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Comparison of osteoblast and cardiomyocyte differentiation in the embryonic stem cell test for predicting embryotoxicity *in vivo*

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ABSTRACT

One of the most studied alternative embryotoxicity assays is the embryonic stem cell test, in which the effect of compounds on cardiomyocyte differentiation is evaluated (subsequently termed the ESTc). This single differentiation endpoint may limit the predictive value of the assay. We recently published a novel embryonic stem cell based osteoblast differentiation assay (subsequently termed the ESTo), in which we studied the effect of six embryotoxic compounds. Differentiation is monitored *via* the differential expression of three genes related to osteogenesis (Runx2, SPARC and collagen type I). In the current study, we evaluated the effect of 14 additional compounds in the ESTo, to assess its added value as compared to the ESTc. To this end, we compared the effects of the compounds in the ESTo to their effects in the ESTc and to their published *in vivo* developmental toxicity profiles. The results show that there is a high overall correlation between compound potencies as regards inhibition of osteoblast and cardiomyocyte differentiation. Moreover, the results in both the ESTo and ESTc showed a significant correlation to *in vivo* developmental toxicity potency ranking of compounds tested. Interestingly, the embryotoxic effect of TCDD could only be detected using the ESTo, which can be explained based on its mechanism of action and its known inhibitory effect on osteogenesis. The results of TCDD suggest that incorporating the ESTo into a testing battery together with the ESTc could improve the overall predictive value of the battery.

1. Introduction

It has been three decades since Martin (1981) first showed that mouse embryonic stem (ES) cells could be isolated from blastocysts and cultured *in vitro*, and that their pluripotent nature could be maintained using a combination of teratocarcinoma stem cell conditioned medium and fibroblastic feeder cells [1]. The subsequent discovery that the sole addition of leukemia inhibiting factor (LIF) to culture medium could maintain mouse ES cell pluripotency, together with general improvements of cell culture methods for ES cells, led to a rapid increase in the research on the applications of these ES cells [2]. This research has focused on the use of ES cells in tissue engineering, as a model to study embryonic development, and as an *in vitro* model for developmental toxicity testing (the embryonic stem cell test (EST)) [3]. In the EST the pluripotent nature of ES cells is used to study the effect of chemicals on cardiomyocyte differentiation, as an indication of the *in vivo* embryotoxicity (the ESTc). The EST was developed at the Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET), under contract of European Center for the Validation of Alternative Methods (ECVAM) [4]. The assay has been part of an ECVAM validation study in which 20 chemicals were evaluated in three separate laboratories. The EST showed low inter- and intra-laboratory variation and led to an overall accuracy of embryotoxicity prediction of 78% [5]. However, the results were less favorable when additional studies were done, using other compounds. Paquette et al. [6] developed a slightly modified version of the EST, using a different ES cell line, and evaluated the ECVAM compounds as well as a number of additional compounds. While this modified EST led to an overall accuracy of 83% for the ECVAM compounds, only 58% of the 19 receptor mediated compounds were correctly classified. Moreover, within the ReProTect consortium 13 additional compounds were evaluated in the EST, mainly consisting of drugs, leading to a correct classification for only 2 of the 13 compounds [7].

One suggestion to improve the predictive value of the EST, is to include other differentiation routes besides the classical cardiomyocyte differentiation. If a compound specifically affects neural or

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Table 1
Description of the tested compounds.

Compound	Abbreviation	Type of compound	Postulated teratogenic pathway	Embryotoxic effects
2,3,7,8-Tetrachlorodibenzodioxin	TCDD	Dioxin	Activates the arylhydrocarbon receptor, upregulates vitamin D3 activity [33,58]	Cleft palate, intestinal hemorrhaging [59]
Monobutyl phthalate	MBuP	Phthalate plasticizer	Activates PPARs, P53-mediated osteoblast apoptosis [60–62]	Cleft palate, skeletal [63,64]
Ethoxyacetic acid	EAA	Glycol ether metabolite (solvent)	Unknown	Skeletal [65]
5-Fluorouracil	5-FU	Antineoplastic	Inhibits DNA synthesis by inhibiting thymidylate synthetase [66]	Cleft palate, skeletal [66]
	HU	Antineoplastic	Inhibits DNA synthesis by inactivating ribonucleotide reductase [67]	Craniofacial, microencephaly, skeletal [68]
Retinoic acid	RA	Vitamin A precursor/topical drug	Elevated RARs expression, Hox genes activation [69]	Craniofacial, urogenital, exencephaly, skeletal [70,71]
Dipheylhydantoin	DPH	Anticonvulsant	Oxidative stress [72]	Cleft lip and palate [73]
Lithium chloride	LC	Diverse chemical applications	Interference with the phosphatidyl inositol cycle, inhibits the Wnt pathway [74]	skeletal [75]
Boric acid	BA	Antiseptic, insecticide, flame retardant, a.o.	HDAC inhibition [39]	Skeletal [76]
D-Camphor	DC	Food flavoring, odor	–	None [43]
Thalidomide	THA	Immunomodulatory, anti-angiogenic	Oxidative stress, anti-angiogenesis [77]	Skeletal [45,48,78]
Flusilazole	FLU	Triazole herbicide	Inhibits CYP26 expression [79]	Skeletal, cleft palate, urogenital [80]
Hexaconazole	HEX	Triazole herbicide	Inhibits CYP26 expression [79]	Skeletal [81]
Myclobutanil	MYC	Triazole herbicide	Inhibits CYP26 expression [79]	Skeletal [82]

osteogenic differentiation for example, it could go undetected in the ESTc. This is the case for methylmercury chloride, a compound that was often incorrectly classified in the ESTc, which shows a much higher potency for affecting neural differentiation with effects occurring at concentrations as low as 2.5 nM [5,8,9]. In a previous study, we developed an embryonic stem cell based osteoblast differentiation method (the ESTo). In that study we evaluated the embryotoxic effect of six teratogenic compounds on two different endpoints of differentiation, namely calcium concentrations and gene expression markers, with the aim to determine the most accurate endpoint to quantify osteoblast differentiation in the assay [10]. We showed that the effect of compound exposure on gene expression markers was a more reliable endpoint to quantify osteoblast differentiation compared to calcium concentrations, as the effect on calcium concentration appeared to be primarily related to a general apoptotic effect and not by a specific effect on differentiation. Three markers were selected to quantify differentiation, Runx2, SPARC and collagen type 1, as they represent relatively independent elements that are important in osteoblast differentiation [10]. Runx2 is a transcription factor which plays an essential role in osteoblast differentiation [11]. Collagen type 1 is the major protein component in bone. Besides collagen type 1, 10% of the bone matrix consists of non-collagenous proteins of which SPARC (osteonectin) is the most abundant [12]. The osteoblast inhibiting capacity of a test compound in the assay was defined as the ID50 provided by the most sensitive of the three markers, which might differ with the mode of action of the compound.

The six compounds previously tested, showed little difference in their potency in the ESTo as compared to the ESTc [10]. Therefore, we decided to evaluate the effect of 14 additional compounds in the ESTo for their effect on cytotoxicity, measured as protein content, and their effect on differentiation, measured as changes in the three marker genes. The chemicals selected were expected to affect osteoblast differentiation, based on their mechanism of action and/or the type of effects found *in vivo* (Table 1). Furthermore, we included a non-embryotoxic compound, namely D-camphor. The goal of this study is to determine the added value of the ESTo in comparison to the "classic" ESTc in which the effect of compounds on differentiation is determined by morphological assessment of embryoid bodies for the presence of beating cardiomyocytes. We compared potencies in the ESTo to the potencies of these compounds in the ESTc, as well as to the *in vivo* embryotoxic potencies of the compounds as obtained from literature studies, similar to what was previously done looking at *in vitro–in vivo* correlation for the whole embryo culture (WEC) [13].

2. Materials and methods

2.1. Cell culture

Murine ES-D3 cells (ATCC, Rockville, MD, USA), were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Breda, The Netherlands) supplemented with 20% fetal calf serum (Hyclone, Thermo Scientific, Etten-Leur, The Netherlands), 2 mM glutamine (Invitrogen), 50 U/ml penicillin (Invitrogen), 50 μg/ml streptomycin (Invitrogen), 1% non-essential amino acids (Invitrogen) and 0.1 mM β-mercaptoethanol (Sigma–Aldrich, Zwijndrecht, The Netherlands). To maintain pluripotency 1000 U/ml murine leukemia inhibiting factor (mLIF) (Chemicon, Amsterdam, The Netherlands) was added to the cultures. Routine subculturing was performed three times a week and cultures were kept at 37 °C and 5% CO_2.

2.2. Chemicals

Unless stated otherwise all chemicals were obtained from Sigma–Aldrich. Exposure compounds included, monobutyl phthalate (MBuP, Wako Chemicals, Neuss, Germany), ethoxyacetic acid (EAA), lithium chloride (LC), diphenylhydantoin (DPH), 5-fluorouracil (5-FU), all-trans retinoic acid (RA), 2,3,7,8-tetrachlorodibenzodioxin (TCDD), hydroxyurea (HU), boric acid (BA), thalidomide (THA), D-camphor (DC), flusilazole (FLU), hexaconazole (HEX), and myclobutanil (MYC). All compounds were dissolved in DMSO (0.1% v/v), with the exception of LC, EAA, and HU which were dissolved directly in DMEM and 5-FU which was dissolved in PBS.

2.3. Osteoblast differentiation culture (ESTo)

During the osteoblast differentiation culture similar medium was used as in the maintenance culture but with a reduced fetal calf