Evaluation of the Embryotoxic Potency of Compounds in a Newly Revised High Throughput Embryonic Stem Cell Test

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The ability of murine-derived embryonic stem cells (D3) to differentiate into cardiomyocytes is the basis of the embryonic stem cell test (EST). With the EST, chemicals and pharmaceuticals can be assessed for their embryotoxic potency early on in the development process. In order to come to a higher throughput EST, a 96-well based method was developed based on low attachment well plates that allow for the formation of embryonic bodies from which the stem cells can differentiate. Twelve test compounds were selected based on their reported in vitro and in vivo embryotoxic potency. The 96-well based EST, reportedly strong embryotoxic compounds 5-fluorouracil, 6-aminonicotinamide (6AN), methylmercury chloride, and hydroxyurea were correctly ranked with corresponding Relative Embryotoxic Potency values (REP, based on the EC50 (μM) value of 6AN) of 2.6 ± 2.9, 1, 2.0 ± 3.1, and 0.07 ± 0.05, respectively. Moderately embryotoxic compounds valproic acid, boric acid, methoxyacetic acid, and lithium chloride resulted in a correct ranking with REP values of 0.01 ± 0.003, 0.001 ± 0.001, 0.0007 ± 0.001, and 0.0006 ± 0.0004, respectively. The included nonembryotoxic compounds Penicillin G, acrylamide, and saccharin did not result in an inhibition of D3 cells to differentiate into cardiomyocytes, other than related to cytotoxicity (REP value of 0.00001). However, diphenhydramine resulted in an inhibitory effect similar to the strong embryotoxic compound hydroxyurea, with a REP value of 0.40 ± 0.36. However, further evaluation suggested this was due to direct inhibition of the contractile capacity of the D3 cardiomyocytes, rather than an embryotoxic mechanism. The 96-well based EST is a promising addition to the screening process of newly developed chemicals and pharmaceuticals.

Key Words: embryonic stem cells; D3; EST; Relative Embryotoxic Potency; REP.

Possible harmful effects need to be assessed prior to marketing of compounds. For this, various in vitro and in vivo systems have been established to assess embryotoxicity (developmental toxicity without the observation of maternal toxicity) or teratogenicity (irreversible structural developmental effects without the observation of maternal adversities). In vivo whole animal tests based upon exposure of the fetus by maternal transfer have been in use for years. Still relying on whole animals are the postimplantation rat whole-embryo culture assay (WEC test) and the micromass assay (MM) (Genschow et al., 2002; Piersma, 2004; Piersma et al., 1995), though the exposure in these experiments is performed in vitro. There are alternatives that do not require experimental animals, such as the embryonic stem cell test (EST) (Genschow et al., 2002; Piersma, 2004; Spielmann et al., 1997). The EST is based on murine-derived embryonic stem cells from the blastocyst stage. The pluripotency of embryonic stem cells allows differentiation into all lineages of the three germ layers: mesoderm, ectoderm, endoderm (reviewed in Keller, 2005). The cells remain in an undifferentiated state when cultured on feeder layers or in the presence of murine leukemia inhibitory factor (mLIF; Fig. 1A). In the absence of the feeder layer or mLIF, the cells spontaneously differentiate.

Applying a hanging drop culture or seeding in a low attachment plate ensures the formation of an aggregate of cells, the embryonic body (EB; Fig. 1B). When this cell mass is transferred to a tissue culture coated plastic plate, the cells will attach and result in a monolayer with contractile areas, that is, cardiomyocyte-like cells (Fig. 1C). Test compounds can inhibit the formation of the cardiomyocyte-like cells which can be expressed as embryotoxic potency (Spielmann et al., 1997).

In the validated EST, fibroblast cells (3T3; Mus Musculus) and embryonic stem cells (D3; M. Musculus) are used. Viability data of both cell lines after exposure to a test compound, as well as the effect of a compound on the inhibition of differentiation of the D3 cells, can be entered in a validated prediction model. The outcome of this model, based on the EC50 values, can either be strong, moderate, or nonembryotoxic (Genschow et al., 2002, 2004; Spielmann et al., 2001). In the ECVAM validation study, twenty compounds with known embryotoxic potential were assessed. The EST resulted in
a correct identification of the embryotoxic potential of 78% of the test compounds. When only the strong embryotoxic compounds were taken into account, the outcome was a 100% correct prediction (Genschow et al., 2004).

Reports of differentiation of embryonic stem cells into other lineages have been published, such as osteogenic, chondrogenic, and neural differentiation (Zur Nieden et al., 2004). Different endpoints such as mRNA expression (Bigot et al., 1999), molecular endpoints of differentiation (Seiler et al., 2004; Wei et al., 2005; Zur Nieden et al., 2001), genetic modifications and fluorescent markers have been proposed (Bremer et al., 2001, 2002; Paparella et al., 2002). The potential of the EST to predict embryotoxicity in early development (lead optimization of a drug candidate) and possible use for regulators is an important aspect of the assay. The described adjustments however, still rely on the formation of EBs in a hanging drop culture. Previously, we described attempts to replace the labor-intensive hanging drop culture by a low cell binding well plate (Peters et al., 2007). Additionally, we introduced Relative Embryotoxic Potency (REP) values, based on the toxic equivalency factors (TEFs) concept for dioxin-like compounds (Ahlborg et al. 1992; Van den Berg et al. 1998). This method allows for ranking of the embryotoxic potential of test compounds with REP values based on the EC50 values relative to the positive control. Exposure of the cells to 6-aminonicotinamide (6AN) resulted in the most reproducible strong embryotoxic activity, and was therefore chosen as positive control.

Initial assessment of the REP values with five test compounds (exposure in sixfold) was based on EC10, EC50, or EC90 values. Exposure of the cells to test compounds starting from the seeding of the cells (day 0 of the experiment) was compared with exposure after EB formation (third day of the experiment). The outcome of the REP values based on the EC50 values with start of exposure of the cells on the third day of the experiment appeared an appropriate method. However, the experimental design was not yet solid: it proved difficult to find a well plate that allowed formation of EBs in a 96-well plate, one EB for each well, uniform in size, without attachment of the cells to the plate. When an appropriate 96-well plate was found, some EBs still attached to the wells and assessment in sixfold therefore occasionally resulted in too few replicates per concentration (Peters et al., 2007).

The introduction of a low cell binding 96-well plate and REP values would imply omission of the validated prediction model and consequently discarding the inclusion of the second cell line (3T3), as used in the original EST. Because limits between categories of embryotoxicity (strong, moderate, and non-embryotoxic, respectively) are hard to set, ranking compounds according to their REP value could be a promising alternative.

Based on the compounds used in the ECVAM validation study of the original EST (Brown, 2002; Genschow et al., 2002, 2004; Spielmann et al., 2001), and additional literature (Piersma et al., 2008; Schwetz and Harris, 1993; Smith et al., 1983) 12 compounds were selected to assess the applicability of the novel 96-well based EST with an improved experimental setup (see Table 1). Furthermore, the suitability of ranking compounds according to their REP values was assessed. Based on the reported embryotoxic effects in the literature, the compounds
were selected to cover both strong, moderate, as well as nonembryotoxic effects. As strong embryotoxic compounds 6AN, 5-fluorouracil, hydroxyurea, and methylmercury chloride were selected. Valproic acid, Boric acid, Methoxyacetic acid, and Lithium chloride were chosen as moderately embryotoxic compounds. Selected nonembryotoxic compounds were Penicillin G, acrylamide, saccharin, and diphenhydramine.

**MATERIALS AND METHODS**

**Chemicals.** Chemicals were obtained from the following companies: dimethyl sulfoxide (DMSO) and methoxyacetic acid were purchased from Merck (VWR; Leuven, Belgium), Dulbecco’s modified Eagle’s medium (DMEM supplemented with 1-glutamine, glucose and NaHCO₃), l-glutamine, nonessential amino acids (NEAA), and phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺ were ordered from Gibco (Carlsbad, CA). Fetal calf serum (FCS; stem cell tested) was ordered from Hyclone (Perbio Science; Erembodegem-Aalst Belgium), mLIF (ESGRO 10⁶ U) from Chemicon (Millipore, Brussels, Belgium), and Penicillin/Streptomycin solution (5000 U/5000 µg) from Cambrex (VWR). All other chemicals were ordered at Sigma Aldrich (St Louis, MO). Lithium chloride and Saccharin were dissolved in PBS (final solvent concentration 1%), all other test compounds in DMSO (final solvent concentration 0.25%).

**Cell line and cell culture.** Marine-derived embryonic stem cells (D3 cells) were kindly provided by ECVM (Ispra, Italy), by courtesy of S. Bremer. The D3 cells were cultured in DMEM supplemented with 20% heat inactivated FCS, 2mM l-glutamine, 50 µM penicillin, 50 µg/ml streptomycin, 1% NEAA, 0.1mM β-mercaptoethanol. Additionally, when cells were seeded, 1000 U/ml mLIF was added directly to the culture medium. The cells were cultured in a humidified atmosphere (5% CO₂, 37°C), and routinely subcultured when 70% confluency was reached with a thin layer of nonenzymatic cell dissociation buffer.

**Embryonic stem cell test.** The EST was performed as described previously (Seiler et al. 2006; Spielmann et al. 1997), with modifications (Peters et al., 2007). In short, D3 cells were seeded in 50 µl of medium (300 cells per well) in low attachment 96-well plates (NUNC, Tokyo, Japan; ref. 145399) at day 0. Three days later, 50 µl of medium with test compounds (double concentration) was added. At day 5, the EBs that were formed in the low attachment 96-well plates, were transferred into tissue culture treated 96-well plates with a multi-channel pipette, together with all the medium present in the wells. Additionally, the low attachment 96-well plates were rinsed with 50 µl of medium (with test compounds in the appropriate concentration) to ensure no EBs were left behind. The tissue culture coated 96-well plates were placed on an orbital platform shaker (240 rpm, 20 s) to attempt a central position of the EBs in the wells. After an additional 5 days of incubation, the contractility of the cells was assessed (Zeiss Axiosvert 200, Carl Zeiss, Zaventem, Belgium; 100x enlargement).

**Cell viability.** Cells were seeded at a density of 1000 cells per well in a 96-well plate (tissue culture coated) and exposed to test compounds two hours later. Medium with test compounds was refreshed three days later. After incubation with the designated test compounds for 6 days, medium was replaced with a 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyterazolium bromide) solution (60 min) as described previously (Peters et al. 2007). Subsequently, the plates were spun (1500 x g, 5 min) and MTT medium was removed from the cells. Formazan was extracted from the cells with 100 µl of DMSO per well (15 min shaking at 200 rpm), and the concentration determined spectrophotometrically (570 nm).

**Statistical analysis.** The EST was carried out in three independent experiments (10 EBs concentration, with 1 EB/well) and expressed as “fraction contractile EBs,” with 100% implying all EBs exposed to the same concentration of test compound resulted in contractile areas (as proposed by D. Verhallen and A. Piersma, RIVM, The Netherlands; personal communication).

The in vitro REP was calculated relative to the EC₅₀ value obtained for the positive control (6AN) of each independent experiment (Eq. 1). The EC₅₀ value of a compound is the concentration at which 50% of the maximum effect was induced. With 100% fraction contractility implying no inhibition of differentiation, and 0% fraction contractility a complete inhibition of differentiation, where no contractility was observed. When no EC₅₀ value could be determined in the EST (no inhibition of differentiation), the REP was given an arbitrary value of 0.00001.

The cell viability determination was carried out in three independent experiments (with n = 6), and expressed as “% of control,” with the solvent control as 100% viability.

Statistical analysis of the dose-response curves from the EST and cell viability assay was performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). In order to assess if the observed effects in the EST were due to embryotoxicity or cytotoxicity, the dose-response curves of both assays were compared with one-another with an extra sum-of-squares F-test ($p < 0.05$). Both the slope of the curves, the EC₅₀ values as well as the top and bottom were compared (average from three independent experiments). When there was a statistically significant difference between outcomes of the two assays ($p < 0.05$), the inhibition of differentiation observed in the EST was considered due to embryotoxicity. However, when there was no statistically significant difference between the cell viability and the EST curves ($p < 0.05$), the observed inhibition of differentiation was considered more likely due to cytotoxicity rather than embryotoxicity, and the corresponding REP value was given an arbitrary value of 0.00001 (one decimal lower than the lowest obtained REP value in the current manuscript).

**RESULTS**

**Strong Embryotoxic Compounds**

A concentration-dependent increase in the inhibition of differentiation of D3 cells into contractile cardiomyocytes was observed after the D3 cells were exposed to the compounds that were selected as strong embryotoxic compounds: 6AN, 5-fluorouracil, hydroxyurea, and methylmercury chloride (Fig. 2A). The observed decrease in cell viability for the compounds resulted in statistically significant different outcomes (Fig. 2B), indicating that the observed inhibition of differentiation was not entirely due to decreased cell viability (extra sum-of-squares F-test $p < 0.05$, GraphPad Prism, Table 2).

By applying the REP values, possible interexperimental differences are corrected for (e.g., cell growth). The REP values were calculated relative to the positive control of each individual experiment. For 5-fluorouracil, this resulted in three calculations as described in Equation 1: EC₅₀ (6AN)/EC₅₀ (5-fluorouracil) for three individual experiments. In Table 2, both the individual data as well as the average EC₅₀ (µM) and REP values are presented.

$$
\text{EC}_{50}\text{value (positive control 6AN)} \div \text{EC}_{50}\text{value (test compound)} = \text{REP value (test compound)}
$$

Ranking according to the REP values resulted in the following order of embryotoxic potential: 5-fluorouracil (2.6 ± 2.89) > methylmercury chloride (2.0 ± 3.13) > 6AN (1) > hydroxyurea (0.07 ± 0.05) (Table 2).

Additionally, the test compounds were assessed for their ability to inhibit the contractility of the EBs directly. This was
done by addition of the highest concentrations of test compounds to contracting EBs. Microscopic analysis occurred regularly during 3 h of exposure. None of these test compounds was able to inhibit the contractile capacity of the EBs directly.

**Moderate Embryotoxic Compounds**

As presented in Figure 3, exposure of the EBs to compounds that were selected as moderate embryotoxic (valproic acid, boric acid, methoxyacetic acid, and lithium chloride) resulted in a concentration-dependent decrease in contractility. The outcomes of the EST and cell viability assay were significantly different ($p < 0.05$), with no observed decrease in cell viability in the concentration range tested ($7.2 \text{mM}$) for Methoxyacetic acid (Table 2, Fig. 3B). The observed inhibition of differentiation was therefore considered due to embryotoxicity, and not entirely by a decreased cell viability.

Ranking the REP values resulted in the following order: valproic acid $>$ boric acid $>$ lithium chloride $\approx$ methoxyacetic acid, with corresponding REP values of $0.011 \pm 0.003$, $0.0014 \pm 0.001$, $0.0006 \pm 0.0004$, and $0.0007 \pm 0.001$, respectively (Table 2).

The capacity of the compounds to directly inhibit the contractile capacity of the cells was assessed as described above. None of the moderate embryotoxic compounds resulted in a positive outcome, indicating that the observed inhibition was indeed due to embryotoxicity.

**Nonembryotoxic Compounds**

Exposure of the EBs to Penicillin G did not result in a decrease in contractile capacity. Even at the highest concentration tested ($15 \text{mM}$), no inhibition was observed (Fig. 4A). Similarly, after exposure of the cells to Penicillin G, no EC$_{50}$ value could be determined when the cell viability was assessed (Fig. 4B). Acrylamide and Saccharin did inhibit the differentiation of D3 cells (Fig. 4A, Table 2), though the outcome was not significantly different ($p > 0.05$) from the cell viability data (Fig. 4B, Table 2). This is an indication that the observed effects in the EST were due to cytotoxicity rather than embryotoxicity. Therefore, both Penicillin G (no embryotoxicity observed), as well as Acrylamide and Saccharin (no statistically difference between the curves of the EST and the cell viability assay) were given an arbitrary REP value of 0.00001 (Table 2).
When the EBs were exposed to diphenhydramine, selected as a nonembryotoxic compound, a concentration-dependent inhibition of differentiation was observed. This inhibition was in the same order of magnitude as Hydroxyurea, a strong embryotoxic compound (Table 2, Fig. 4A). There was a significant difference ($p < 0.05$) between the cell viability (EC$_{50}$ 255.77 ± 27.7 µM) data and the outcome of the EST (EC$_{50}$ 7.89 ± 2.7 µM), indicating the observed inhibition was caused by embryotoxicity of the test compound.

This resulted in the following order of embryotoxicity for the selected nonembryotoxic compounds: diphenhydramine $>$ Penicillin G $=$ saccharin $=$ acrylamide, with REP values of 0.4 ± 0.36, and 0.00001, respectively (Table 2).

However, when the nonembryotoxic compounds were assessed for their ability to inhibit the contractile capacity of the EBs directly (as described above), the highest concentration of diphenhydramine resulted in 0% fraction contractility (no contractile EBs observed) within 30 min of exposure. Repeating the experiment with a full dose-response curve of diphenhydramine revealed clear direct inhibition of the contractile capacity of the EBs in a concentration-dependent manner (Fig. 5). The EC$_{50}$ value of the direct inhibition was 61.16 µM (range 54.25–68.96 µM) after three hours of exposure, compared with 7.89 ± 2.7 µM as was calculated in the main experiment (Table 2). The direct inhibition was partly reversible when the exposure medium was replaced with fresh medium after three hours of exposure (data not shown).

**DISCUSSION AND CONCLUSION**

Based on available literature, 12 compounds were assessed for their embryotoxic potency in the modified EST (Table 1). This novel approach to the EST allows for higher throughput and simplification of the experimental procedures in a 96-well based assay, as described previously (Peters et al., 2007).

When the outcome of the 96-well EST as described in the current manuscript, was compared with that of the “classic EST” (ECVAM-validated 24-well EST), only exposure to diphenhydramine resulted in a statistically significant different outcome (based on EC$_{50}$ values [µM], two-tailed Student $t$-test, $p < 0.004$) (Table 3). The other eleven test compounds resulted in a similar EC$_{50}$ value as reported in the literature (Genschow et al., 2004; Spielmann et al., 1997) (Table 3).

In order to correct for interexperimental differences, the outcome of the EST is presented as REP values, relative to the positive control 6AN (based on the TEF concept; Ahlborg et al., 1992; Van den Berg et al., 1998). The validated prediction model of the 24-well based EST classifies compounds as either strong, moderate, or nonembryotoxic (Genschow et al., 2002; Piersma, 2004; Scholz et al., 1998). Ranking compounds however, allows for comparison between the compounds, instead of between the categories chosen.
Ranking the compounds by their REP values, as shown in Table 2, resulted in the following order of embryotoxic potential: 5-fluorouracil (2.6 ± 2.89) > methylmercury chloride (2.0 ± 3.13) > 6AN (1) > diphenhydramine (0.40 ± 0.36) > Hydroxyurea (0.07 ± 0.05) > valproic acid (0.011 ± 0.003) > boric acid (0.0014 ± 0.001) > methoxyacetic acid (0.0007 ± 0.001) > lithium chloride (0.0006 ± 0.0004) > Penicillin G = saccharin = acrylamide = lithium chloride (0.0001). The order of embryotoxic potential for these 12 compounds was as expected, with the compounds chosen from the literature to cover strong, moderate, and nonembryotoxic compounds clustered, though not for diphenhydramine. This reportedly nonembryotoxic compound (Brown, 2002; Genschow et al., 2002, 2004; Spielmann et al., 2001) resulted in a REP value in the order of magnitude of the strong embryotoxic compound Hydroxyurea. This would imply the compound was more likely to be classified as a moderate to strong embryotoxic compound than a non. Previously, exposure to diphenhydramine in the “classic EST” resulted in a misclassification of 100% (Genschow et al., 2002). Reported EC_{50} values for diphenhydramine in the “classic EST” (24 wells) are presented in Table 3: 46.05 ± 16.3 μM (Genschow et al. 2004), and 94.26 μM (Spielmann et al., 1997), compared with 7.89 ± 2.70 μM from the current manuscript. Comparing these values to other compounds assessed in the 24-well based EST as summarized in Table 3, the EC_{50} values for diphenhydramine are similarly to that of the strong to moderate embryotoxic compounds hydroxyurea and valproic acid (Table 3).

Diphenhydramine was previously reported as both a nonembryotoxic compound (Brown, 2002; Genschow et al., 2002, 2004; Spielmann et al., 2001) and moderate embryotoxic compound (Spielmann et al., 1997). Here, we present a possible explanation for the observed effects; diphenhydramine was able to inhibit the contractile capacity of the EBs in a concentration-dependent manner with an EC_{50} value of 61.16 μM (range 54.25–68.96 μM) (Fig. 5). The direct inhibition was partly reversible when the exposure medium was replaced with fresh medium after 3 h. This is an indication that the compound does not alter the EBs functionally. However, refreshing the medium
Inhibition of Differentiation of Embryonic Stem Cells (D3) into Contractile Cardiomyocytes

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC_{50} (µM)</th>
<th>REP(^{a})</th>
<th>EST average EC(_{50}) (µM)</th>
<th>Cell viability average EC(_{50}) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6AN</td>
<td>3.8(^{a})</td>
<td>1.97(^{b})</td>
<td>0.65(^{c})</td>
<td>3.12(^{d})</td>
</tr>
</tbody>
</table>

Note. Inhibition of differentiation of D3 cells into cardiomyocytes after exposure to 12 test compounds is presented as EC\(_{50}\) value (µM) of each individual experiment (n = 3), as well as the average EC\(_{50}\) value (± SD). The corresponding individual REP values were calculated relative to EC\(_{50}\) value of the positive control of that individual experiment\(^{1}\) (as described in Eq. 1), and also presented as the average REP\(^{2}\) (as described in Eq. 2). Results from the EST were compared with the outcome of the cell viability assay (n = 3, presented as average EC\(_{50}\) value ± SD) with an extra-sum-of-squares F-test (p < 0.05, *significantly different, **no cytotoxicity observed). When no EC\(_{50}\) could be determined in the EST (no embryotoxicity), or when there was no statistical significant difference between the cell viability data and the outcome of the EST, an arbitrary REP value of 0.00001 was given. Ranking the compounds according to their embryo toxic potency resulted in the following order (based on the average REP values): 5-fluorouracil > methylmercury chloride > 6AN > diphenhydramine > hydroxyurea > valproic acid > boric acid > lithium chloride ≈ methoxyacetic acid > Penicillin G = acrylamide = saccharin.

could have been damaging to the EBs and be partly responsible for the incomplete recovery of the EBs.

In the WEC test, and MM, exposure to diphenhydramine also resulted in a misclassification of 100% for both assays (prediction model 1 for the WEC) (Genschow et al., 2002). The outcome parameters in the WEC test are morphological, developmental, functional and growth scores (Genschow et al., 2002). If diphenhydramine is able to directly inhibit heart beating in the WEC test, could this be a reason for the observed misclassification (with the first prediction model)? Two

Inhibition of Differentiation of Embryonic Stem Cells (D3) into Contractile Cardiomyocytes

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC_{50} (µM)</th>
<th>EC_{50} (µM) (Genschow et al., 2004)(^{1})</th>
<th>EC_{50} (µM) (Scholz et al., 1999)</th>
<th>EC_{50} (µM) (Spielmann et al., 1997)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6AN</td>
<td>2.33 ± 1.18</td>
<td>15.95 ± 23.1</td>
<td>0.26 ± 0.07</td>
<td>0.22</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>0.38 ± 0.11</td>
<td></td>
<td>0.26 ± 0.07</td>
<td>0.22</td>
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<tr>
<td>Methylmercury chloride</td>
<td>0.36 ± 0.07</td>
<td>0.47 ± 0.15</td>
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<tr>
<td>Hydroxyurea</td>
<td>31.43 ± 7.6</td>
<td>38.46 ± 49.2</td>
<td></td>
<td>22.35</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>270.5 ± 121.4</td>
<td>353 ± 57.6</td>
<td></td>
<td>22.35</td>
</tr>
<tr>
<td>Boric acid</td>
<td>1754 ± 53.6</td>
<td>2600 ± 1463</td>
<td></td>
<td>22.35</td>
</tr>
<tr>
<td>Methoxyacetic acid</td>
<td>3422 ± 716</td>
<td>2734 ± 911.5</td>
<td></td>
<td>22.35</td>
</tr>
<tr>
<td>Lithium chloride</td>
<td>5381 ± 2041.3</td>
<td>3597 ± 1565</td>
<td></td>
<td>22.35</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>nd</td>
<td></td>
<td>4257 ± 673</td>
<td>10317</td>
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<tr>
<td>Saccharin</td>
<td>7800</td>
<td></td>
<td></td>
<td>8961</td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td>7.89 ± 2.7(*)</td>
<td>46.05 ± 16.3</td>
<td></td>
<td>94.26</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>1053</td>
<td>1159 ± 511</td>
<td></td>
<td>94.26</td>
</tr>
</tbody>
</table>

Note. Inhibition of D3 cells into cardiomyocytes after exposure to 12 test compounds is presented as EC\(_{50}\) (± SD). The outcome of the 96-well EST (left panel) was compared with data adapted from literature (24-well “classic EST”), converted from µg/ml to the µM range. The data is presented as the average of the reported data (± SD). This resulted in the average from three laboratories with n = 6 (Genschow et al., 2004); n = 3 (Scholz et al., 1999), and the reported data from 1 laboratory with n ≥ 3 (Spielmann et al., 1997). *There was a statistically significant difference between the reported data from the literature and the outcome obtained in the current manuscript after exposure to diphenhydramine (Student t-test, p < 0.004).
prediction models were used for the validation of the WEC test, both use malformedations (this includes dead embryos) as an endpoint (Genschow et al., 2002). In the EST, the EBs were not damaged functionally and were able to regain their contractile capacity after the exposure to diphenhydramine ended. If this is similar in the WEC test, and there is no functional damage, removal of the exposure medium could alter the outcome of the experiment. However, when the second prediction model for the WEC test validation was used (identical to the first prediction model, with addition of cytototoxicity data), the compound was correctly classified as nonembryotoxic (Genschow et al., 2002). This is an indication that the observed misclassification of diphenhydramine in the WEC test was not due to direct inhibition by diphenhydramine. The observed direct inhibition of contractility can also not explain the misclassification of diphenhydramine in the MM test, because the outcome parameter in the MM assay is the inhibition of differentiation of primary limb bud cells into chondrocytes (Genschow et al., 2002).

Stem cells have been in use to evaluate cardiotoxicity (as reviewed by Baharvand et al., 2006; Boehler et al., 2002; Davila et al., 2004; Hescheler et al., 1997). These human and/or murine-derived embryonic and adult stem cell-derived functional cardiomyocytes display the correct morphology, protein expression and rhythmic contraction. Nonexposed EBs with foci of contractility as used in the current study, can be regarded as containing cardiomyocytes. Thus, inhibition of the contractile capacity of the cells could be a demonstration of cardiotoxicity. Diphenhydramine is in fact an arrhythmic drug with sodium channel blockage as one of the mechanisms of action (Khalifa et al., 1999; Sharma et al., 2003). In future research, all test compounds in the EST should be tested for their direct inhibitory capacities.

Previously, it was mentioned that the low cell binding 96-well plates used in the currently described novel EST, resulted in some undesired binding of the EBs (Peters et al., 2007). This was mostly avoided in the current experimental setup by addition of a washing step (the well plate was rinsed). Furthermore, in the current approach, 10 EBs were used for each concentration instead of six. However, occasionally an EB did attach to the wells. These were transferred to the tissue culture treated 96-well plate (day 5 of the experiment), similar to the nonattached EBs. When the contractility of the cells was assessed at day 10, a minimum of eight EBs for each concentration was considered acceptable.

In the validation study of the 24-well based EST, methymercury chloride was misclassified in 50% of the experiments, hydroxyurea 12.5%, and acrylamide 25% (Genschow et al., 2002). The presented REP values in the current manuscript resulted in an order of embryotoxicity as was expected, with clustering of the compounds that were selected as either strong, moderate, or nonembryotoxic. As described previously (Peters et al., 2007), introduction of REP values, omission of the 3T3 cells, higher throughput and a less extensive experimental procedure make the novel 96-well based EST a promising alternative method to animal experimentation.

Future research should assess acceptance criteria of the novel EST and its applicability domain further. For the applicability domain, care should be taken with compounds that do not cross the placental barrier in vivo. These compounds could result in false-positive outcomes in the EST. Test compounds that are metabolized to their active form in vivo cannot be picked up in the EST either, because D3 cells have little or no metabolic capacity (Bremer and Hartung, 2004). Thus, data regarding adsorption and half-life of the compounds, as well as the ability to cross the placental barrier should be regarded for risk assessment.

Furthermore, the outcome of the EST should be correlated to the achievable in vivo plasma concentration (both fetal as well as maternal), because the compounds most likely will be assessed at far higher concentrations in the in vitro EST than achievable in the in vivo situation. As was observed for diphenhydramine, literature search for known mechanisms of action is essential for the determination of the applicability domain of the EST.

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REFERENCES


