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A higher throughput method to the Embryonic Stem cell Test (EST), to detect embryotoxicity in early development

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Abstract
The *in vitro* embryonic stem cell test (EST) allows for categorisation of the embryotoxic potential of chemicals and drug candidates. For classification, a validated prediction model was developed based on the inhibition of differentiation of murine embryonic stem cells (D3 cells) into cardiomyocytes, and the cytotoxicity data of D3 cells and murine fibroblasts (3T3 cells). Alterations were made in order to simplify the experimental procedures of the EST; a low-cell-binding 96-well plate was used to obtain the embryonic bodies instead of the original hanging drop culture. Furthermore, we assessed the need to include the 3T3 cells and developed a new ranking system. D3 cells were exposed to test compounds either from day 0 or day 3 onwards and the compounds were ranked by their Relative Embryotoxic Potency (REP), relative to the positive control 6-Aminonicotinamide. This resulted in the following REP order; 6-Aminonicotinamide > Hydroxy urea > Valproic acid > Methoxyacetic acid > Penicillin G. A similar outcome was obtained when the validated prediction model was used. Exposure of cells from day 0 or day 3 onwards did not have any effect on the outcome as calculated with both methods. We propose a simplification of the *in vitro* EST procedure and REP values to rank compounds.

Keywords: murine embryonic stem cells, D3, EST, embryotoxicity, relative embryotoxic potency

Introduction

The embryonic stem cell test (EST) was developed to assess the possible embryotoxic potential of chemicals and drug candidates in an *in vitro* system [1]. The application of the EST for chemical testing reduces time, testing costs and the amount of animal experimentation for embryotoxicity tests.

For the test, murine embryonic stem cells (D3) are used. D3 cells are derived from the inner cell mass of pre-implantation embryos or blastocysts at day 3,5 of mouse development [2]. These pluripotent cells can differentiate into various tissue types of the three germ layers; endo-, meso-, and ectoderm. The pluripotent nature of D3 cells makes it an appropriate candidate for many *in vitro* embryotoxicity experiments. The cells spontaneously differentiate when cultured in the absence of murine leukaemia inhibitory factor (mLIF) and presence of foetal bovine serum. As a result, a culture of differentiated D3 cells will consist of a heterogeneous population with cells from various lineages (reviewed in [3]).

The EST is based on the principles of formation of embryonic bodies (EB's) when D3 embryonic stem cells are placed in a 'hanging drop' culture. When subsequently seeded in tissue culture plates, the cardiomyocyte lineage is the predominant differentiation route, resulting in contractile areas in the EB [1,4]. These contractile areas can be quantified manually by microscopic analysis.

A validated prediction model based on three representing endpoints: inhibition of proliferation of the embryonic D3 cells, as well as adult 3T3 cells (murine fibroblasts), and the inhibition of differentiation of ES cells into contracting EB's can be used to classify compounds as either *non*-embryotoxic, *moderate* embryotoxic, or *strong* embryotoxic [5-7].

Recent modifications to the 'classic EST' include addition of molecular markers as endpoints [8-11], assessment of extra cellular matrices [12], upscaling and alterations of the production of embryonic stem cell-derived cardiomyocytes [13,14].

The above-described 'classic' EST includes time-consuming steps in the experimental procedure, such as setting up the 'hanging drop' cultures and seeding the EB's to tissue culture plates. In our laboratory, research was done to omit these steps, and thus ensuring a higher throughput of the *in vitro* system.

Addition of the 3T3 cell line in the 'classic EST' is used to mimic maternal toxicity [4]. But the inhibition of proliferation in this mouse embryo fibroblasts cell line does not represent a relevant *in vivo* situation. As the validated prediction model can not be applied anymore after omission of the 3T3 cell line with this new test design, a new method to define *in vitro* embryotoxicity was also developed. Relative Embryotoxic Potency (REP) values are introduced as a novel method of ranking compounds relative to their inhibition of differentiation of D3 cells.

The test compounds chosen were known embryotoxicants, selected from the ECVAM validation study of the 'classic EST'. In the original study, these compounds were selected on their *in vivo* and *in vitro* potency and because they do not need inclusion of a metabolising system [4-7,15]. As examples of strong embryotoxic compounds, 6-Aminonicotinamide and Hydroxy urea were chosen. In the ECVAM validation study, these compounds had a 12.5% mis-classification (*moderate* instead of *strong* embryotoxicity), while the reported *in vivo* classification was *strong* embryotoxicant. Valproic acid and Methoxyacetic acid were included as moderate embryotoxicants, and Penicillin G as a non-embryotoxic compound. The latter compounds did not result in any false classifications in the ECVAM validation study, with identical reported *in vivo* classifications [4-7,15].

Materials and methods

Chemicals

The chemicals used, were obtained from the following companies: DMSO and Methoxyacetic acid were purchased from Merck (VWR; Leuven, Belgium). Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine, glucose and $NaHCO_3$, L-glutamine, non essential amino acids (NAA), and phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} were ordered from Gibco (Carlsbad, CA, USA). Foetal Calf Serum (FCS; stem cell tested) was ordered from Hyclone (Perbio Science; Erembodegem-Aalst Belgium), mLIF (ESGRO® 10^6 U) from Chemicon (Millipore; Brussels, Belgium), and Penicillin/Streptomycin solution (5000 U/5000 µg) from Cambrex (VWR; Leuven, Belgium). All other chemicals were ordered at Sigma Aldrich (St. Louis, MO, USA).

Cell line and cell culture

D3 embryonic stem cells (*mus musculus)* and BALB/3T3 fibroblasts (clone A31; *mus musculus*) were acquired from ECVAM (Ispra, Italy), by courtesy of S. Bremer. The D3 cells were cultured in DMEM supplemented with 20% heat inactivated FCS, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 1% non essential amino acids, 0.1 mM β-mercaptoethanol and 1000 U/ml mLIF. BALB/3T3 cells were culture in DMEM, supplemented with 10% heat inactivated FCS, 4 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin. Both cell lines were cultured in a humidified atmosphere with 5% CO_2 at 37°C.

Embryonic stem cell test

Cells were seeded (300 cells/well, either with or without test compounds) in various low-cell-binding 96-well plates: NUNC U-shaped Low-cell-binding 96-well plate (ref. 145399), BD Gentest enhanced recovery 96-well plate (ref 453603), Greiner suspension culture 96-well plate (ref. M3562), Falcon 96-well plate (ref. 3531918), Falcon microtest 96-well plate (ref. 351177), Corning V-shaped 96-well plate (ref 3894).

At day 3, fresh medium with test compounds was added (solvent DMSO < 0.25%). If embryonic bodies were formed, they were transferred to a tissue culture treated 96-well plate at day 5, with microscopic assessment of contractility 5 days later (Zeiss Axiovert 200, 100X).

Cytotoxicity assay

The cytotoxicity assay in the 'classic EST' was based on the methods described by Spielmann et al. [1], with slight modifications. In short, 3T3 and D3 cells were seeded in 96-well plates (500 cells/well), and medium with test compounds added two hours later. Medium was refreshed at day 3 and 5 of the experiment. At the designated end of the exposure (10 days), medium was removed and serum-free MTT medium (0.5 mg/ml, 37°C) was added to the cells for one hour. Subsequently, the plates were spun (1500g, 5min) and MTT medium was removed from the cells. After washing the cells with PBS (37°C), formazan was extracted from the cells with 100 µl DMSO/well, and the concentration determined spectrophotometrically (570 nm) after 15 min shaking (200 rpm).

Statistical Analysis – adjusted 96-well EST

Experiments were carried out three times with 8 EB's per concentration (n=8). Statistical differences among different treatments were analysed with a two-tailed Student t-Test, with a level of statistical significance of 95% ($p< 0.05$). Data was plotted using XLfit 2.0.9 software (IDBS, Guildford, United Kingdom), model 205 (Y=A+((B-A)/(1+((C/x)^D)))), and always checked manually. The *in vitro* Relative Embryotoxic Potency (REP) was calculated relative

to the IC value obtained for the positive control (6-Aminonicotinamide; IC value positive control/ IC value test compound). The IC_{10} value of a compound is the concentration where 10% of the maximal observed effect was obtained, with the maximal effect being 100% inhibition of D3 cell differentiation into cardiomyocytes (no contractility observed). Subsequently, the IC_{90} value reflects the concentration where 90% of the maximal observed effect was obtained.

Statistical Analysis – Classic EST

The IC values of the cytotoxicity determination in D3 cells and 3T3 cells, as well as the IC value of the inhibition of differentiation of D3 cells into contracting cardiomyocytes were calculated using XLfit 2.0.9 software (IDBS, Guildford, United Kingdom), model 205 (Y=A+((B-A)/(1+((C/x)^D))))). For the classification of the compounds according to the 'classic EST', the validated prediction model was applied [4,7].

When no cytotoxicity IC_{10} or IC_{90} could be calculated, the highest or lowest concentration tested (for the calculation of IC_{10} and IC_{90} respectively) was used in the validated prediction model.

Results

Formation of embryonic bodies in a 96-well plate

Various 96-well plates were used to obtain embryonic bodies. However, size and amount of embryonic bodies varied with most plastics used (Fig. 1A, B, C) compared to the hanging drop culture as used in the 'classic EST' (Fig. 1D). Uniformity in EB formation is necessary when a degree of reproducibility is desired. From the plastics tested, only the NUNC plates resulted in a uniform sized EB, with only one EB/well. However, in one batch of the NUNC plates, we observed attachment of EB's to the well plate (Fig. 1E). This occurred only in the lower rows.

Exposure from day 0 or day 3 onwards

When the D3 cells were exposed to known embryotoxic test compounds, a dose related reduction in the size of the EB could be observed as shown for 6-Aminonicotinamide (Fig. 2). To ensure all EB's had a similar size at the start of exposure, D3 cells were exposed to test compounds not only from the start of the experiment (day 0), but when the embryonic body had already formed in the well (day 3) as well. The IC_{10}, IC_{50} and IC_{90} values of the inhibition of differentiation of D3 cells are presented in Table 1 (left panel). The IC_{50} values were comparable between duration treatments. However, more variation was observed between the exposure groups in the IC_{10} and IC_{90} values.

Relative embryotoxic potency (REP) values compared to the validated prediction model of the 'classic EST'

The outcome of the adjusted high throughput EST method is presented using both the REP values (Table

Table 1. D3 cells were exposed on day 0 (upper panel) or day 3 (lower panel). Data described is from one representative experiment out of three. Relative effect potency (REP) values indicate embryotoxicity from strong to non-embryotoxic in the following order; 6-Aminonicotinamide> Hydroxy urea > Valproic acid > Methoxy acetic acid > Penicillin G. The REP value was calculated relative to the IC value obtained for the positive control (*); When values larger then the maximal tested concentration were indicated by XL fit, the highest concentration tested was used for the REP calculation. (**); When values smaller then the maximal tested concentration were indicated by XL fit, the lowest concentration tested was used for the REP calculation.

Effects of test compounds on inhibition of differentiation of D3 cells into cadio-myocytes
Exposure to test compound on day 0
adjusted 96-well protocol

Compound	IC_{90} (µg/ml)	IC_{50} (µg/ml)	IC_{10} (µg/ml)	REP value (IC_{90} 6AN)	REP value (IC_{50} 6AN)	REP value (IC_{10} 6AN)
6-Aminonicotinamide	0.59	0.45	0.36	1	1	1
Hydroxy urea	2.53	1.18	0.56	0.2	0.4	0.6
Valproic acid	29.17	19.84	13.49	0.02	0.02	0.03
Methoxyacetic acid	283.48	105.36	31.44	0.002	0.004	0.011
Penicillin G	>298.10	>298.10	>298.10	0.0006*	0.0005*	0.0004*

Effects of test compounds on inhibition of differentiation of D3 cells into cadio-myocytes
Exposure to test compound on day 3
adjusted 96-well protocol

Compound	IC_{90} (µg/ml)	IC_{50} (µg/ml)	IC_{10} (µg/ml)	REP value (IC_{90} 6AN)	REP value (IC_{50} 6AN)	REP value (IC_{10} 6AN)
6-Aminonicotinamide	0.7	0.65	0.63	1	1	1
Hydroxy urea	4.28	1.32	0.31	0.2	0.5	2
Valproic acid	71.73	24.14	<8.26	0.01	0.03	0.08**
Methoxyacetic acid	470.67	121.65	31.44	0.001	0.005	0.020
Penicillin G	>298.10	>298.10	>298.10	0.0007*	0.0007*	0.0006*

Table 2 D3 cells were exposed on day 0 (left panel) or day 3 (right panel). Data described is from one representative experiment out of three. Classifications presented are calculated with the validated prediction model of the 'classic EST' with IC-values of D3 and 3T3 cells after ten days of exposure to the test compounds. '*Strong*': strong embryotoxic compound, '*Moderate*': moderate embryotoxic compound, '*Non*'; non-embryotoxic compound.

	Exposure to test compound on day 0			Exposure to test compound on day 3		
Compound	IC_{90} (μg/ml)	IC_{50} (μg/ml)	IC_{10} (μg/ml)	IC_{90} (μg/ml)	IC_{50} (μg/ml)	IC_{10} (μg/ml)
6-aminonicotinamide (6AN)	strong	strong	strong	strong	strong	strong
hydroxy urea	moderate	strong	moderate	moderate	strong	moderate
valproic acid	moderate	moderate	moderate	moderate	moderate	moderate
methoxy acetic acid	moderate	moderate	moderate	moderate	moderate	moderate
penicillin G	non	non	non	non	non	non

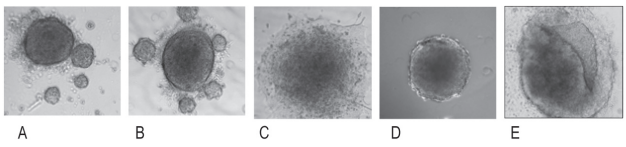

Fig. 1. Embryonic bodies formed using various plastics compared to the 'classic EST'. **A)** Falcon plate, **B)** Falcon culture plate, **C)** BD high recovery plate, **D)** an EB formed using the 'classical EST' hanging drop culture, **E)** unexpected binding of D3 cells to the NUNC low attachment plates. Photographs were taken at day 3 of exposure (100X).

1), as well as the validated prediction model of the 'classic EST' (Table 2).

For the REP values, the IC values obtained from the inhibition of differentiation of D3 cells were calculated relative to the positive control 6-Aminonicotinamide (Table 1, right panel). Both after exposure from day 0 or day 3 onwards, as well as with the different IC values, the order of embryotoxic potency was: 6-Aminonicotinamide > Hydroxy urea > Valproic acid > Methoxyacetic acid > Penicillin G.

The validated prediction model of the 'classic EST' was used to classify the test compounds, using the IC values obtained from the inhibition of differentiation of D3 cells and the IC values obtained from the cytotoxicity experiments with D3 and 3T3 cells. The classification of the test compounds was in the same order of magnitude as was obtained with the REP values; 6-Aminonicotinamide > Hydroxy urea > Valproic acid > Methoxyacetic acid > Penicillin G (Table 2). However, when IC_{10} and IC_{90} values were used, Hydroxy urea was classified as a moderate embryotoxic compound, compared to a classification of *strong* embryotoxicant based on the IC_{50} data.

Discussion and conclusion

A novel approach for the EST was set up, to detect embryotoxicity in a more high throughput manner. In contrast to the 'classic EST', the hanging drop culture was omitted with the introduction of a low binding 96-well plate to generate embryonic bodies (EB's). Not only do the NUNC low binding 96-well plates allow for the formation of a single embryonic body, the size is also uniform throughout the well plate and the cells are capable of differentiation into contracting cardiomyocytes. This simplification of the experimental procedure compared to the 'classic EST' [16] will result in significant time-reduction. Furthermore, using 96-well plates, the amount of test compounds and medium is reduced enormously compared to the 'classic EST' (5 ml/concentration range compared to 35 ml/single test concentration).

While the NUNC plates resulted in the best uniform EB's, further experiments clearly demonstrated attachment of D3 cells to the wells (Fig. 1E). This was only observed at the lower rows of the 96-well plate. Future experiments with a different batch

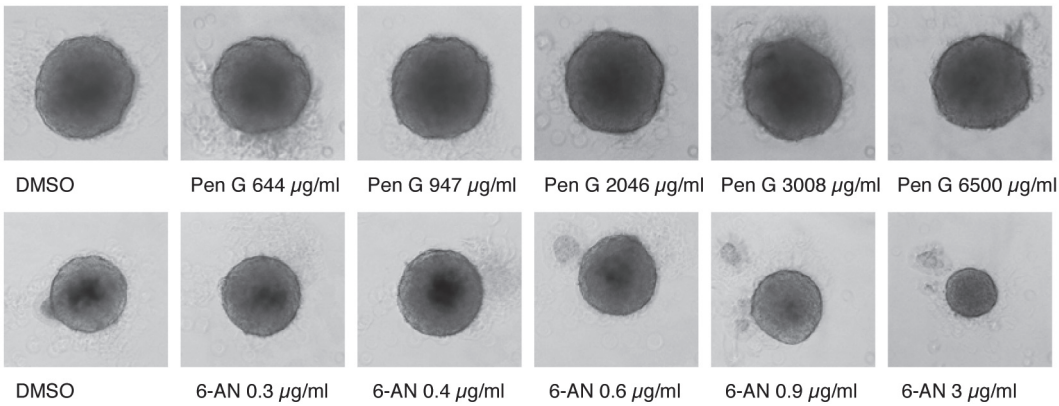

Fig. 2. Exposure of D3 cells in 96-well low attachment plates (NUNC) to Penicillin G, a *non*-embryotoxic compound (upper row), and to 6-aminonicotinamide (6-AN), a *strong* embryotoxic compound (lower row). Photographs were taken at day 3 of exposure (100X).

number of NUNC plates should determine the cause of attachment.

Exposure form day 0 or day 3 onwards did not alter the outcome of either the validated prediction model ('classic EST') or the Relative Embryotoxic Potency (REP) values. However, when the validated prediction model was used, there was an under-estimation of the embryotoxic potency of Hydroxy urea, compared to the data obtained in the validation study of the EST [15]. This under-prediction occurred when IC_{10} and IC_{90} values were used instead of the IC_{50} values. Therefore, the use of IC_{50} values should be recommended when using the prediction model. In comparison with the REP values (Table 1), calculations with different IC values do not alter the outcome of the relative potency.

Both the classification of the compounds, as well as ranking them in order of embryotoxic potency, does not differ between exposure of the cells from day 0 or day 3 onwards. Comparing exposure schemes, the IC_{50} values differ maximally 1.2 times for Methoxyacetic acid, while the lowest observed difference is only 1.1 times for Hydroxy urea. However, more compounds should be tested before a certain exposure scheme can be applied throughout the experiment.

When the REP values are used, the cytotoxicity data of the D3 cells is only used as a range determinator, while the 3T3 cells are no longer included in the experimental procedure. As seen in Table 1 and Table 2, both methods allow for a similar ranking of the compounds. The exclusion of the 3T3 cell line, will result in a further simplification of the experimental procedure.

The described experimental procedures will be repeated with a new batch of NUNC plates and the replicate number (currently n=8) as well as acceptance criteria (how many contracting EB's are needed minimally for the non-treated control) and exposure scheme (day 0 or day 3 onwards) determined. The REP values and inclusion of a classification method based on more test compounds will be assessed further for risk assessment purposes.

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