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Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryotoxicity test and comparison with their *in vivo* potencies

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ABSTRACT

The zebrafish embryotoxicity test (ZET) is a fast and simple method to study chemical toxicity after exposure of the complete vertebrate embryo during embryogenesis *in ovo*. We developed a novel quantitative evaluation method to assess the development of the zebrafish embryo based on specific endpoints in time, the general morphology score (GMS) system. For teratogenic effects a separate scoring list was developed. The relative effects of eight glycol ethers and six 1,2,4-triazole anti-fungals were evaluated in this system and results were compared with *in vivo* developmental toxicity potencies.

Methoxyacetic acid and ethoxyacetic acid appeared as the most potent glycol ether metabolites, inducing growth retardation and malformations. Other glycol ethers showed no developmental toxicity. Flusilazole appeared the most potent triazole, followed by hexaconazole, cyproconazole, triadimefon, myclobutanil and triticonazole, respectively.

In general, the potency ranking of the compounds within their class in the ZET was comparable to their *in vivo* ranking.

In conclusion, the ZET with the GMS system appears an efficient and useful test system for screening embryotoxic properties of chemicals within the classes of compounds tested. This alternative test method may also be useful for the detection of embryotoxic properties of other classes of chemicals.

1. Introduction

A variety of alternative assays for developmental toxicity testing in animals has been developed over the years, including the zebrafish embryotoxicity test (ZET). This test is gaining popularity, since it is a unique alternative that enables the study of the initial stages of a complete and well characterized developmental period of a vertebrate embryo (Gilbert, 2000; Hill et al., 2005) in a simple and fast culture system (Kimmel et al., 1995; Nüsslein-Volhard and Dahm, 2002). Alternative low vertebrate whole embryo cultures include Japanese medaka (*Oryzias latipes*), fathead minnow (*Pimephales promelas*) and *Xenopus laevis*. Each of these models has their pros and cons (Braunbeck et al., 2005; Fort and Paul, 2002). Zebrafish embryos develop independently of the maternal fish, are simply kept in water and development until hatching takes only three days. All these advantages make the zebrafish embryo suitable for relatively high-throughput tests. In addition, at the embryonic stages used in the ZET, zebrafish embryos are not considered as experimental animals under European legislation (European Commission, 1986).

For evaluation of development and malformations of embryos, standardization of the scoring system will enhance reproducibility and thus improve comparison among experimental groups. One of the current methods is based on the scoring of several developmental and lethal endpoints in a binomial way to derive the EC_{50} and LC_{50} (Bachmann, 2002; Braunbeck et al., 2005; Nagel, 2002; Seok et al., 2008). Additionally, these data can be used to calculate the teratogenic index to predict the teratogenic potency of the compound (Nagel, 2002; Selderslaghs et al., 2009; Ton et al., 2006). However, the endpoints monitored may differ between studies and are scored as all or nothing events without taking severity of effects into account. To overcome this problem a more quantitative method has been introduced by Brannen et al. (2010). They assigned severity scores for several endpoints. Furthermore, body length and head–trunk angle were measured, the distance between eye and otic vesicle was estimated and somite pairs were counted, which makes this method relatively labor intensive.

In order to improve the standardization of the scoring, we developed a novel evaluation method for screening zebrafish embryo development allowing a relatively fast, but semi-quantitative

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assessment which is comparable to a scoring system commonly used in the rat whole embryo culture (WEC) (Brown and Fabro, 1981). Scores are assigned to readily observable specific developmental endpoints at different points in time. Specific teratogenic effects are separately recorded. This allows us to monitor developmental delay and retardation as well as teratogenicity.

In this study we used our general morphology score (GMS) system to evaluate the relative embryotoxic effects of compounds within two different classes of chemicals to evaluate the applicability of the ZET for these classes of compounds. We compared the ZET relative potencies within each class with their *in vivo* developmental toxicity ranking. This category approach assumes that if the ranking of the compounds in the ZET corresponds to the *in vivo* ranking, there is a high likelihood that the embryotoxic potency of new compounds within the same class can be predicted with the test system (de Jong et al., 2009; Hefter et al., 1999).

Both classes of compounds were selected based on the availability of *in vivo* data and the presence of embryotoxic as well as non-embryotoxic class members. To this end, a series of structural homologous glycol ether alkoxy acid metabolites and two of their parent compounds were tested. Glycol ethers are widely used as solvents in inks and paints. Some of them have been shown to have embryotoxic properties after exposure through several routes of administration in mice, rats and rabbits (Brown et al., 1984; Feuston et al., 1990; Hanley et al., 1984; Hardin et al., 1984; Nagano et al., 1981). Embryotoxic effects, mainly caused by ethylene glycol monomethyl ether (EGME) and its metabolite methoxyacetic acid (MAA), and ethylene glycol monoethyl ether (EGEE) and its metabolite ethoxyacetic acid (EAA), include visceral and skeletal malformations as well as resorptions (Hanley et al., 1984; Hardin et al., 1984).

Furthermore, we tested a series of six triazole derivatives. These compounds are used as fungicides and some of them also exhibit developmental toxic effects in rats and mice (Farag and Ibrahim, 2007; Machera, 1995; Menegola et al., 2005). These teratogenic effects include craniofacial and axial skeletal malformations. Specific teratogenic effects on the level of the branchial apparatus, such as reduction, agenesis and fusion between the arches were observed in rat whole embryo culture (Menegola et al., 2000, 2001) and in the amphibian *X. laevis* embryos (Groppelli et al., 2005; Papis et al., 2006).

2. Materials and methods

2.1. Maintenance of fish and egg spawning

Danio rerio adults were commercially obtained (Ruinemans Aquarium BV, Montfoort, The Netherlands) and maintained and bred in our facilities for more than 3 years. Adult zebrafish were kept in 7.5 l ZebTEC aquaria at 27 °C ± 1 °C with a photoperiod of 14 h light: 10 h dark. They were fed twice daily with dry flakes (Special Diet Services, Tecnilab-BMI BV, The Netherlands) and once daily with defrosted *Artemia* (Landman BV, The Netherlands) in a quantity that was consumed within 5 min. Three days before spawning, females were separately housed and fed only thawed *Artemia*, both to optimize egg production. Males and females were paired in spawning boxes the day before spawning in a ratio of 2:2. Spawning was triggered once the light was turned on and was usually completed within 30 min.

2.2. Compounds

All compounds were obtained from Sigma–Aldrich unless stated otherwise. A series of glycol ether 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), phenoxyacetic acid (PAA, cat. No. 77740), butoxyethoxyacetic acid (BEAA, Tokyo Chemical Industries, cat. No. D2491) and methoxyethoxyacetic acid (MEAA, cat. No. 407011) were selected. Furthermore, ethylene glycol monomethyl ether (EGME, cat. No. 360503) and ethylene glycol monoethyl ether (EGEE, cat. No. 128082), the two parent compounds of MAA and EAA, respectively, were tested. These compounds were diluted directly in Dutch Standard Water (DSW; demineralized water supplemented with $NaHCO_3$ (100 mg/l), $KHCO_3$ (20 mg/l), $CaCl_2{\cdot}2H_2O$ (200 mg/l), and $MgSO_4{\cdot}7H_2O$ (180 mg/l) and then aerated for 24 h at 27 °C).

In addition, a series of six triazole derivatives was tested: flusilazole (FLU, cat. No. 45753), hexaconazole (HEX, cat. No. 34348), cyproconazole (CYP, mixture of diastereomers, cat. No. 46068), triadimefon (TDF, cat. No. 45693), myclobutanil (MYC, cat. No. 34360) and triticonazole (TTC, cat. No. 34172). All triazoles were dissolved in DMSO and further diluted in DSW (0.2% DMSO vol/vol final concentration). 0.2% DMSO was used as solvent control.

As negative and positive control 3,4-dichloroaniline (cat. No. 35827) was used at concentrations of 6.2 and 48.4 μM respectively, to verify the sensitivity of the embryos. At the lower concentration embryos developed normally as opposed to the high exposure which caused coagulation of all embryos within 24 h. Sensitivity of the embryos remained the same during all tests (data not shown).

The pH of all test media ranged from or was adjusted to 7.4–8.4, and O_2-concentration was at least 6.5 mg/l before and after the test.

2.3. Exposure

Fertilized eggs were collected 30 min after spawning (approximately 2–8 batches per test) and rinsed a few times in DSW before exposure. Fertilization rate of the batch of eggs used was at least 90%. After rinsing, the eggs were evenly distributed among the test concentrations. Hereafter, embryos within the 4- to 32-cell stage were selected and transferred to a 24-well plate containing 2 ml of test medium per well. One embryo was transferred to one well and 10 embryos per test concentration were used. Each experiment was performed in triplicate. Four control embryos were present on each plate and if necessary solvent controls were included. Embryos were kept in an incubator at 26.5 °C ± 1 °C with a photoperiod of 14 h light: 10 h dark.

2.4. Evaluation of embryos

Morphological evaluation of the embryos was performed at 72 h post fertilization (hpf) using a Leica Labovert FS microscope. GMS was recorded using the GMS system (Fig. 1). This scoring system is developed similar to the one used for rat WEC (Brown and Fabro, 1981) and comprises the normal development of a zebrafish embryo up to 72 hpf as described by Kimmel et al. (1995). The semi-quantitative assessment of specific developmental endpoints supports standardization of the evaluation. An experimental embryo is compared to the reference embryo in the scoring matrix and receives points for each developmental hallmark dependent on its stage of development. All deviations, for instance incomplete detachment of the tail, will result in a lower point score which corresponds to a certain extent of developmental retardation. Malformations and other teratogenic effects are separately recorded as present or absent according to the list in Table 1. The test was considered valid if <10% of the control embryos showed coagulation or effects.

2.5. Benchmark concentration and benchmark dose determination

The results of the ZET data were analyzed using the benchmark dose (BMD) approach (Slob, 2002), in which the benchmark

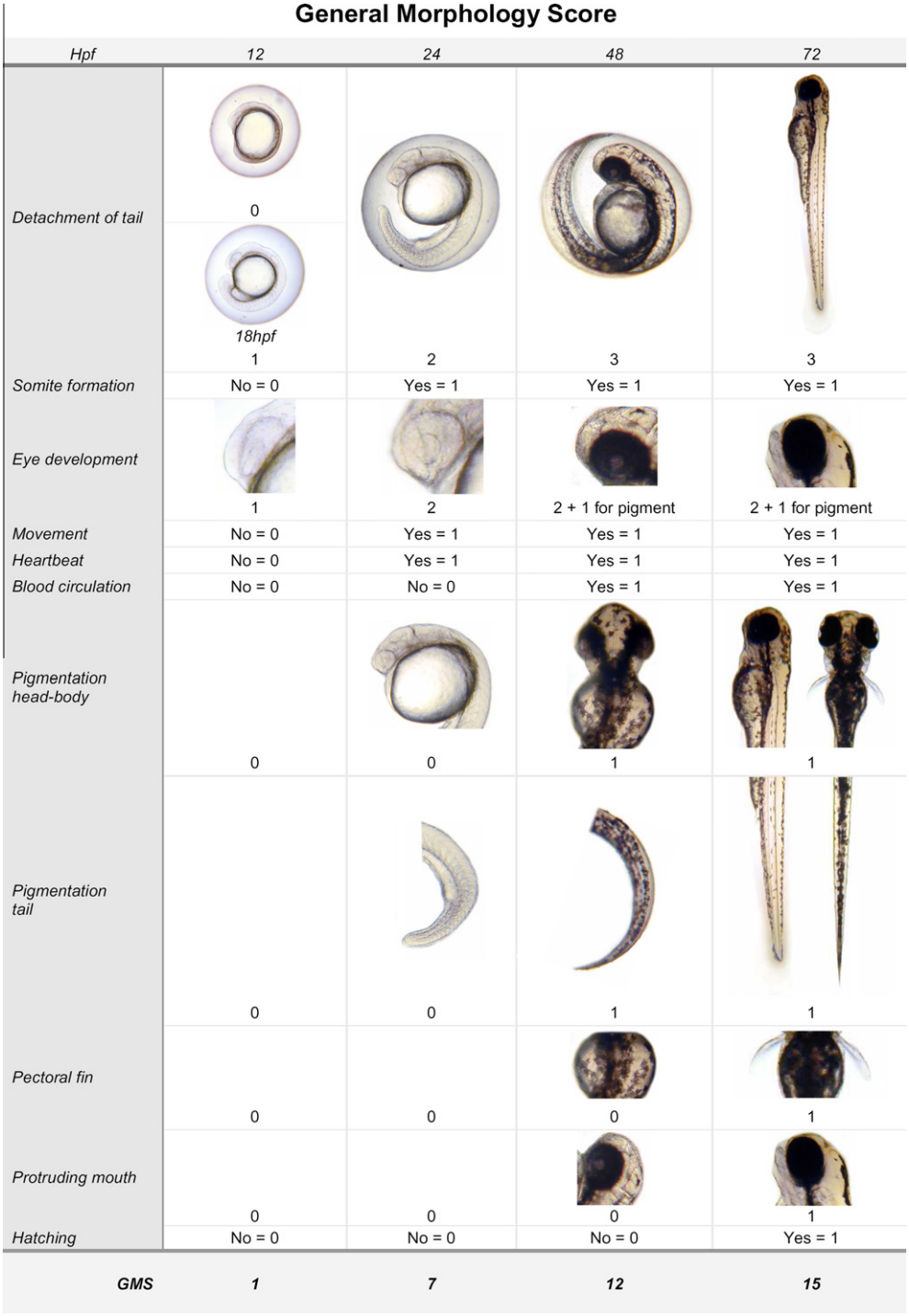

Fig. 1. General morphology scoring system showing normal development of a zebrafish embryo up to 72 h post fertilization with different scores assigned to specific developmental endpoints in time.

concentration (BMC) at a predefined benchmark response (BMR) was calculated using a fitted dose–response curve.

For the tested compounds a decrease of 5% in GMS was defined as the BMR for calculating the corresponding BMC (BMC_{GMS}). This BMR level was arbitrarily selected to obtain the concentration related to the threshold of effect outside the normal variation. The model used to fit these data was selected according to a previously described method (Piersma et al., 2008; Slob, 2002). Briefly, in this procedure a nested family of concentration–response curves with an increasing number of parameters is fitted and the log likelihood of each model is calculated to determine its goodness of fit. The model with the lowest number of parameters which gave the best fit was selected to calculate the BMC_{GMS}.

The BMC for teratogenicity (BMC_T), with teratogenicity defined as the fraction of embryos with one or more teratogenic effects, was calculated with a BMR defined as a 5% increase in the fraction of affected embryos. This level was also arbitrarily selected in the same manner as for the BMC_{GMS}. For these quantal data, four models with statistically similar goodness of fit were fitted, namely log–logistic, Weibull, log-probit and gamma. The model with the

Table 1
List of teratogenic effects scored in zebrafish embryos.

Pericardial edema
Yolk sac edema
Eye edema
Malformation of the head
Malformation of sacculi/otoliths
Malformation of tail
Malformation of heart
Modified chorda structure
Scoliosis
Rachischisis
Yolk deformation

Effects are scored as present or absent.

lowest BMC outcome was chosen. However, compounds within the same class are expected to have similar mechanisms of action. Therefore, based on the analysis of individual compounds the most conservative model per class of compounds was selected for final BMC calculation (DPR-MT1, 2004; DPR-MT2, 2004). For the group of glycol ethers and their metabolites the gamma model was used, as for the triazole anti-fungals the Weibull model was selected to fit the concentration–response curves.

2.6. In vivo data

A literature survey was performed for each of the glycol ether compounds to map their embryotoxic and developmental toxic effects *in vivo*. For the glycol ether metabolites, which are regarded as the proximate teratogens (Brown et al., 1984; Giavini et al., 1993; Yonemoto et al., 1984), no relevant literature on *in vivo* studies was available and for that reason studies using the parent glycol ethers were included. Furthermore, only studies with multiple exposure times, multiple doses and an oral exposure route were taken into account.

Model selection and BMD derivation was performed in the same way for the *in vivo* data as was done for the ZET data. The endpoints for *in vivo* data were fetal body weight (BMD_{BW}) and incidence of malformations (BMD_M). The corresponding BMRs were set at 10% decrease in fetal body weight and a 10% increase in incidence of malformations, which were judged to be close to the threshold of detection of adverse effects. Fetal body weight was analyzed as a continuous endpoint and the incidence of malformations as a quantal one.

The effect levels for the *in vivo* as well as for the ZET data were chosen such that they could be estimated within each of the selected studies and could be distinguished from the background variation. This approach has previously been used by Piersma et al. (2008). Proast curve-fitting software was used to derive the BMCs and BMDs.

In vivo data for all the triazole anti-fungals was obtained from the Toxicity Reference Database (ToxRefDB (US Environmental Protection Agency)). This database provides detailed toxicity data including the results of developmental toxicity studies. The developmental lowest effect levels (dLEL) of the triazoles were used to compare with our ZET data. *In vivo* and ZET data were correlated using Proast software and a maximum correlation was calculated using the model which fitted a straight line on a double logarithmic scale ($y = ax^b$) (Bokkers and Slob, 2005; Piersma et al., 2008).

3. Results

3.1. Glycol ethers – ZET

After conducting the ZET, BMC_{GMS} and BMC_T were derived for the group of glycol ethers and their metabolites (Table 2). Results showed that only MAA and EAA resulted in a concentration-dependent decrease in GMS, with a BMC_{GMS} of 2.7 and 3.1 mM, respectively (Fig. 2(A and B)). The other glycol ether metabolites did not reduce the GMS as compared to the controls up to the highest concentration that could be tested. Furthermore, embryos exposed to MAA and EAA showed comparable dysmorphology after exposure (Fig. 3, left panel). Several teratogenic effects were observed following exposure, among which heart, head and tail malformations, including scoliosis, were the most pronounced. The corresponding BMC_T for MAA and EAA were 4.6 and 2.9 mM, respectively. Unlike their metabolites, the parent compounds EGME and EGEE did not show any effect on general morphology and teratogenicity.

Table 2
Benchmark concentrations for the endpoints GMS and teratogenicity for different glycol ethers in the ZET.

Glycol ethers	BMC_{GMS}[a] (mM)	BMC_T[b] (mM)
MAA	2.7 (1.9–3.6)	4.6 (2.5–5.7)
EAA	3.1 (2.6–3.7)	2.9 (2.2–3.5)
BAA	–	–
PAA	–	–
MEAA	–	–
BEAA	–	–
EGME	–	–
EGEE	–	–

– No effect.
[a] Benchmark concentration for general morphology score at a 5% benchmark response.
[b] Benchmark concentration for teratogenicity at a 5% benchmark response.

3.2. Glycol ethers – in vivo

From literature, *in vivo* studies were selected to calculate the benchmark dose for body weight effects (BMD_{BW}) and for malformations (BMD_M) for the different compounds. To facilitate comparison, selection criteria included similarity of species, exposure route and exposure timing and duration. Table 3 presents the details of the studies selected and the calculated BMD_{BW} and BMD_M. Rat gavage studies with complete prenatal developmental exposure were predominant, although for some compounds only a mouse study or a rat dietary exposure could be identified. EGME and EGEE, parent compounds of MAA and EAA (also indicated in Table 3), appeared as the most potent compounds *in vivo* both with regard to fetal body weight reduction and malformations. The respective $BMDs_{BW}$ were 0.2 and 0.7 mmol/kg bw/day and the respective $BMDs_M$ were 0.5 and 0.8 mmol/kg bw/day. EGME and EGEE were followed by EGBE and diEGME (the parent compound of MEAA), which had similar BMDs. However, for EGBE it should be noted that the confidence interval exceeded the highest concentration tested, and its developmental effects occurred at doses toxic to pregnant female rats. For EGPE just one study was available from which only a BMD_{BW} could be derived. However, it must be noted that the slight decrease in fetal body weight that was observed occurred at the relatively high dose of 4000 mg/kg bw/day and that the BMD_{BW} exceeded the highest concentration tested. For diEGBE (BEAA) no observed effects subsequent to exposure were described *in vivo*.

3.3. Triazoles – ZET

In Fig. 2(C and D) the concentration–response curves for the six triazoles tested are presented. Using these curves the BMC_{GMS} was determined. In this study, FLU and HEX were the most potent triazole anti-fungals tested (Table 4). A reduction of 5% in GMS was found for FLU at 4.8 μM and for HEX at 7.0 μM. CYP, TDF and

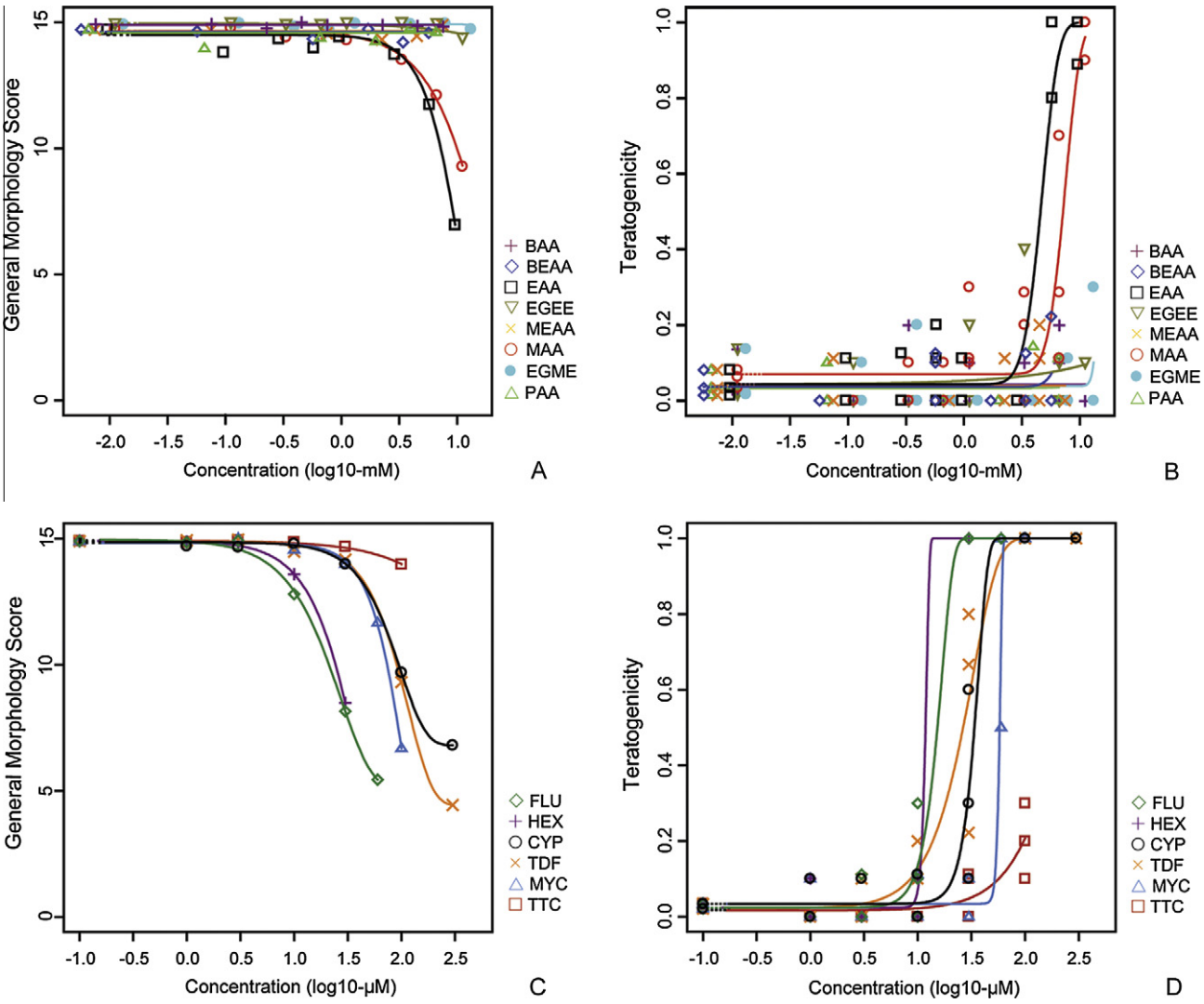

Fig. 2. Concentration–response curves of general morphology score and teratogenicity (fraction of embryos with at least one teratogenic effect) scored at 72 h post fertilization for the glycol ether metabolites (A and B) and 1,2,4-triazoles (C and D).

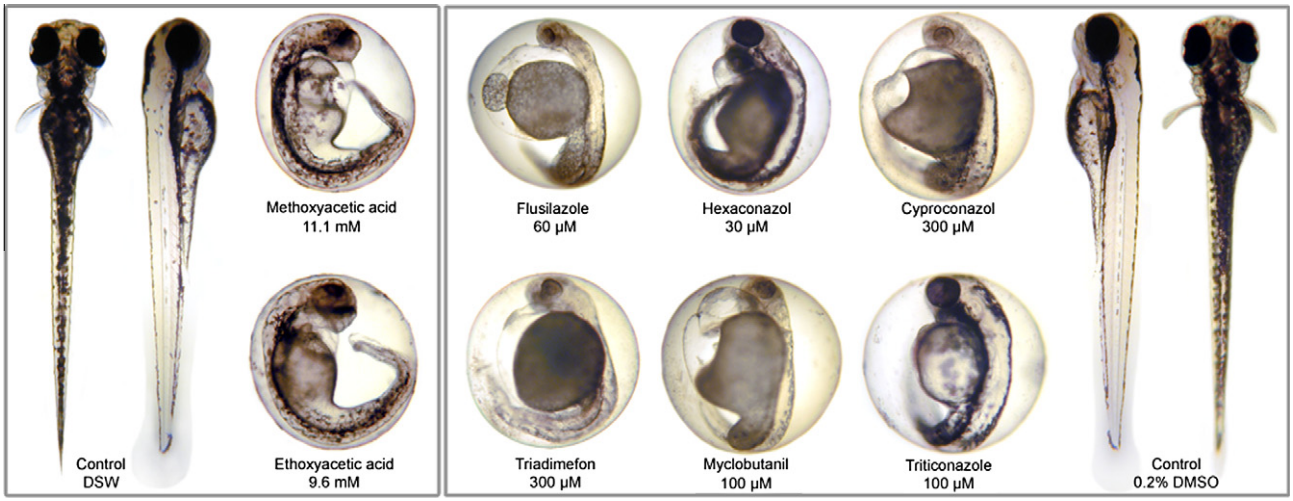

Fig. 3. Representative pictures of zebrafish embryos at 72 h post fertilization exposed to DSW (control) and glycol ether metabolites (left panel), and DMSO (control) and 1,2,4-triazoles (right panel).

MYC showed a lower but similar potency with a BMC_{GMS} ranging between 27.7 and 30.2 µM. TTC showed minor effects only in the highest concentration tested and was indicated as the least potent triazole with a BMC_{GMS} of 80.5. Furthermore, it should be noted

Table 3
Developmental toxicity of glycol ethers in rodents.

Parent compound	Species and strain	Route	Days	Dose (mg/kg bw/day)	BMD_{BW}[a] (mmol/kg bw/day)	BMD_M[b] (mmol/kg bw/day)	References
EGME (MAA)	SD rat	Diet	GD7–18	0–16–31–73	0.2 (0.1–0.3)	0.5 (0.5–0.7)	Nelson et al. (1989)
EGEE (EAA)	Wistar rat	Diet	GD1–21	0–12–23–46–93–186–372	0.7 (0.6–0.8)	0.8 (0.8–0.9)	Stenger et al. (1971)
EGBE (BAA)	CD-1 mice	Diet	GD8–14	0–350–650–1000–1500–2000	12.0 (7.9–24.4[c])	12.8 (9.7–45.1[c])	Wier et al. (1987)
diEGME (MEAA)	SD rat	Gavage	GD7–16	0–1000–1495–2235–3345	16.3 (11.9–20.6)	10.4 (4.3–14.5)	Hardin et al. (1986)
EGPE (PAA)	CD-1 mice	Diet	7 PM–B	0–400–2000–4000	30.7[c] (24.4–41.5[c])	NA	Heindel et al. (1990)
diEGBE (BEAA)	Wistar rat	Diet	GD0–20	0–25–115–633	No effect	No effect	Ema et al. (1988)

PM = premating, GD = gestational day, B = birth, NA = data not available.
Metabolite is indicated between brackets.
[a] Benchmark dose for fetal bodyweight reduction at a benchmark response of 10%.
[b] Benchmark dose for the increase in fetal malformations at a benchmark response of 10%.
[c] Exceeding highest dose tested.

Table 4
Benchmark concentrations for the endpoints GMS and teratogenicity for different triazoles in the ZET.

Triazoles	BMC_{GMS}[a] (μM)	BMC_T[b] (μM)	dLEL[c] (μmol/kg bw/day)
FLU	4.8 (4.3–5.4)	8.1 (5.4–11.3)	1.3
HEX	7.0 (6.1–7.9)	10.1 (7.1–19.0)	8.0
CYP	27.7 (22.3–34.7)	19.8 (8.4–29.7)	41.1
TDF	29.2 (23.1–37.5)	6.6 (3.5–12.3)	170.2
MYC	30.2 (28.0–32.5)	51.4 (25.8–53.8)	1083.9
TTC	80.5 (66.7–101.5[d])	40.0 (16.2–96.2)	3146.5

[a] Benchmark concentration for general morphology score at a 5% benchmark response.
[b] Benchmark concentration for teratogenicity at a 5% benchmark response.
[c] dLEL: lowest effect level for any developmental effect derived from the ToxRefDB.
[d] Exceeding highest concentration tested.

that the confidence interval of the TTC BMC_{GMS} exceeded the highest tested concentration.

Comparable patterns of teratogenic effects were observed for all triazoles, however, at different concentrations, indicative of differences in potency. TDF most potently induced teratogenic effects, showing a 5% increase in the fraction of affected embryos at a concentration of 6.6 μM. Next in line were FLU and HEX, with a BMC_T of 8.1 and 10.1 μM, respectively, followed by CYP with a BMC_T of 19.8 μM. MYC was found to have a BMC_T of 51.4 μM. TTC showed a BMC_T of 40.0 μM, however, even at the highest tested concentration TTC did not cause 100% teratogenicity in contrast to the other compounds.

Despite the different concentrations at which the various triazoles exerted their effects, the patterns of teratogenic effects appeared very similar (Fig. 3, right panel), mostly comprising head and heart malformations, scoliosis, yolk deformation and edema in exposed embryos.

3.4. Triazoles – *in vivo*

Similar to our ZET results, the lowest effect level for developmental effects (dLEL), as obtained from the ToxRefDB, showed that FLU is the most potent triazole antifungal (1.3 μmol/kg bw/day) (Table 4). In addition, the dLEL of HEX is in the same order of magnitude at 8 μmol/kg bw/day. CYP, TDF and MYC have lower potencies with a dLEL of 41.1, 170.2 and 1083.9 μmol/kg bw/day, respectively. TTC showed to be the least potent compound (3146.5 μmol/kg bw/day). In general, the ranking of these compounds with the ZET is comparable to the ranking *in vivo*.

Fig. 4 shows the correlation between the *in vivo* dLEL and the ZET BMC_{GMS} for the triazoles. On a double logarithmic scale a straight line can be fitted with a slope of 2.6 and with a maximum correlation (r^2) of 0.88.

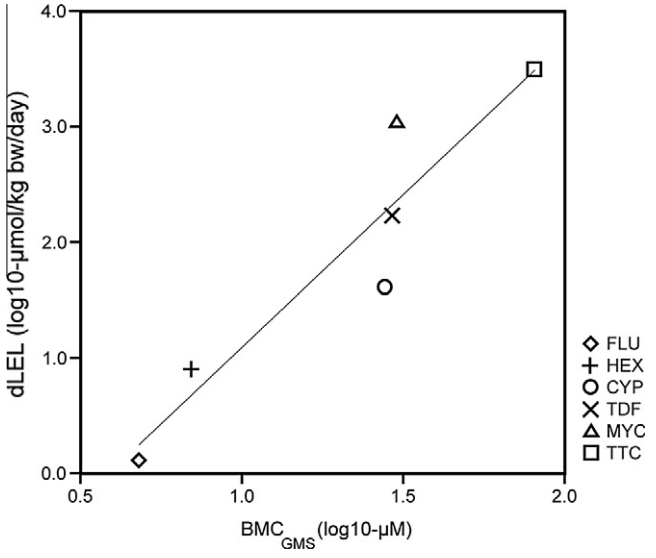

Fig. 4. *In vivo* dLEL values plotted against *in vitro* BMC values for GMS. Line shows correlation with $y = ax^b$. Slope $b = 2.6$ and correlation $r^2 = 0.88$.

4. Discussion

In this study we employed a novel evaluation method for morphologically screening zebrafish embryo development. The GMS system was based on the normal developmental hallmarks of a zebrafish embryo up to 72 hpf. Scores were assigned to well-defined and easily observable morphological endpoints characterized by a distinct developmental progression in time which leads to a standardized and semi-quantitative assessment of (mal)development. The GMS system has a similar design as the scoring system developed for WEC (Brown and Fabro, 1981) albeit that GMS includes fewer endpoints and more limited score levels. Different methods for evaluation of zebrafish embryos are available, for instance the one developed by Nagel (2002). They score twenty-one endpoints in a binomial way to derive the LC_{50} and EC_{50} (Nagel, 2002). In addition, the teratogenic index (LC_{50}/EC_{50}) can be calculated to give an indication of the teratogenicity of a compound (Nagel, 2002; Selderslaghs et al., 2009). However, the severity of effects for the endpoints used is not taken into account. Brannen et al. (2010) use a more quantitative assessment of the zebrafish embryos by assigning points to the evaluated parameters, and several endpoints are measured or counted, giving quantitative results, which is quite labor intensive. Our system uses a semi-quantitative assessment, which is relatively faster, and measures development in time as well as teratogenic effects. Our results

indicate that the GMS system is sensitive to detect effects on development and allows us to discriminate between compounds within a class of chemicals, with different embryotoxic potencies.

Within the class of glycol ether compounds, our results indicate that MAA and EAA were the most potent glycol ether metabolites inducing growth retardation. The ranking of the metabolites based on BMC_{GMS} was found to be in good agreement with the *in vivo* BMD_{BW} of the parent compounds. The same held true for malformations in the ZET and *in vivo* for these compounds; in the ZET MAA and EAA both most potently caused teratogenic effects, and *in vivo* both EGME and EGEE were also found to be the most potent teratogenic compounds. These compounds decreased the GMS in developing zebrafish in a concentration-dependent manner. Furthermore, they induced several distinct teratogenic effects. The differences in relative potencies of metabolites *in vitro* and their parent compounds *in vivo* can be explained at least in part by differences in *in vivo* kinetics of the compounds. After intravenous or intraperitoneal injection in the rat the elimination half-life was estimated to be 14–18.6 h for MAA and 7.6–10.1 h for EAA (Aasmoe and Aarbakke, 1997; Aasmoe et al., 1999). The slower elimination of MAA suggests increased exposure of the embryo to this compound compared to EAA, which might explain its relatively higher embryotoxic potency.

In addition, other studies showed growth retardation and malformations in embryos exposed *in utero* to MAA and EGME (Brown et al., 1984; Feuston et al., 1990; Hanley et al., 1984; Nagano et al., 1981). Skeletal defects were among the most frequently found malformations caused by MAA and EGME (Brown et al., 1984; Hanley et al., 1984; Nagano et al., 1981; Sleet et al., 1988; Stenger et al., 1971), which are comparable to one of the most frequent malformations observed in this study in the ZET, namely tail malformations including scoliosis.

The relative potencies in the ZET were also comparable to observations in *in vitro* tests. In the embryonic stem cell test MAA and EAA were also found to be the most potent compounds of the glycol ether metabolites in inhibiting the differentiation of stem cells into beating cardiomyocytes (de Jong et al., 2009). In addition, a concentration-related decrease in total morphological score, indicating growth retardation, was observed in the rat WEC after exposure to MAA and EAA (Giavini et al., 1993; Rawlings et al., 1985; Yonemoto et al., 1984), which is comparable to our results for GMS in the ZET.

In vivo, parent compounds EGME and EGEE are thought to exert their effects via their alcohol dehydrogenase (ADH) mediated embryotoxic metabolites MAA and EAA, respectively (Brown et al., 1984; Giavini et al., 1993). However, in the ZET these parent compounds do not seem to have an effect, which indicates a lack of metabolism. In WEC the rat embryo is also not affected by the parent compounds probably due to a lack of ADH activity (Yonemoto et al., 1984). For zebrafish embryos it has been found that ADH8A and ADH8B mRNA were expressed as early as 24 hpf (Reimers et al., 2004), which is part of the time window in the ZET. However, ADH8A showed considerably lower expression in 24–96 hpf zebrafish embryos compared to adults, suggestive of a limited ability to metabolize compounds during the first hours of development (Reimers et al., 2004).

In contrast to MAA and EAA, BAA and PAA did not show any effects in the ZET. *In vivo*, their parent compounds EGBE and EGPE appear to reduce fetal body weight in mice. However, for EGPE the BMD_{BW} exceeded the highest concentration that was tested, which was indicated as the maximally tolerated dose (4000 mg/kg bw/day) (Heindel et al., 1990). In rabbits, dermally exposed to EGPE, neither embryotoxicity nor teratogenic effects were observed (Scortichini et al., 1987), which concurs with our results in the ZET as well. EGBE also induced some teratogenic effects *in vivo*. However, the observed effects occurred at maternally toxic doses, which might explain the lower body weight of the fetuses at these doses (Wier et al., 1987). Thus, the absence of observed effects in the zebrafish embryo, in which maternal toxicity does not play a role, indeed may be in line with inactivity of EGBE and EGPE in mouse and rabbit embryos. This finding stipulates the advantage of the ZET, in that effects are always directly on the embryo and no uncertainty can arise about possible maternally mediated embryotoxicity.

Also BEAA and MEAA did not change the GMS and the fraction of embryos with teratogenic effects compared to the controls. As well as in the ZET, the parent compound of BEAA did not have an effect *in vivo* in rats (Ema et al., 1988; Nolen et al., 1985) or rabbits (Nolen et al., 1985) exposed during gestation. For diEGME, *in vivo* effects were found in contrast to no observed effects in zebrafish embryos exposed to MEAA. In a developmental toxicity study, Hardin et al. observed effects of diEGME in rats after exposure from GD7–16 (Hardin et al., 1986). However, the potency of diEGME was considerably lower than that of EGME and EGEE which might be the reason why we did not measure any effects in the ZET with MEAA.

In summary, for the chemical class of glycol ethers and their metabolites, the ZET was able to distinguish and rank compounds as to their embryotoxic potencies *in vivo*, although the ZET apparently lacked the required metabolic activation capacity and the interpretation was based on prior knowledge of proximate embryotoxic metabolites *in vivo*.

The ranking of triazole derivatives based on BMC_{GMS} showed that FLU and HEX were the most potent compounds in the ZET. These compounds were also found to be the most potent *in vivo*, with FLU and HEX having the lowest dLEL. FLU and HEX were followed by the less potent CYP, TDF and MYC. These three compounds had a similar potency in the ZET. The least potent triazole derivative in the ZET as well as *in vivo* was TTC. *In vivo*, the triazole ranking was comparable to the ranking in the ZET, however, the relative potencies were different. These variations may be explained by differences in uptake, distribution and elimination between the models. Anyway, the overall correlation between the *in vivo* and ZET data appeared to be good ($r^2 = 0.88$).

Based on teratogenicity, TDF was found to be very potent in the ZET, comparable with FLU and HEX, which is in contrast with the ranking *in vivo*. However, the number of effects observed in one embryo caused by FLU and HEX was higher than the number of effects of TDF at similar low doses. Mainly heart malformations or pericardial edema were found after exposure to TDF, in contrast to head malformations, yolk sac edema and yolk deformations which were observed after exposure to FLU and HEX.

In a developmental toxicity study using mice, craniofacial malformations and axial skeletal defects were found following exposure to TDF (Menegola et al., 2005). In addition, Machera et al. showed delayed ossification of the skull bones and cleft palate in rat embryos exposed during gestation to CYP (Machera, 1995). *X. laevis* studies showed also craniofacial malformations in embryos exposed to triazoles; mainly branchial arch malformations were found after exposure to TDF, which precedes craniofacial defects (Groppelli et al., 2005; Papis et al., 2006). Similar defects were also found in rat embryos exposed to FLU (Menegola et al., 2001). It can be concluded that in the ZET all tested triazoles, except TTC, showed teratogenic effects of a comparable nature, although at different doses, indicative of differences in potency. In addition, the potency ranking appeared very favorably comparable to the *in vivo* potencies, especially when considering that the correlation was based on toxicodynamics only.

As stated before, the teratogenic effects found in one class of chemicals appeared very similar between the compounds in that class. Moreover, as shown in Fig. 3, the effects found in glycol ether exposed zebrafish embryos were very different from the effects

observed in zebrafish embryos exposed to the triazoles. This is indicative of different mechanisms of embryotoxic action between these classes. For instance, MAA appears to have an endocrine disruptive effect; it potentiates the ligand-dependent activity of multiple nuclear receptors by targeting a common pathway in nuclear receptor-mediated signaling (Henley and Korach, 2006). The triazoles are thought to inhibit the cytochrome P450 isoenzyme CYP26 (Menegola et al., 2006). In early development of zebrafish these enzymes are already present (Dobbs-McAuliffe et al., 2004; Gu et al., 2005). It is possible that in the zebrafish embryo the different mechanisms of action of these classes of compounds may lead to different patterns of malformations. Thus, in addition to embryotoxic potency determination, the ZET allows the identification of specific malformation patterns that may be used to further elucidate mechanisms of embryotoxicity.

The wealth of transgenic zebrafish models in addition to siRNA, morpholino and transciptomics approaches currently being developed adds to the elaborate toolbox available for the study of embryotoxicity in the zebrafish embryo model (Bill et al., 2009; Hill et al., 2005; Nasevicius and Ekker, 2000; Weil et al., 2009; Yang et al., 2007, 2009), and could be employed in combination with the GMS.

In this study, the category approach was applied, which assumes that a series of compounds with similar structure will show coherent trends in their toxicological effects, generally associated with a common mechanism of action (Hefter et al., 1999; OECD, 2007). If the *in vitro* ranking of the compounds within a class corresponds to the *in vivo* ranking there is a high likelihood that embryotoxicity of new compounds within the same class can be reliably predicted with the test system. We demonstrated that the ranking of glycol ether alkoxy metabolites and 1,2,4-triazole derivatives with the ZET are very comparable to the *in vivo* ranking. This approach may help to further assess the applicability domain of the ZET regarding additional chemical classes.

Conflict of interest

The authors declare that they do not have a conflict of interest.

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