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Research article

Human neuron-astrocyte 3D co-culture-based assay for evaluation of neuroprotective compounds

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ABSTRACT

Introduction: Central nervous system drug development has registered high attrition rates, mainly due to the lack of efficacy of drug candidates, highlighting the low reliability of the models used in early-stage drug development and the need for new *in vitro* human cell-based models and assays to accurately identify and validate drug candidates. 3D human cell models can include different tissue cell types and represent the spatiotemporal context of the original tissue (co-cultures), allowing the establishment of biologically-relevant cell-cell and cell-extracellular matrix interactions. Nevertheless, exploitation of these 3D models for neuroprotection assessment has been limited due to the lack of data to validate such 3D co-culture approaches.
Methods: In this work we combined a 3D human neuron-astrocyte co-culture with a cell viability endpoint for the implementation of a novel *in vitro* neuroprotection assay, over an oxidative insult. Neuroprotection assay robustness and specificity, and the applicability of Presto Blue, MTT and CytoTox-Glo viability assays to the 3D co-culture were evaluated.
Results: Presto Blue was the adequate endpoint as it is non-destructive and is a simpler and reliable assay. Semi-automation of the cell viability endpoint was performed, indicating that the assay setup is amenable to be transferred to automated screening platforms. Finally, the neuroprotection assay setup was applied to a series of 36 test compounds and several candidates with higher neuroprotective effect than the positive control, Idebenone, were identified.
Discussion: The robustness and simplicity of the implemented neuroprotection assay with the cell viability endpoint enables the use of more complex and reliable 3D *in vitro* cell models to identify and validate drug candidates.

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1. Introduction

There is an increased social, clinical and economical need for new therapies targeting central nervous system (CNS) neurodegenerative diseases. CNS drug development slowed down since 1990 and high attrition rates have been registered, with CNS drugs more likely to fail in late-stage clinical trials than others (Kesselheim, Hwang, & Franklin, 2015). The most common reason for failure was the lack of efficacy, highlighting the low reliability of the models used in early-stage development and the need for new preclinical *in vitro* cell-based models and assays to accurately identify and validate new drug candidates (Astashkina & Grainger, 2014; Kesselheim et al., 2015).

Human neural *in vitro* models typically consist of 2D cultures of neuronal-like cells, such as neuroblastoma cell lines (Choi et al., 2011; Del Barrio et al., 2011) or, more recently, pluripotent stem cell-derived neurons (Avior, Sagi, & Benvenisty, 2016). However, to achieve a significant level of mimicry of the *in vivo* tissue, cell models must include the different tissue cell types and be able to represent the spatiotemporal context of the original tissue.

Human *in vitro* 3D models allow the establishment of biologically relevant cell-cell and cell-extracellular matrix interactions, that recapitulate the tissue microenvironment (Edmondson, Broglie, Adcock, & Yang, 2014). In the last years, 3D models have undergone rapid development and gained increased attention as complementary tools for pre-clinical research which bridge the gap between animal models and human clinical trials (Breslin & Driscoll, 2013; Astashkina & Grainger, 2014). Nevertheless, exploitation of these 3D models for neuroprotection assessment has been limited due to the lack of validated, robust and user-friendly assays.

Cell viability assays have been developed and widely used in standard 2D cultures as simple and robust endpoints (Burroughs et al.,

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2012). The most commonly used cell viability assays are based on cell metabolic activity, such as tetrazolium- and resazurin-based methods. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is an endpoint tetrazolium-based assay that quantifies the cellular metabolic activity through the formation of blue formazan crystals after the cleavage of the tetrazolium ring by mitochondrial dehydrogenases; such crystals can then be solubilized and quantified in cell lysates (Riss et al., 2004). Assays based on tetrazolium reduction that originates water-soluble tetrazolium salts, such as MTS, XTT and WTS are also commercially available. Nevertheless, all of these assays are toxic to the cells (Riss et al., 2004). Resazurin-based methods quantify the cellular metabolic activity by measuring the fluorescence of the water soluble, non-toxic resofurin. This compound is formed through the metabolization of resazurin by live cells and diffuses from the cells into the surrounding medium; therefore cell lysis is not required and cell cultures can be maintained after the measurement (Riss et al., 2004; Sonnaert, Papantoniou, Luyten, & Schrooten, 2014). Alternatively, cell viability can be evaluated by plasma membrane integrity assays that include trypan blue or nucleic acid staining, by ATP determination or by assays that measure the activity of an intracellular enzyme that has been released from cells with damaged membrane integrity (Niles et al., 2007); these latter can be lactate dehydrogenase (LDH), or cytosolic, lysosomal or transmembrane-bound proteases whose activities are measured using luminogenic peptide substrates. However, the use of these assays in 3D cultures for drug screening requires its validation.

Herein we describe the implementation of an assay setup for neuroprotection evaluation in a 3D human co-culture of neurons and astrocytes over an oxidative insult. We describe the evaluation of several cell viability assays and semi-automation of the endpoint as a proof of concept that the neuroprotection assay setup is amenable for high-throughput screening platforms. We have used a scalable 3D human neuron-astrocyte co-culture previously developed, based on the 3D differentiation of the pluripotent embryonic carcinoma-derived NTera-2/clone D1 (NT2) cell line, in agitation-based culture systems (Terrasso et al., 2015). This 3D co-culture is an amenable and cost-effective cell model to feed high-throughput screening platforms and to evaluate human neuronal and astrocytic toxicity with improved sensitivity due to the functional neuron-astrocyte metabolic interactions (Simão et al., 2016; Terrasso et al., 2015).

Finally, we have applied the neuroprotection assay setup implemented in this work to a series of 36 compounds, where several candidates with higher neuroprotective effect than the positive control, Idebenone, were identified.

2. Materials and methods

2.1. Cell culture and 3D neural differentiation

Undifferentiated NTera-2/clone D1 (NT2) cells from the American Type Culture Collection (ATCC) were routinely propagated in 2D culture systems (Brito et al., 2007); 3D neural differentiation was performed in an agitation-based culture system as previously described (Terrasso et al., 2015). Briefly, undifferentiated NT2 cells were inoculated as a single cell suspension in 125 mL spinner vessels equipped with a ball impeller (Wheaton) in DMEM, 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (P/S; all from Life Technologies). After 3 days of aggregation, neuronal and astrocytic differentiation was induced by addition of 10 μM RA, with a 50% media exchange every 2–3 days for 21 days. Following this period, a 3D neuron-astrocyte co-culture was obtained and maintained in DMEM, 5% (v/v) FBS, 1% (v/v) P/S up to day 50.

2.2. Cell viability endpoint

Presto Blue™ cell viability assay (Life Technologies) was performed following the manufacturer's instructions. Briefly, Presto Blue cell viability reagent was diluted 1:10 in culture media and incubated with aggregates for 40 min. at 37 °C and 5% CO_2. The fluorescence intensity was evaluated using a FluoroMax®-4 spectrofluorometer, with excitation and emission wavelengths of 580/595 nm, respectively.

For MTT assay a 5 mg/mL stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich)in PBS was diluted 1:10 in culture media and incubated with aggregates for 3 h, at 37 °C and 5% CO_2. Afterwards, blue formazan crystals were dissolved in dimethyl sulfoxide (DMSO), for 15 min. at RT, under shaking conditions, and the absorbance was read at 570 nm.

CytoTox-Glo™ Cytotoxicity Assay (Promega) was performed accordingly to manufacturer's instructions. Briefly, CytoTox-Glo ™ cytotoxicity assay reagent was added to the aggregates and incubated for 15 min. at RT, in an orbital shaker. Afterwards, the lysis reagent was added and further incubated for additional 15 min. in agitation before luminescence measurement.

2.3. Semi-automation of cell viability endpoint

Semi-automation of cell viability endpoint was performed using a Hamilton® Microlab STARlet workstation (Hamilton) equipped with 8 independent pipetting channels, a reagent container carrier, a plate carrier (5 positions) and a disposable tip carrier (5 trays with 5 racks). The workstation has an integrated plate storage module with 64 positions and a Biotek Synergy 2 multimode microplate reader controlled by a computer using the MicroLab Star software. A program for the automation of the steps of (1) addition of Presto Blue reagent, (2) transfer of medium from assay plate to fluorescence reading plate and (3) fluorescence reading was implemented.

2.4. Neurotoxicity and neuroprotection assays

3D neuron-astrocyte aggregates were collected from spinner vessels between days 38 and 50 of culture, where aggregate diameter was kept stable, typically with approximately 180 μm, distributed in 96-well plates at 10 aggregate/well and incubated in DMEM, 5% (v/v) FBS, 1% (v/v) P/S, before carrying out neurotoxicity or neuroprotection assays. Six wells were used per test condition and the culture medium was used as an untreated control. Cell viability, evaluated by the Presto Blue assay, was used as endpoint.

The neurotoxicity of Idebenone (provided by Grupo Tecnimede) was evaluated for concentrations between 0.01 nM-100 μM.

Thirty-six (36) test compounds were evaluated for their neuroprotective effects on 3D neuron-astrocyte cultures over an oxidative insult. The test compounds were added to the cultures under different regimens: 24 or 48 h pre-incubation, 24 or 48 h pre-incubation followed by 48 h of co-incubation together with tert-butyl hydroperoxide (tBHP) or chloramphenicol, 48 h co-incubation, 48 h co-incubation followed by 24 h post-incubation (Fig. 1). tBHP- and chloramphenicol-induced insults were performed by exposure to its half maximal inhibitory concentration (IC_{50}; 280 μM and 4.3 mM, respectively) for 48 h (Terrasso et al., 2015). Negative (medium), insult and test compound neurotoxicity controls have been performed. Cell viability was accessed before exposure to test compound, before tBHP insult and 48 h after insult. Final cell viability was calculated as a percentage of cell viability before insult.

2.5. Immunofluorescence microscopy

3D neuron-astrocyte co-cultures were collected at day 38 and processed for immunofluorescence staining, accordingly to Terrasso et al. (2015). Briefly, the aggregates were fixed in 4% (w/v) paraformaldehyde (Sigma) solution in PBS with 4% (w/v) sucrose and processed directly for immunostaining. The primary antibodies used for cell characterization were anti-βIII-tubulin (Millipore) and anti-glial fibrillary acidic protein (GFAP, DAKO). The secondary antibodies used were

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