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An evaluation of a human stem cell line to identify risk of developmental neurotoxicity with antiepileptic drugs

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ABSTRACT

Determination of the impact of a drug on human brain development relies instead on surrogate animal studies. Here we have exploited the human stem cell line, TERA2.cl.SP12 to differentiate into neurons and addressed their value as an *in vitro* model to evaluate the risk of developmental neurotoxicity with antiepileptic drugs (AEDs). The effects of four AEDs were investigated on cell viability, cell cycle and neural differentiation. Exposure to either phenobarbital (10–1000 μM), valproic acid (10–1000 μM), lamotrigine (1–100 μM) or carbamazepine (1–100 μM) for 3 days reduced viability in non-differentiating cells only at the highest concentrations tested. Viability was also reduced with lower concentrations of all AEDs in cells undergoing neural differentiation. Valproic acid and carbamazepine increased DNA fragmentation and reduced cell cycle progression. 3 days exposure at the start of neural differentiation to phenobarbital, valproic acid or lamotrigine also significantly reduced the proportion of stem cells that subsequently differentiated into neurons at 15 days *in vitro*. The two control agents tested, ciprofloxacin and perfluorooctanoic acid had no impact on neurogenesis *in vitro*. These new data show that modelling neurogenesis *in vitro* using a human stem cell line may be a powerful method to predict risks of developmental neurotoxicity *in vivo* with psychotropic drugs.

1. Introduction

Since the first successful isolation and culture *in vitro* of human embryonic stem (ES) cells (Thompson et al., 1998), a number of alternative sources of human stem cells have been identified which broadly includes adult (or tissue)-derived stem cells, induced pluripotent stem cells (iPSCs) and fetal stem (FS) cells. Enormous excitement surrounds pluripotent stem cells because of their unique potential to expand *ad infinitum* and, under specific conditions, differentiate to any of the more than 200 cell types in the human body. They may therefore be of great value in regenerative medicine, drug discovery and toxicology.

The embryonic stem cell test (EST) was developed as an *in vitro* tool to evaluate the potential of compounds to be embryotoxic and thus reduce the numbers of animals needed in toxicity screening (Genschow et al., 2004). However, this model uses mouse ES cells to measure the effects of chemicals on stem cell viability and their potential to differentiate into beating cardiomyocytes. The EST can therefore miss the effects of chemical agents in the human because of species differences and it can also miss the impact of these agents on the development of other tissues and organs, including the nervous system (see Marx-Stoelting et al., 2009).

The use of cultured human stem cells in developmental neurotoxicology is also emerging (Bosnjak, 2012) with early studies reporting on the impact of heavy metals, including mercury, lead and cadmium as well as organic pesticides, such as chlorpyrifos on cell viability, proliferation, apoptosis, neurite growth and neural differentiation (e.g. Buzanska et al., 2009; Zychowicz et al., 2014). Few studies to date, however, have determined the validity or predictive value of human stem cells for the risk of developmental neurotoxicity with the use of psychotropic agents, such as the anticonvulsants, antipsychotics and antidepressant drugs.

Abbreviations: AEDs, antiepileptic drugs; β-III, βIII tubulin; CBZ, carbamazepine; DMSO, dimethyl sulphoxide; CIP, ciprofloxacin; CNS, central nervous system; Cy3, cyanine 3; DAPI, 4′-6-diamino-2-phenylindole; DMEM, Dulbecco's modified eagle's medium; ES, embryonic stem; EST, embryonic stem cell test; FITC, fluorescein isothiocyanate; FS, fetal stem; GABA, gamma aminobutyric acid; GFAP, glial fibrillary acidic protein; hECSC, human embryonal carcinoma stem cells; EC, embryonal carcinoma; iPSCs, induced pluripotent stem cells; LTG, lamotrigine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; PFOA, perfluorooctanoic acid; PHB, phenobarbital; PI, propidium iodide; RA, retinoic acid; TTX, tetrodotoxin; TERA2.cl.SP12, teratoma cell line clone 12; VPA, valproic acid.

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Antiepileptic drugs (AEDs) reduce and inhibit seizures by acting on diverse targets in the central nervous system (CNS) including ion channels and neurotransmitter receptors (Das et al., 2012). The targets for AEDs also mediate a range of physiological processes in the developing brain and therefore their use can impact neurodevelopment (Costa et al., 2004). Consistent with this notion, epidemiological studies show that AEDs during pregnancy are associated with an increased risk of fetal malformations including neural tube defects, growth retardation and microcephaly (Gedzelman and Meador, 2012; Meador et al., 2013; Tomson et al., 2011; Vajda, 2014). Moreover, *in utero* exposure in humans to AEDs increases the risk of cognitive dysfunction in later life (Meador et al., 2013) and in animals can damage domains critical for learning, memory and attention in the developing brain (Ikonomidou and Turski, 2010; Shi et al., 2010; Wu and Wang, 2002).

An important source of human neurons can be derived from human pluripotent embryonal carcinoma (EC) stem cells, originally isolated from germ cell tumors (Andrews, 2002; Przyborski et al., 2004). Human EC stem cells (hECSCs) are considered the adult counterparts of ES cells (and their study gave rise to many of the protocols to derive human ES cells) and provide a well-established model to study cell differentiation throughout embryogenesis (Andrews, 2002) and neurogenesis (Park et al., 2007; Przyborski et al., 2000; Przyborski, 2001). The human EC stem cell line, TERA2.cl.SP12, is easy to grow in cell culture, does not require feeder layers and has enhanced propensity to differentiate into neurons and glia using a simple differentiation protocol (Przyborski, 2001). Specifically, when TERA2.cl.SP12 stem cells are exposed to retinoic acid the pattern of gene and protein expression, along with morphological changes *in vitro*, parallel the early stages of neural differentiation during embryonic development and, within 3–4 weeks, they develop into functional neurons expressing a range of voltage- and ligand-gated ion channels (Andrews, 2002; Coyne et al., 2011; Stewart et al., 2004).

In the present report therefore, we have begun to explore the value of TERA2.cl.SP12 cells in developmental neurotoxicity studies, since EC stem cell lines have already proven useful addressing the impact of metals such as mercury, lead and aluminum (Laurenza et al., 2013; Stern et al., 2014). The specific objectives of our study were to begin to assess the predictive validity of hECSCs in neurotoxicology by determining the impact of four major AEDs, namely phenobarbital, valproic acid, carbamazepine and lamotrigine and two control drugs, ciprofloxacin and perfluorooctanoic acid on cell viability, cell cycle and their differentiation into neurons. Perfluorooctanoic acid is an environmental contaminant associated with developmental cardiotoxicity and ciprofloxacin is a widely used antimicrobial agent associated with damage to immature joint cartilage in animals but neither is an established developmental neurotoxin (Jiang et al., 2012; Stahlmann and Lode, 2013).

By addressing the impact of these diverse drugs on the parameters of cell viability, proliferation, apoptosis and neural differentiation, all of which could contribute to the impairment of fetal growth, we also sought to determine the value of human stem cells as an *in vitro* model that distinguishes between general cytotoxicity and developmental neurotoxicity.

2. Methods

2.1. Cell culture

TERA2.cl.SP12 stem cells were prepared, maintained and differentiated according to methods previously described (e.g. Stewart et al., 2004). Briefly, stem cells were maintained in 75 cm^2 tissue culture flasks at 37 °C, 5% CO_2, 100% relative humidity in Dulbecco's Modified Eagle Medium (DMEM, Sigma–Aldrich) supplemented with fetal bovine serum (FBS, 10%, v/v; Invitrogen), L-glutamine (2 mM; Sigma–Aldrich) and penicillin–streptomycin (100 U/ml, 100 μg/ml; Invitrogen). When cells reached approximately 80% confluence, they were detached from the flask base using microglass beads (Fisher Scientific), removed and re-seeded at a density of 1.5×10^4 cells/cm^2 into 12 well plates for monolayer culture. Retinoic acid (RA, 10 μM in dimethyl sulphoxide [DMSO]; Sigma Aldrich) was used to differentiate stem cells towards neuronal phenotypes with differentiation media refreshed on alternate days.

2.2. Drug treatment

Stem cells seeded for monolayer culture were allowed to attach for 24 h before antiepileptic drugs (AED) were applied over the following concentration ranges: phenobarbital (PHB, Sigma–Aldrich), 10–1000 μM; valproic acid (VPA, Sigma–Aldrich) 10–1000 μM, lamotrigine (LTG, Tocris) 1–100 μM and carbamazepine (CBZ, Sigma–Aldrich) 1–100 μM; stock solutions of PHB (500 mM) and LTG (100 mM) were made up in water, a stock solution of VPA (1 M) was made up in phosphate buffered saline (PBS) and a stock solution of CBZ (100 mM) was dissolved in DMSO. Drug concentration ranges were based on plasma levels of therapeutic doses for epilepsy (Eadie, 2001). Control drugs were tested over the following concentration ranges: ciprofloxacin (CIP, Cellgro) 1–100 μM and perfluorooctanoic acid (PFOA, Sigma–Aldrich) 1–100 nM; stock solutions of CIP (100 mM) and PFOA (100 μM) were made up in water. CIP concentrations were based on those for quinolones measured in animals and patients (Halliwell et al., 1993) and PFOA concentrations were based on average serum concentrations in humans (Ehresman et al., 2006). All compounds and solvent controls (⩽0.2% v/v) were applied to cells diluted in culture media at their test concentrations.

2.3. MTT cell viability assay

A tetrazolium dye reduction assay (CellTiter 96 MTT®; Promega) was used to determine compound effects on cell viability. Stem cells were treated with AEDs or control drugs for 3 days in the absence or presence of RA with phenol red-free media 24 h after seeding. Following drug exposure, cells were harvested and re-suspended in medium free of drug. 90 μl of cell suspension was dispensed into each well of a 96-well tissue culture plate. 15 μl of dye solution was added to each well of the plate, which was then incubated for 4 h at 37 °C, 5% CO_2 and 100% relative humidity. After 4 h, 100 μl of solubilization solution/stop mix was added to each well. Absorbance at 540 nm was recorded using the Synergy HT multi-mode microplate reader (BioTek) or the Multiskan MCC microplate reader (Fisher Scientific).

2.4. Cell cycle

Cell cycle analysis was performed using DNA content analysis of fixed cells stained with propidium iodide (PI; Sigma–Aldrich). Stem cells were synchronized by serum starvation for 24 h before culturing in serum supplemented medium and treatment with AEDs or controls for 3 days in the absence or presence of RA. Cells were then harvested and fixed in 70% ethanol and stored for 24 h at −20 °C. Fixed cells were rinsed with PBS and stained with a PI solution (20 μg/ml PI, 0.1% Triton X-100 and 200 μg/ml DNase-free RNase A in PBS) that was freshly prepared before use. The flow cytometer was adjusted for detection of PI through a 630/22 nm bandpass filter from 488 nm laser excitation using a BD FACSCalibur flow cytometer. The pulse width (FL2-W)-pulse area (FL2-A) signal was used to discriminate between G_2 cells and cell doublets and the latter were gated out. A total of 7000 gated events were