BMC_d25 values for each of the compounds at both labs (Table 2). The choice for BMC_d25 instead of BMC_d50 values did not affect the ranking within the group of compounds. Furthermore, using an even lower BMR of 10% and using the BMC_d10 values for comparison also did not affect the ranking of the compounds (data not shown). In addition, the effect of the choice of the fitted model was evaluated by calculating the BMC_d50 values with different models for each of the compounds (Table 3). For the BMC_d50 values it was shown that the choice of model did not significantly affect the values obtained. Furthermore, the

TABLE 2
BMC_d25 and BMC_d50 of Differentiation of ES Cells into Beating Cardiomyocytes after Exposure to Four Glycol Ether Alkoxy Acid Metabolites and Two Structural Analogs

	BMC_d25 (mM), Lab 1	BMC_d50 (mM), Lab 1	BMC_d25 (mM), Lab 2	BMC_d50 (mM), Lab 2
MAA	1.7 (1.5–2.0)	2.3 (2.1–2.6)	2.0 (1.9–2.2)	2.5 (2.3–2.7)
EAA	2.1 (1.8–2.5)	2.9 (2.6–3.2)	3.2 (2.9–3.5)	3.9 (3.7–4.2)
BAA	3.4 (3.0–3.8)	4.5 (4.1–5.0)	4.8 (4.5–5.2)	5.9 (5.5–6.2)
PAA	3.7 (3.4–4.0)	4.6 (4.6–5.0)	6.4 (6.1–6.8)	7.8 (7.5–8.2)
BuA	0.4 (0.4–0.5)	0.6 (0.5–0.6)	0.3 (0.2–0.3)	0.4 (0.3–0.4)
TCAA	2.8 (2.4–3.1)	3.8 (3.4–4.1)	3.2 (2.9–3.4)	3.9 (3.7–4.1)

Note. The 90% confidence interval is given between brackets. BMC_d25 = 25% reduction of the fraction of wells containing beating EBs; BMC_d50 = 50% reduction of the fraction of wells containing beating EBs.

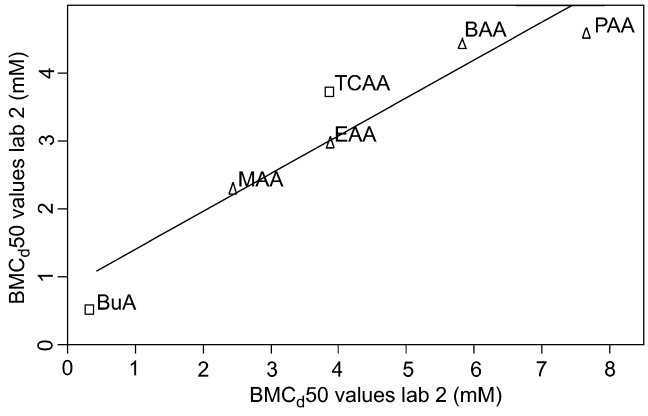

FIG. 3. Scatter plot for the interlaboratory comparison of BMC_d50 values of the six compounds tested in the EST. open triangle = glycol ether alkoxy acid metabolites, open square = structural analogs.

TABLE 3
Effect of Fitted Model on EST BMC_d50 Values of Four Glycol Ether Alkoxy Acid Metabolites and Two Structural Analogs in Two Laboratories

	Gamma	Loglogistic	Logprobit	Weibull
Lab 1				
MAA	2.4 (2.1–2.7)	2.5 (2.3–2.7)	2.5 (2.3–2.7)	2.3 (2.1–2.6)
EAA	2.7 (2.4–3.0)	2.4 (2.2–2.6)	2.6 (2.4–2.8)	2.9 (2.6–3.2)
BAA	4.5 (4.1–4.9)	4.5 (4.1–4.8)	4.5 (4.2–4.9)	4.5 (4.1–5.0)
PAA	4.6 (4.3–5.0)	4.6 (4.3–4.9)	4.6 (4.3–4.9)	4.6 (4.3–5.0)
BuA	0.6 (0.5–0.6)	0.6 (0.5–0.6)	0.6 (0.5–0.6)	0.6 (0.5–0.6)
TCAA	3.9 (3.6–4.3)	4.0 (3.7–4.4)	4.0 (3.7–4.3)	3.8 (3.4–4.1)
Lab 2				
MAA	2.5 (2.3–2.6)	2.3 (2.1–2.5)	2.5 (2.3–2.6)	2.5 (2.3–2.7)
EAA	3.7 (3.4–4.0)	3.4 (3.1–3.8)	3.6 (3.3–4.0)	3.9 (3.7–4.2)
BAA	5.7 (5.4–6.1)	5.5 (5.1–5.9)	5.7 (5.3–6.1)	5.9 (5.5–6.2)
PAA	7.7 (7.4–8.1)	8.2 (7.6–8.9)	7.7 (7.4–8.1)	7.8 (7.5–8.2)
BuA	0.3 (0.3–0.4)	0.3 (0.3–0.4)	0.3 (0.3–0.4)	0.4 (0.3–0.4)
TCAA	3.8 (3.6–4.0)	3.4 (3.2–3.7)	3.8 (3.5–4.0)	3.9 (3.7–4.1)

Note. The 90% confidence interval is given between brackets.

ranking of the compounds was not affected when using different models. Similarly, the choice of model did not significantly affect the BMC_d10 or BMC_d25 values and did not modify their ranking (data not shown).

BMD Derivation from In Vivo *Data*

Table 4 provides the details of the animal studies used to establish the BMD values. Studies were selected to represent the most uniform study design available between the different studies with respect to the animal species used, exposure route and exposure duration. For all compounds a mouse developmental toxicity study with the parent glycol ethers was available with exposure from gestation days 7–8 to day 14 by oral exposure with the exception of ethylene glycol monophenyl ether (EGPE). For EGPE, a mouse study with exposure from premating day 7 to birth was available. BMD values were determined that correspond with a 10% reduction in fetal body weight and with a 10% additional incidence of malformations. EGME was the most potent embryotoxic compound followed by EGEE. Ethylene glycol monobutyl ether (EGBE) exposure did lead to an increase in malformations and a reduction of fetal body weight with BMD values slightly higher than those of EGEE. However, these effects occurred only at EGBE doses that were maternally toxic, and thus they may be in part secondary to maternal toxicity. In the available *in vivo* study for EGPE, a slight decrease in fetal weight only occurred after exposure to a relatively high dose of 4 g/kg bw/day indicating that this glycol ether has the lowest developmental toxicity potency within the series.

DISCUSSION

The present study showed that all glycol ether alkoxy acid metabolites tested in the EST inhibited the differentiation of ES cells into beating cardiomyocytes in a concentration-dependent way. MAA appeared to be the most potent compound, followed by EAA, BAA, and PAA, respectively. The EST results were compared with *in vivo* embryotoxicity data for the corresponding parent glycol ethers, based on the BMC_d50 values for the *in vitro* effect of the glycol ether alkoxy acid metabolites on cell differentiation in the EST and the BMD10 values for fetal weight and malformations derived from the *in vivo* data for the parent glycol ethers.

The *in vitro* potency ranking of the glycol ether alkoxy acid metabolites was found to be the same as the *in vivo* potency ranking based on the BMD10 values of the parent glycol ether compounds. However, the relative differences between the *in vitro* BMC_d50 levels of the four compounds were smaller than the relative differences in BMD10 levels of the compounds found *in vivo*. In view of the observed effects on malformations in the animal studies, EGME (the parent compound of MAA) is 17-fold more potent than EGEE (the parent compound of EAA). In contrast, there was only a 1.4-fold difference between MAA and EAA in the *in vitro* study. In addition, the BMC_d50s for BAA are twofold higher in the *in vitro* EST compared with

TABLE 4
Developmental Toxicity of Glycol Ethers in Mice

Compound	Animal	Route	Days	Dose (mg/kg bw/day)	Reference	$BMD_{fetal\ weight}$ (mmol/kg bw/day)	$BMD_{malformations}$ (mmol/kg bw/day)
EGME (MAA)	JCL-ICR mice	G	GD7-14	0, 31.25, 62.5, 125, 250	Nagano *et al.* (1981)	1.3[a]	0.8[a]
EGEE (EAA)	CD-1 mice	G	GD8-14	0, 1000, 1800, 2600, 3400, 4200	Wier *et al.* (1987)	4.9[a]	13.9[b]
EGBE (BAA)	CD-1 mice	G	GD8-14	0, 350, 650, 1000, 1500, 2000	Wier *et al.* (1987)	12.0[a]	14.8[b]
EGPE (PAA)	CD-1 mice	D	7 PM-B	0, 400, 2000, 4000	Heindel *et al.* (1990)	30.7[a]	NA

Note. $BMD_{fetal\ weight}$ = dose (mmol/kg bw/day) at 10% decrease in fetal body weight; $BMD_{malformation}$ = dose (mmol/kg bw/day) at a 10% increase in malformations; G = gastric incubation; D = diet; GD = gestational day; PM = premating; B = birth; NA = data not available.

[a]BMDs were calculated from a model dose-response.

[b]BMDs were calculated from a model does-response using constrained fit parameters.

those for MAA, whereas in the *in vivo* animal studies there is about a 19-fold difference between the BMD10 values for EGME and EGBE (the parent compounds of MAA and BAA). With respect to the *in vivo* BMD10 values it should be mentioned that EGBE only induced malformations at maternally toxic doses. EGBE has been shown to induce severe hemolytic anemia following gavage and dermal exposure (Ghanayem *et al*., 2001). In general, a possible role of maternal toxicity in the mediation of developmental effects should be kept in mind, but a useful *in vitro* correlate is not easily defined. The developmental toxicity study on EGPE in mice that we used for comparison used oral exposure and reported effects of EGPE on fetal body weight only, but did not report malformations. In a dermal exposure developmental toxicity study with pregnant rabbits, EGPE showed no effect on the incidence of malformations up to a dose of 1000 mg/kg bw/day (Scortichini *et al*., 1987). Higher doses could not be achieved because of the occurrence of maternal toxicity. An explanation for the lack of malformations occurring after EGPE exposure as opposed to other glycol ethers could be the difference in exposure route and as a result a difference in internal concentrations. However, dermal exposure to EGME has been shown to induce malformations in rats at similar doses as those used in the EGPE study (Feuston *et al*., 1990), indicating that dermal exposure to glycol ethers can result in internal concentrations that are sufficiently high to induce malformations. Furthermore, EGEE has been shown to induce malformations in rabbits after inhalation indicating that rabbits are also sensitive for glycol ether induced malformations via exposure routes different from oral exposure (Doe, 1984). It is, therefore, unlikely that the lack of malformations upon dermal exposure to EGPE is caused by a difference in study design. The results rather reflect the lower embryotoxicity of EGPE as compared with the other glycol ethers.

The differences between the relative potencies *in vitro* and *in vivo* can, at least in part, be explained by differences in the kinetics of these compounds in the *in vivo* model as compared with the *in vitro* model system. Differences in *in vivo* kinetics of the glycol ethers and their metabolites are reflected in their half-life values. After intravenous injection MAA was shown to have a low elimination rate with a half-life in blood ranging between 14 and 18.6 h, whereas EAA and BAA displayed a shorter half-life ranging between 7.6 and 10.1 h for EAA and ranging between 1.5 and 3.2 h for BAA (Aasmoe and Aarbakke, 1997; Aasmoe *et al*., 1999; Ghanayem *et al*., 1990). This indicates that MAA is eliminated more slowly than EAA and BAA, resulting in higher plasma levels, which may explain the relatively higher embryotoxic potency of its parent glycol ether *in vivo*. The *in vitro* system does not simulate the *in vivo* kinetic processes and, therefore, the *in vitro* levels should be extrapolated to *in vivo* levels by integrating information on *in vivo* kinetics of the compounds. This also includes the differences in protein binding and rate of degradation affecting actual exposure concentrations. To overcome these issues, physiologically based kinetic modeling may prove to be a way to ultimately link the *in vitro* BMC_d50 values to BMD10 values from *in vivo* studies (Piersma *et al*., 2008; Verwei *et al*., 2006). Currently work is in progress to develop such *in silico-in vitro* models for the developmental toxicity of glycol ethers.

The two structurally related nonglycol ether compounds, TCAA and BuA, also caused a concentration-dependent inhibition of the differentiation of embryonic stem cells into cardiomyocytes. However, these effects appeared at concentrations at which the viability of cells in culture was also reduced indicating that the effect on differentiation may have been caused by the cytotoxic effect of the compounds. This observation demonstrates that it is informative to compare BMC_d50 values with the viability concentration-response of the test compounds for the interpretation of results obtained with the EST, whereas both endpoints may be relevant in predicting embryotoxicity *in vivo*. Our combined BMC_d50 and BMC_v50 results show that TCAA and BuA behave differently from the alkoxy acid metabolites, suggesting that the latter have a specific effect patterns which is not shared by both structurally related organic acids. TCAA and its parent compound trichloroethylene (TCE) have been linked to an adverse effect on the development of the heart in rats (Johnson *et al*., 1998). Furthermore, Drake *et al*. (2006) suggested that TCE induced cardiac malformations at a late stage of development, namely during the formation of cushion-like thickenings of the endocardium, which ultimately leads to the development of the valves. This late developmental process involving the endocardium and not the myocardium is apparently not detected in the EST. These observations emphasize that the applicability domains of *in vitro* tests with respect to the biological processes involved are important aspects to be taken into account when results of *in vitro* studies are translated to the *in vivo* situation.

In the present study the possible sources of uncertainties influencing the results were also investigated. First, we have studied interlaboratory variation. The high correlation between the BMC_d50 values of the two laboratories with an R^2 of 0.89 demonstrates the interlaboratory reproducibility of the EST. The 1.3-fold difference in the sensitivity of the EST observed between laboratories in BMC_d50 values is probably due to subtle differences in culture conditions. However, we show that this difference did not interfere with the overall conclusions in regard to the relative potency of the glycol ether metabolites. Furthermore, the results have shown that the selection of different models for the curve fitting did not affect the overall outcome. Finally, changing the arbitrarily chosen *in vitro* BMR of a 50% decline in the number of beating EBs to a 25 or 10% decline did not affect the overall ranking of the potency of the compounds either. These findings could be expected on the basis of the data structure in our experiments, because with an adequate distribution of test concentrations over the entire concentration-response range, the concentration-response

models will deliver similar fits and BMC_d50 values, as illustrated here.

In conclusion, the EST is a useful tool for the identification of the relative embryotoxic potencies of glycol ethers as a chemical class. However, the different kinetic processes in the EST as compared with *in vivo* affect the interpretation and should be taken into account in the quantitative extrapolation of the *in vitro* results to the *in vivo* situation. Combining the EST BMC_d50 values with a physiologically based kinetic model describing the *in vivo* kinetic properties of the glycol ethers using *in vitro* parameters could lead to a more accurate link of the *in vitro* results from the EST to the data from the *in vivo* studies. To our knowledge this is the first study to evaluate the EST for embryotoxic potencies within a chemical class. This study shows that the EST can be used to set priorities for developmental toxicity testing within classes of related compounds. Prior knowledge on major metabolites of the parent compound of interest can focus the choice of derivatives to be tested in the EST. A similar approach could be applied to other chemical classes to further define the applicability domain of the EST in terms of the groups of chemicals for which the test can be used and to set priorities in future animal testing for developmental toxicity. This approach can be useful in regulatory settings such as the European chemicals legislation (REACH), which anticipates significant additional animal testing while at the same time promoting the use of alternatives wherever appropriate (Van der Jagt *et al*., 2004).

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