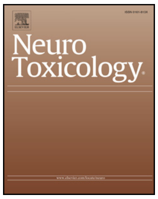
Neurotoxicology 73 (2019) 258–264

Contents lists available at ScienceDirect

Neurotoxicology

journal homepage: www.elsevier.com/locate/neuro



An *in vitro* developmental neurotoxicity screening assay for retinoic acid-induced neuronal differentiation using the human NT2/D1 cell line

Matthew A. Taylor*, H. Lynn Kan, B. Bhaskar Gollapudi[1], M. Sue Marty

The Dow Chemical Company, Midland, MI, USA

A R T I C L E I N F O

Keywords:
NT2
NTera 2
In vitro
Developmental neurotoxicity
Assay development
Retinoic acid
Neuron
Differentiation
Flow cytometry
β3-tubulin

A B S T R A C T

Traditional approaches (e.g., neurobehavior, neuropathology) can detect alterations in apical endpoints indicative of developmental neurotoxicity (DNT). However, there is an increasing desire to understand mode-of-action (MOA) for DNT effects; thus, this short communication describes initial work on a neuronal differentiation assay. Basically, our laboratory used the human NT2/D1 cell line to develop an assay to evaluate toxicants for effects on all-trans retinoic acid (RA)-induced neuronal differentiation. Based on literature reports, we selected a neuronal protein, neuronal class III β-tubulin (β3-tubulin), as a marker of differentiation. For this assay, cultured RA-treated NT2 cells were trypsinized to individual cells, methanol fixed, and labeled with a β3-tubulin specific monoclonal antibody (TUJ1). Characterization studies using 100,000 cells/sample showed that NT2 cells had appreciable expression of β3-tubulin starting around day 7 of the differentiation process with a peak expression noted around day 12. Methylmercury, 22(R)-hydroxycholesterol, N-(4-hydroxyphenol)retinamide (4HPR), and 9-cis retinoic acid were selected as initial test compounds. Of these, only 9-cis RA, which is known to affect the RA pathway, was positive for specific impacts on differentiation. These results demonstrate the feasibility of using a flow cytometry method targeting specific cellular biomarkers for evaluating effects on neuronal differentiation. Additional assays are needed to detect compounds targeting other (non-RA) neuronal differentiation pathways. Ultimately, a battery of *in vitro* assays would be needed to evaluate the potential MOAs involved in altered neuronal differentiation.

1. Introduction

Historically, toxicologists have focused on characterization of chemical hazard with attention focused on defining no observed adverse effect levels (NOAELs) for toxicants. While this is still critical work, there is an increased desire to identify mode-of-action (MOA) to better understand the relevance of a toxicant's effects to humans and other species.

One area in which additional MOA assays are needed is developmental neurotoxicity (DNT). In an effort to facilitate development of alternative DNT assays, the Center for Alternatives to Animal Testing (CAAT) at Johns Hopkins University and EU regulatory bodies have hosted DNT workshops (e.g., TestSmart conferences from 2006 to 2016: CAAT, 2006, 2008, 2014; European Commission Joint Research Centre - EC JRC, 2011; and Organisation for Economic Co-operation and Development/European Food Safety Authority - OECD/EFSA, 2016). These conferences identified seven developmental processes which are integral for normal neurodevelopment and could present targets for developmental neurotoxicity (DNT). These processes include proliferation, differentiation, migration, axon/dendrite outgrowth, synaptogenesis, myelination, and apoptosis. Ultimately, the goal is to develop a battery of assays that can evaluate toxicant effects on each of these key developmental processes to screen for potential activity or identify MOA.

For neuronal differentiation, assays examining spontaneous neuronal differentiation are being developed (e.g., Tamm et al., 2006, 2008). This short communication describes an assay system that can be used to investigate chemical-mediated effects on induced neuronal differentiation. In our inducible model, the retinoic acid (RA) signaling pathway was selected as a potential differentiation target. RA is known to play a critical role in neurodevelopment and neuronal differentiation (Wilson and Maden, 2005; Dhara and Stice, 2008). Early in neurodevelopment, when the neural tube forms from the neural plate, fibroblast growth factors (FGFs), WNT, and RA form a gradient which imparts hindbrain character to the neural tube (i.e., antero-posterior development of the neural tube). The dorsal and ventral patterning of the brain

---

* Corresponding author at: Electronics & Imaging, DowDuPont Specialty Products (DuPont) Division, 455 Forest Street, Marlborough, MA, 01752, USA.
E-mail address: matthew.taylor@dupont.com (M.A. Taylor).

[1] Current Address: Exponent, Inc., 1800 Diagonal Road, Suite 500, Alexandria, VA, USA.

https://doi.org/10.1016/j.neuro.2019.04.005
Received 20 October 2018; Received in revised form 4 March 2019; Accepted 8 April 2019

and spinal cord are determined by opposing gradients of bone morphogenetic protein (BMP) from the roof plate and sonic hedgehog (SHH) from the notochord and floor plate, respectively. RA plays a role in motor neuron differentiation and the induction of different motoneuron subtypes, as well as the formation of some inter-neurons in the hindbrain and spinal cord. It is hypothesized that RA interacts with its nuclear receptors, stimulating phospholipase A_2, C and D activities, which leads to arachidonic acid release and redistribution as part of the neuronal differentiation process (Farooqui et al., 2004). Thus, the RA pathway was selected as a suitable target for altered neuronal differentiation and the focus of this *in vitro* MOA assay.

The *in vitro* model that was selected for assay development was the human NTera2 (NT2) cell line. NT2 cells were derived from a metastatic testicular teratoma (Fogh and Trempe, 1975), which was subcloned in nude rodents to create the D1 subline (Andrews et al., 1984). This subclone was shown to differentiate into functional neurons in response to all-trans retinoic acid administration (Andrews, 1984). While this cell line displays some chromosomal abnormalities, the resulting neurons are of sufficient quality that they have been used in human clinical trials (Nelson et al., 2002).

Neuronal differentiation was evaluated using a neuron-specific biomarker, neuronal class III β-tubulin (β3-tubulin) (Katsetos et al., 2003; Matus, 1988; Pleasure et al., 1992). β3-tubulin is a microtubule-associated protein that contributes to the neuronal cytoskeleton. The expression of unphosphorylated β3-tubulin indicates a commitment by neuroepithelial cells to a neuronal lineage (Sarnat, 2013). The selected antibody used in these studies did not identify β-tubulin found in non-neuronal-fate NT2 cells (Abcam, 2015), a finding that was confirmed with fluorescence microscopy (data not shown).

The characteristics of NT2 cells cultured in our laboratory were examined and NT2 neuronal differentiation was verified using antibodies directed against β3-tubulin with detection by flow cytometry. Factors critical to the neuronal differentiation efficiency of NT2 cells (RA dose, RA exposure period, cell density, etc.) were determined in hydrophobic-plate cultures. Once optimal assay conditions were identified, NT2 cellular differentiation and cytotoxicity were examined for four test compounds intended as 1) a positive control (9-cis retinoic acid) to alter RA signaling; 2) a negative control (an apoptotic compound, N-(4-hydroxyphenyl)retinamide) to examine non-specific effects on differentiation; 3) a compound reported to stimulate NT2 cellular differentiation (22(R)-hydroxycholesterol); and 4) a non-specific developmental neurotoxicant (methylmercury) reported by some to inhibit neuronal differentiation (Tamm et al., 2006, 2008; Ceccatelli et al., 2010).

2. Materials and methods

2.1. Cell culture

NT2/D1 cells were obtained from American Type Culture Collection (ATCC; cat# CRL-1973, Manassas, VA) and grown in standard tissue culture flasks using Dulbecco's modified eagle medium (DMEM; ATCC cat # 30–2002) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, cat# 16000) and 1% penicillin/streptomycin solution (Gibco, cat# 15140). Cells were maintained in a humidified incubator at 37 °C and 5% CO_2. For differentiation experiments, cells were used between passages 6 and 50 as NT2 cell cultures have been shown to be stable in chromosome count and behavior over this passage range (Fogh and Trempe, 1975). Initial investigation of the NT2 cells was performed in adherent culture, progressing to suspension culture, then 'hydrophobic well' cultures to address logistical challenges with assay development. The final hydrophobic culture conditions are reported below. Stability of the cells under all three culture conditions was confirmed in our laboratory as differentiation efficiency and growth rates were consistent across the 6–50 passage range within each culture paradigm. Following RA differentiation (described below), neuronal character of adherent NT2 cell cultures was confirmed by colocalization of differentiated neuronal morphology and β3-tubulin expression using fluorescence microscopy with an anti-β3-tubulin primary antibody and fluorescent secondary antibody. Concurrently, undifferentiated cells were examined for the absence of β3-tubulin labeling.

2.2. Hydrophobic plate cultures used for differentiation and β3-tubulin labeling

Differentiation conditions for NT2 cells were adapted from methods described by Hill et al. (2008). Hydrophobic 6-well plates (Ultra-low attachment plates; Corning Inc., Lowell, Massachusetts) seeded with NT2 cells (450k) were maintained under standard culture conditions (described above). After two days in the hydropobic plates, medium was replaced with retinoic acid (+RA)-containing medium (designated "RA day 0"). RA-containing medium (+RA) was base medium supplemented with 1 µl/ml of 10 mM all-trans retinoic acid solution in dimethyl sulfoxide (DMSO; 0.1% final concentration). Two days later (RA day 2), medium was refreshed 100% with + RA medium. Thereafter, +RA medium was refreshed daily. The differentiation assay was based on labeling with a monoclonal antibody from the clone line TUJ-1 directed at β3-tubulin to label differentiated neurons in RA-treated NT2 cells. Cells were harvested for β3-tubulin labeling on RA day 9–10 based on a time course study examining maximum β3-tubulin expression after RA exposure on Days 7–10.

A series of experiments (data not shown) were conducted to optimize neuronal differentiation efficiency (8–12%). Experiments indicated that cell density impacted differentiation (i.e., seeding 300k to 500k cells in 6-well hydrophobic plates was optimum). Daily media refreshes starting on RA Day 2 also were important. The most sensitive period for RA differentiation was determined using a series of temporal experiments in which RA was removed from the medium for different periods to identify the critical window for maximum differentiation efficiency, which was identified as RA Days 2–3. Thus, to ensure maximum assay sensitivity to toxicant effects, a 48-h dosing period was selected in which cells would be exposed to toxicants on RA Days 2–3 with subsequent evaluation of the proportion of differentiated neurons on RA Days 9–10. The concentration of RA during the critical window of neuronal differentiation was maintained at 10 µM RA as reported in the literature (Serra et al., 2007).

2.3. Toxicant selection and dosing of hydrophobic plate NT2 cultures

Four test compounds (Table 1) were chosen for initial testing based on literature reports of effects on neuronal differentiation or due to interaction with the retinoic acid pathway by which NT2 cells differentiate. Concentrations were designed to extend from non-cytotoxic to cytotoxic levels, 100 µM, or to the limit of solubility. 9-cis RA was included as a positive control to demonstrate inhibitory effects on neuronal differentiation, which is dependent on all-trans-RA (Redfern et al., 1994; Zeller and Strauss, 1995; Lovat et al., 1997). N-(4-hydroxyphenyl)retinamide (4HPR) was included as a negative control as this compound was reported to induce apoptosis without specific effects on differentiation (Kitareewan et al., 1999). Methylmercury (II) chloride was included as a nonspecific neurotoxicant that purportedly alters neuronal differentiation via an unknown MOA (Tamm et al., 2006, 2008; Ceccatelli et al., 2010). 22(R)-hydroxycholesterol has been reported to increase neuronal differentiation in NT2 cells (Yao et al., 2007).

Toxicants were applied with each media refresh on RA day 2 and RA day 3 (48 h total exposure), the critical period for RA-induced differentiation. Cells were cultured in fresh + RA media without test substance beginning on RA day 4 with daily media replacement until cells were evaluated on RA day 9–10. Toxicants were prepared as a DMSO stock at 1000x the highest final concentration and tested across the

Table 1
Test Compounds Used to Characterize NT2 Neuronal Differentiation Assay.

Compound (CAS no.)	Reference	Reference Test System and Concentrations	Concentrations in NT2 Cultures (µM)	Notes
9-cis RA (5300-03-8)	Redfern et al., 1994	Human neuroblastoma SH SY 5Y cells; 0.1-100 nM	0, 1, 3, 10, 30, 100	Planned maximum concentration (100 µM)
4HPR (65646-68-6)	Kitareewan et al., 1999	NT2 cells; 0.5-5 µM	0, 0.1, 0.3, 1, 3, 10	Maximum based on cytotoxicity
Methylmercury (115-09-3)	Tamm et al. (2006, 2008)	Neural stem cells (line C17.2 and embryonic cortical cells); 2.5-10 nM	0, 0.01, 0.1, 1, 2.5, 5	Maximum based on cytotoxicity
22(R)-hydroxycholesterol (17954-98-2)	Yao et al., 2007	NT2 cells; 1-50 µM	0, 0.3, 1, 3, 10, 25	Maximum based on solubility

concentrations shown in Table 1. Lower doses were prepared by serial dilution in media with additional DMSO added to control wells to ensure a constant 0.2% DMSO concentration (0.1% from all-trans RA, 0.1% from toxicant) during toxicant dosing. Inclusion of the same DMSO percentage in each well removed DMSO concentration as a variable impacting differentiation efficiency. Each exposure was conducted in triplicate wells and the proportion of differentiated cells within these samples was averaged (n = 1). Each exposure scenario was repeated at least three times in three different passages of NT2 cells to verify that findings were reproducible.

2.4. *Flow cytometry to assess β3-tubulin expression as a marker of neuronal differentiation*

On RA day 9–10, suspensions of NT2 cells were trypsinized to break up clusters, fixed, and labeled with conjugated primary antibody to β3-tubulin. Cells were centrifuged, resuspended, and analyzed with a Coulter Epics XL-MCL (Beckman Coulter, Brea, CA) flow cytometer controlled by Expo32 ADC v1.1 software to examine the proportion of β3-tubulin-labeled cells. Approximately 100,000 cells were counted for each of the triplicate wells per dose group. The resulting data was gated based on size and granularity to eliminate the majority of debris and multicellular aggregates. Identical size/granularity gating was used on all samples to aid in the identification of individual neuronal cells. Flow cytometry spectra were gated at the visually identified low point between baseline and signal peaks to distinguish the proportion of unlabeled (undifferentiated) cells from those labeled for β3-tubulin (differentiated cells).

2.5. *Cytotoxicity*

In an effort to distinguish selective effects on cell differentiation from indirect effects from cytotoxicity, two different cytotoxicity assays were employed with the understanding that the best indicator of cytotoxicity may vary by test compound. In neuronal differentiation experiments, cytotoxicity was measured at 24 h and/or 48 h after test compound exposure using a separate population of cells handled identically to those which were labeled for β3-tubulin detection. First, a lactate dehydrogenase (LDH) release assay was utilized to test for membrane leakage. Media samples were collected at 24 and 48 h to determine LDH content. LDH assays were performed according to manufacturer instructions (LDH-Cytotoxicity Colorimetric Assay Kit; Cat# K311-400; BioVision Inc.; Milpitas, CA). After data analysis, all significant cytoxicity effects on the first day of toxicant dosing (24 h) were reproduced on the second day (48 h), so only the 48 h LDH results were used for statistical analysis.

In addition to the LDH release assay, a mitochondrial function assay (MTS reduction, 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) was employed 48 h after test compound exposure. This assay was performed according to manufacturer instructions (CellTiter 96 Aq$_{\text{ueous}}$ One Solution Cell Proliferation Assay kit; Promega, Madison Wisconsin, Cat# G3580) with volumes adjusted to account for the larger well size in 6-well plates vs. the 96-well format used in the manufacturer's protocol. The quantity of formazan product, as measured by the amount of 490 nm absorbance, is directly proportional to the mitochondrial activity rate of viable cells in culture. Compound-induced differentiation effects in the absence of significant cytotoxicity were deemed to be selective effects.

2.6. *Statistics*

Data were tested for equality of variance using Bartlett's test at alpha = 0.01. Data were analyzed using a one-way ANOVA. If significant dose effects were determined in the one-way ANOVA at alpha = 0.05, then individual dose groups were compared to controls using Dunnett's test at alpha = 0.05 (Winer, 1971).

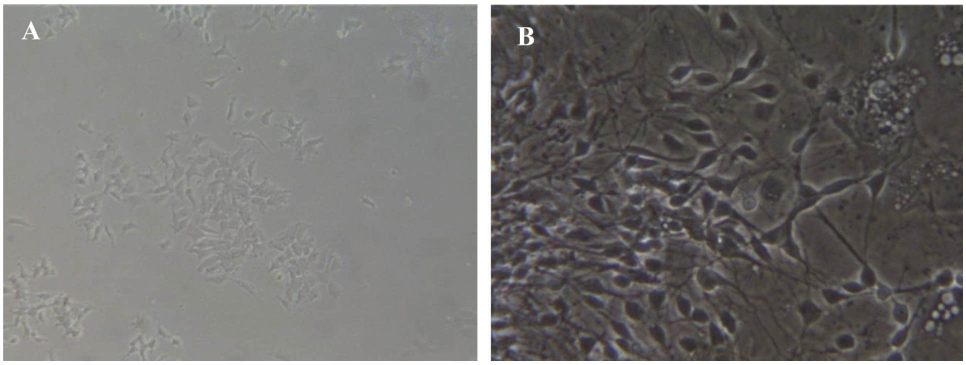


Fig. 1. Undifferentiated NT2 cells exhibit a flattened morphology (A), whereas neuronally differentiated NT2 cells (representative image on adherent plate) have a characteristic ovoid or triangular cell body with axons and dendrites present (B).

3. Results

3.1. Hydrophobic plate cultures used for differentiation (β3-tubulin labeling)

Cell populations were evaluated to optimize β3-tubulin-labeling conditions, verify labeling sensitivity and confirm that labeling was specific for differentiated cells. All cells with a neuronal morphology (ovoid or triangular cell body with axons and dendrites present) displayed pronounced β3-tubulin labeling; cells lacking neuronal morphology were negative for β3-tubulin labeling (Fig. 1A and B shows cell morphology without fluorescent staining). Thus, β3-tubulin was confirmed as a specific marker of neuronal differentiation and an appropriate target for assay development as previously reported in the literature (Pleasure et al., 1992).

Cultures were dosed daily with RA and samples were collected to evaluate the efficacy of RA treatment to increase the number of TUJ1-labeled cells. TUJ1-positive cells were detected as a right shift in the flow cytometry pattern (Fig. 1C, D, and E), which was interpreted as an increase in the proportion of differentiated neuronal cells. With 100,000 cells counted per sample, these results were reproducible across experiments (mean differentiation in controls 9%, CV 13% across 15 experiments, average CV 10% within experiments) which falls within the differentiation efficiency previously reported for adherent and suspension cultures (Serra et al., 2007).

3.2. Toxicant effects on differentiation

To confirm that differentiation of NT2 cells was mediated via activation of the trans-RA receptor pathway and that differences in differentiation efficiency could be measured with the current experimental design, experiments were conducted with 9-cis RA, which altered signal transduction via the RA pathway (Redfern et al., 1994; Zeller and Strauss, 1995; Lovat et al., 1997). 9-cis RA caused a 30 and 60% decrease in cell differentiation at both the 30 μM and 100 μM doses, respectively (Fig. 3A). Cell cytotoxicity was not increased at 30 μM using either the LDH or MTS assays; however, a slight increase in cytotoxicity (+10%) was discernable at 100 μM 9-cis RA using LDH, whereas the MTS assay did not detect an increase in cytotoxicity. Thus, 9-cis RA was effective as a positive control compound to confirm that the disruption of RA signaling results in inhibitory effects on NT2 neuronal differentiation.

As overt cytotoxicity may be expected to impair cellular processes such as differentiation, a negative control compound, 4HPR, was used to examine the effects of cell cytotoxicity on NT2 differentiation. 4HPR-treated cells displayed significant cytotoxicity by both LDH and MTS assays at 3 and 10 μM doses (Fig. 3B). The highest dose of 4HPR left only a minimal cell population alive for labeling at RA Day 9. A minor increase in cytotoxicity was observed at the 1 μM dose, which was significant using the MTS assay, but not the LDH assay. Relying upon the work of Kitareewan, these data were used to establish a relationship between cytotoxicity and impaired differentiation to distinguish specific anti-differentiation effects from indirect effects due to general cytotoxicity.

Methylmercury, a known developmental neurotoxicant (tested as the chloride salt), significantly decreased β3-tubulin labeling by 52% at 2.5 μM and 63% at 5 μM (Fig. 3C). The fluorescence curve was more difficult to quantify due to a shift from a secondary signal peak to an extended baseline curve (Fig. 2D). NT2 cells displayed significant cytotoxicity at the 5 μM concentration, which was detected as significant decreases (˜30%) in viability as measured by both LDH and MTS assays. Visually, cell populations were decreased in wells treated with 5 μM methylmercury, further confirming significant cytotoxicity. The LDH assay also detected significant cell cytotoxicity (8%) at the 2.5 μM dose, whereas there was no change in cell viability using the MTS assay. These results may reflect the non-discriminant nature of methylmercury effects through interaction with sulfhydryl groups. However, the markedly higher reduction in differentiation efficiency with a minor increase in cytotoxicity compared to the other test compounds is suggestive of a potential anti-differentiation effect.

22(R)-hydroxycholesterol, which was reported to induce neuronal differentiation in NT2 cells (Yao et al., 2007), did not alter differentiation at any tested concentration, though cytotoxicity was observed at 10 μM in the LDH assay and at 25 μM in both the LDH and MTS assays. (Fig. 3D). Thus, while 22(R)-hydroxycholesterol did not show increased neuronal differentiation, it was able to maintain differentiation efficiency even at doses that caused ˜30-40% cytotoxicity. The highest 22(R)-hydroxycholesterol concentration (25 μM) represented the maximum soluble concentration in DMSO that remained within the vehicle limit for NT2 cultures; thus, testing at higher concentrations of 22(R)-hydroxycholesterol was not feasible.

4. Discussion

This paper describes the characterization and development of an NT2 cell flow cytometry assay that can examine DNT MOA for toxicants that may affect RA-mediated neuronal differentiation. Identification of early molecular initiating events (MIEs), like interference with neuronal differentiation, will enable the development of more complete adverse outcome pathways (AOPs). These AOPs can then be evaluated using integrated approaches to testing and assessment (IATAs) to link early events with downstream outcomes. This approach will aid interpretation of *in vivo* neurobehavioral/neuropathological results and enable better cross-species extrapolation.

For this assay, NT2 cells were treated with 10 μM RA-containing medium on days 2–10 *in vitro* with test material treatment for 48 h on days 2–3. Differentiated NT2 cells were fluorescently labeled with the TUJ1 antibody, which was detected by a right shift in the flow

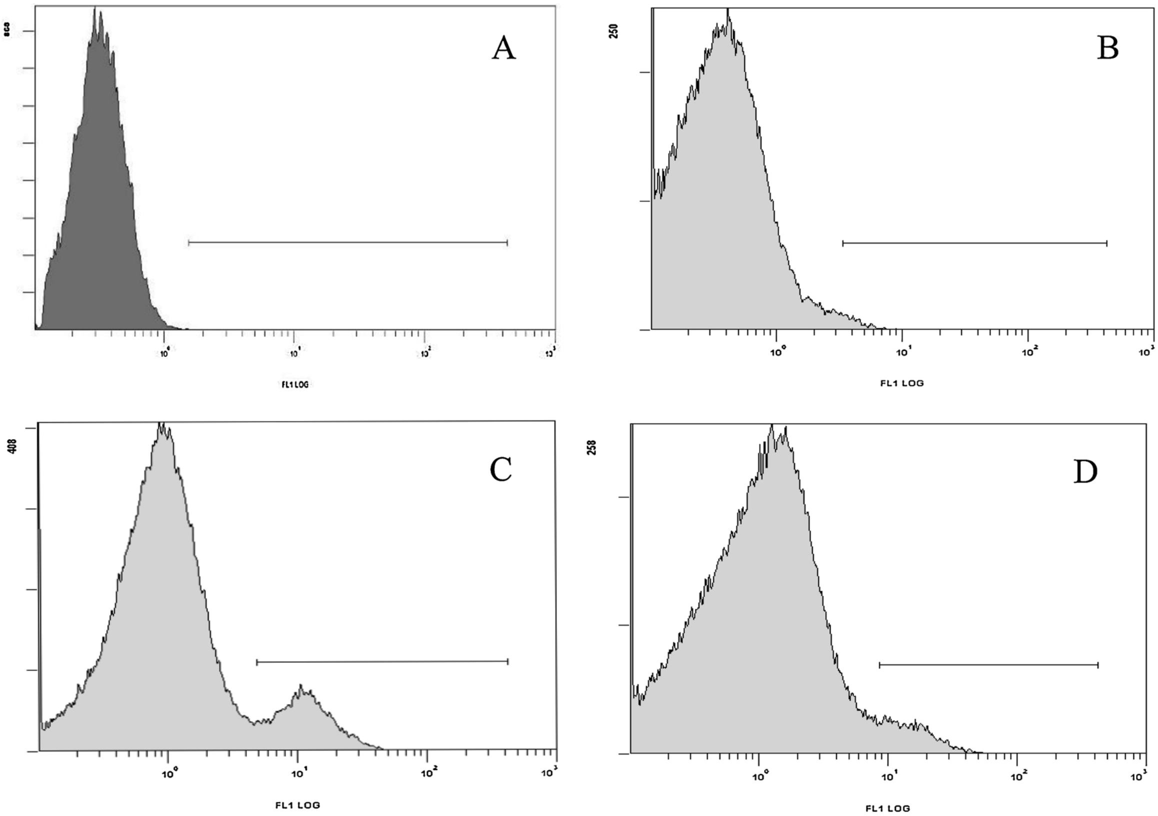


Fig. 2. Flow cytometry pattern showing data from individual wells of undifferentiated NT2 cells (A), differentiating NT2 cells on Day 7 after retinoic acid administration (B) and differentiated NT2 cell cultures on Day 10 after retinoic acid administration (C). Note the shift to the right and secondary peak of β3-tubulin-labeled, differentiated NT2 cells in the 10-day control. Flow cytometry pattern with 5 μM methylmercury exposure showing a less distinct differentiation signal (D) compared with (C).

cytometry pattern. Approximately 100,000 cells were counted per sample and treatment groups were run in triplicate during each experiment along with concurrent controls. Generally, approximately 9% of control cells have differentiated to neurons; with an inter-assay CV of about 13% and an average within-experiment CV of about 10%. The use of flow cytometry allows the counting of large numbers of cells per well, improving the precision of generated data compared to well-based fluorescence assays. The use of hydrophobic plates to encourage three-dimensional aggregates improves differentiation efficiency in NT2 cells, but poses additional challenges to high content image analysis.

The retinoic acid pathway is a key signaling pathway for neuronal differentiation; therefore, assays that examine this pathway have utility to identify a potential DNT MOA. Given its direct inhibition of trans-RA signaling, 9-cis RA gave a specific response for altered neuronal differentiation in the absence of cell cytotoxicity. Experiments with both the addition of 9-cis RA and the removal of all-trans RA (single experiment during assay development, data not shown) served as mechanistic positive controls for disruptions in neuronal differentiation. In both cases, impairment of differentiation could be detected as reductions of 19% (RA removal) or 29–62% (9-cis) in β3-tubulin expression. These experiments show that it is possible to interrupt the process of neuronal differentiation of NT2 cells in the absence of cell cytotoxicity and that changes of ˜20% can be reliably detected using the current processes. This assay was designed for mechanistic exploration, and the biological significance of specific percent changes in differentiation is uncertain.

The effect of 9-cis RA on neuronal differentiation was expected based on previous studies. Kitareewan et al. (1999) have reported that all-trans RA is effective at inducing NT2 differentiation as selective RA receptor (RAR) γ agonists are preferred for differentiation in NT2/D1 cells, while RARa/b, RARb, retinoid X receptor (RXR) agonists and an RAR pan-antagonist do not trigger differentiation, even when used at 10 mM dosages. Terminal differentiation of NT2 cells is induced by over-expression of RARγ (Moasser et al., 1995) with cooperation by RXRβ (Spinella et al., 1998). In NT2 cells, RA treatment has been shown to increase transcripts for a number of developmentally related pathways, including transforming growth factor (TGF) β, homeobox D1 (HoxD1), insulin-like growth factor (IGF), Hedgehog, Notch, and Wnt signaling (Freemantle et al., 2002), which have been associated with neuronal differentiation.

To facilitate use as an MOA assay, the specificity of NT2 cell differentiation for the RA pathway was examined. 4HPR, an apoptotic agent, was included as a negative control to demonstrate that decreases in differentiation efficiency would not occur prior to cytotoxicity with nonspecific toxicants. Increased cell cytotoxicity was initially detected at 1 μM 4HPR via the MTS assay, but was subsequently detected by both cytotoxicity assays at 3 and 10 μM 4HPR. Differentiation of NT2 cells was decreased at 3 and 10 μM 4HPR, concentrations within the cytotoxic range. In a previous report (Kitareewan et al., 1999), 4HPR was shown to increase apoptosis in NT2 cells at concentrations ≥ 1 μM through a RARγ-independent pathway.

The 4HPR example also establishes the need to include parallel cytotoxicity testing of test compounds, because significant cytotoxicity will cause a decrease in neuronal differentiation. Given that "general cytotoxicity" may occur through many pathways, we utilized assays to detect both disruption of cell membranes (LDH) and impairment of cellular metabolism (MTS). As shown in our examples, certain classes of toxicants may have a disproportionate effect on one of these two general cytotoxicity mechanisms, and utilizing both will aid in interpreting specific effects with this assay system.

With methylmercury and 22(R)-hydroxycholesterol, the NT2 assay failed to detect a specific change in neuronal differentiation (i.e.,

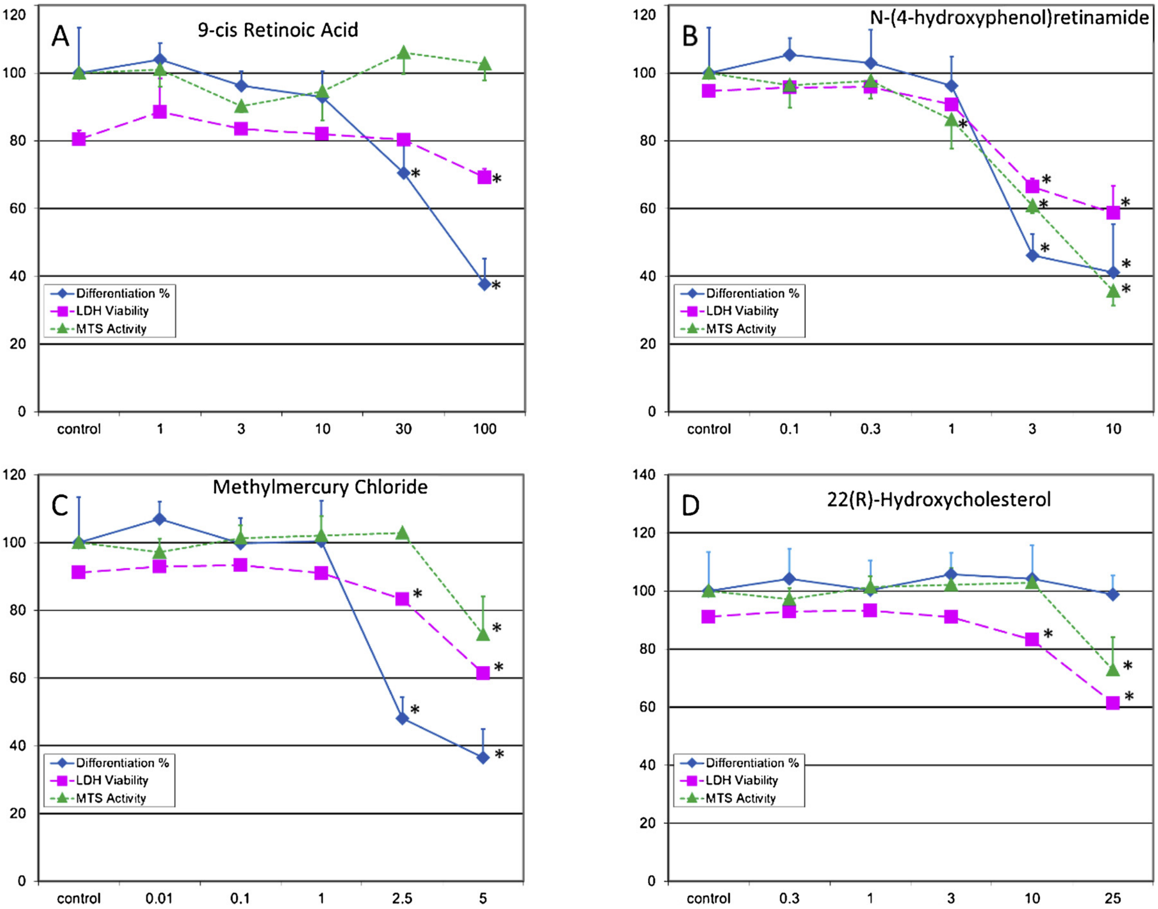


Fig. 3. Effects of 9-Cis Retinoic Acid (A), N-(4-hydroxyphenol)retinamide (B), Methylmercury Chloride (C) and 22(R)Hydroxycholesterol (D) on NT2 Differentiation and Cytotoxicity. Concentrations in μM. Error bars are standard deviations with each individual experiment of 3 wells considered an N of 1. Points indicate means.

differentiation was only affected in the presence of cytotoxicity); however, it is unclear whether these compounds should have been detected with this assay. Methylmercury chloride (2.5 and 5 μM) showed a significant decrease in NT2 differentiation; however, there was increased cytotoxicity at these dose levels. In a previous report (Tamm et al., 2006), methylmercury (2.5–5 nM) was shown to alter neuronal differentiation in spontaneously differentiating cortical neural stem cells at lower concentrations than those inducing apoptosis (25 nM methylmercury; the lowest concentration examined for apoptosis). Alternatively, these differences may be related to differences in the differentiation pathway being assessed, sensitivity of spontaneous vs. induced differentiation, or possibly due to neuronal differentiation not being the primary target for methylmercury DNT; instead, differentiation may be one of several simultaneous targets of methylmercury toxicity (Ho et al., 2013; Castoldi et al., 2001, 2008).

In the case of 22(R)-hydroxycholesterol, there was no effect on NT2 cell differentiation at doses up to 25 μM, although some increases in cytotoxicity were seen at the 10–25 μM doses. 22(R)-Hydroxycholesterol (5–25 μM) has been shown to induce differentiation in undifferentiated (unstimulated) NT2 cells and is associated with increased expression of neurofilament protein NF200, microtubule associated proteins type II (MAP2) a and MAP2b, glial fibrillary acidic protein (GFAP) and glial cell line-derived neurotrophic factor receptor-alpha 2 (GFRα2) (Yao et al., 2007). It seems likely that β3-tubulin would be similarly increased during this differentiation process. However, the 2-day dosing period in the current study was less than the minimum 3 days of dosing used in the Yao et al. (2007) study, which may have impacted the ability to detect an increase in neuronal differentiation. Alternatively, despite up to 40% cytotoxicity at the high dose (25 μM), there was no decrease in neuronal differentiation; thus, 22(R)-hydroxycholesterol may have had a positive effect on maintenance of neuronal differentiation efficiency.

This paper describes an NT2 neuronal differentiation assay that can be used to examine toxicant interference with the RA signaling pathway. Testing of additional chemicals is needed to further characterize the specificity and sensitivity of this assay; however, the general approach (immunostaining and flow cytometry) can be adapted to other assays where cell surface markers of differentiation have been identified. This may be useful as multiple signaling pathways are involved in neuronal differentiation (e.g., IGF - Vogel, 2013; Duenas et al., 1996; Tumor necrosis factor (TNF) α/Nuclear factor (NF) κB – Zhang and Hu, 2013; Wnt/β-catenin – Hirabayashi et al., 2004, etc.); therefore, a battery of *in vitro* DNT assays or a multi-pathway assay are needed to evaluate the spectrum of MOAs that can alter neuronal differentiation. While this assay was conducted in 6-well plates, there is no fundamental barrier to utilization of plates more suitable to high throughput screening and robotic manipulation. With the time available for this project, no work on further miniaturization was possible. With this additional optimization, the assay may be suitable to HT screening. Otherwise, it, and similar assays relying on markers sensitive to other differentiation pathways, may be suitable for mechanistic explorations where overt neurodevelopmental effects are found in animal assays.

Conflict of interest

None.

References

Abcam (2015). http://www.abcam.com/beta-iii-tubulin-antibody-tuj-1-alexa-fluor-488-ab169556.html (Accessed 5 May 2015).

Andrews, P.W., 1984. Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro. Dev. Biol. 103, 285–293.

Andrews, P.W., Damjanov, I., Simon, D., Banting, G.S., Carlin, C., Dracopoli, N.C., Fogh, J., 1984. Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Differentiation *in vivo* and *in vitro*. Lab. Invest. 50, 147–162.

CAAT, 2006. TestSmart Developmental Neurotoxicity. Creating a Humane and Efficient Approach to Developmental Neurotoxicity Testing. March 13-15, 2006. Sponsored by the Center for Alternatives to Animal Testing, Johns Hopkins University, Reston, VA.

CAAT, 2008. TestSmart Developmental Neurotoxicity 2. Creating a Humane and Efficient Approach to Developmental Neurotoxicity Testing. November 12-14, 2008. Sponsored by the Center for Alternatives to Animal Testing, Johns Hopkins University, Reston, VA.

CAAT, 2014. Fourth International Conference on Alternatives for Developmental Neurotoxicity Testing (DNT4). May 12-14 2014. Sponsored by the Center for Alternatives to Animal Testing, Johns Hopkins University, Philadelphia, PA.

Castoldi, A.F., Coccini, t., Ceccatelli, S., Manzo, L., 2001. Neurotoxicity and molecular effects of methylmercury. Brain Res. Bull. 55, 197–203.

Castoldi, A.F., Onishchenko, N., Johansson, C., Coccini, T., Roda, E., Vahter, M., Ceccatelli, S., Manzo, L., 2008. Neurodevelopmental toxicity of methylmercury: laboratory animal data and their contribution to human risk assessment. Regul. Toxicol. Pharmacol. 51, 215–229.

Ceccatelli, S., Daré, E., Moors, M., 2010. Methylmercury-induced neurotoxicity and apoptosis. Chem. Biol. Interact. 188, 301–308.

Dhara, S.K., Stice, S.L., 2008. Neural differentiation of human embryonic stem cells. J. Cell. Biochem. 105, 633–640.

Duenas, M., Torres-Aleman, I., Naftolin, F., Garcia-Segura, L.M., 1996. Interaction of insulin-like growth factor-1 and estradiol signaling pathway on hypothalamic neuronal differentiation. Neuroscience 74, 531–539.

EC JRC, 2011. Third International Conference on Alternatives for Developmental Neurotoxicity Testing (DNT3). May 10-13 2011. Sponsored by the European Commission Joint Research Centre Institute for Health and Consumer Protection, Varese, Italy.

Farooqui, A.A., Antony, P., Ong, W.-Y., Horrocks, L.A., Freysz, L., 2004. Retinoic acid-mediated phospholipase A_2 signaling in the nucleus. Brain Res. Rev. 45, 179–195.

Fogh, J., Trempe, G., 1975. New human tumor cell lines. In: Fogh, J. (Ed.), Human Tumor Cells In Vitro. Plenum Press, New York, pp. 115–159.

Freemantle, S.J., Kerley, J.S., Olsen, S.L., Gross, R.H., Spinella, M.J., 2002. Developmentally-related candidate retinoic acid target genes regulated early during neuronal differentiation of human embryonal carcinoma. Oncogene 21, 2880–2889.

Hill, E.J., Woehrlinga, E.K., Prince, M., Coleman, M.D., 2008. Differentiating human NT2/D1 neurospheres as a versatile in vitro 3D model system for developmental neurotoxicity testing. Toxicology 249, 243–250.

Hirabayashi, Y., Itoh, Y., Tabata, H., Nakajima, K., Akiyama, T., Masuyama, N., Gotoh, Y., 2004. The Wnt/β-catenin pathway directs neuronal differentiation of cortical neural precursor cells. Development 131, 2791–2801.

Ho, N.Y., Yang, L., Legradi, J., Armant, O., Takamiya, M., Rastegar, S., Strähle, U., 2013. Gene responses in the central nervous system of zebrafish embryos exposed to the neurotoxicant methyl mercury. Environ. Sci. Technol. 47, 3316–3325.

Katsetos, C.D., Legido, A., Perentes, E., Mork, S.J., 2003. Class III beta-tubulin isotype: a key cytoskeletal protein at the crossroads of developmental neurobiology and tumor neuropathology. J. Child Neurol. 18, 851–866.

Kitareewan, S., Spinella, M.J., Allopenna, J., Reczek, P.R., Dmitrovsky, E., 1999. 4HPR triggers apoptosis but not differentiation in retinoid sensitive and resistant human embryonal carcinoma cells through an RARγ independent pathway. Oncogene 18, 5747–5755.

Lovat, P.E., Irving, H., Annicchiarico-Petruzzelli, M., Bernassola, F., Malcolm, A.J., Pearson, A.D., Melino, G., Redfern, C.P., 1997. Retinoids in neuroblastoma therapy: distinct biological properties of 9-cis- and all-trans-retinoic acid. Eur. J. Cancer 33, 2075–2080.

Matus, A., 1988. Microtubule-associated proteins: their potential role in determining neuronal morphology. Annu. Rev. Neurosci. 11, 29–44.

Moasser, M.M., Reuter, V.E., Dmitrovsky, E., 1995. Overexpression of the retinoic acid receptor gamma directly induces terminal differentiation of human embryonal carcinoma cells. Oncogene 10, 1537–1543.

Nelson, P.T., Kondziolka, D., Wechsler, L., Goldstein, S., Gebel, J., DeCesare, S., Elder, E.M., Zhang, P.J., Jacobs, A., McGrogan, M., Lee, V.M., Trojanowski, J.Q., 2002. Clonal human (hNT) neuron grafts for stroke therapy: neuropathology in a patient 27 months after implantation. Am. J. Pathol. 160, 1201–1206.

OECD/EFSA DNT workshop, 2016. Brussels, Belgium. (October 18-19, 2016)Organisation for Economic Co-operation and Development/European Food Safety Authority Developmental Neurotoxicity Workshop2016. Organisation for Economic Co-operation and Development/European Food Safety Authority Developmental Neurotoxicity Workshop. https://www.efsa.europa.eu/en/events/event/161018b (Accessed 27 December 2016).

Pleasure, S.J., Page, C., Lee, V.M., 1992. Pure, postmitotic, polarized human neurons derived from NTera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. J. Neurosci. 12, 1802–1815.

Redfern, C.P., Lovat, P.E., Malcolm, A.J., Pearson, A.D., 1994. Differential effects of 9-cis and all-trans retinoic acid on the induction of retinoic acid receptor-beta and cellular retinoic acid-binding protein II in human neuroblastoma cells. Biochem. J. 304, 147–154.

Sarnat, H.B., 2013. Clinical neuropathology practice guide 5-2013: markers of neuronal maturation. Clin. Neuropathol. 32, 340–369.

Serra, M., Leite, S.B., Brito, C., Costa, J., Carrondo, M.J., Alves, P.M., 2007. Novel culture strategy for human stem cell proliferation and neuronal differentiation. J. Neurosci. Res. 85, 3557–3566.

Spinella, M.J., Kitareewan, S., Mellado, B., Sekula, D., Khoo, K.S., Dmitrovsky, E., 1998. Specific retinoid receptors cooperate to signal growth suppression and maturation of human embryonal carcinoma cells. Oncogene 16, 3471–3480.

Tamm, C., Duckworth, J., Hermanson, O., Ceccatelli, S., 2006. High susceptibility of neural stem cells to methylmercury toxicity: effects on cell survival and neuronal differentiation. J. Neurochem. 97, 69–78.

Tamm, C., Duckworth, J.K., Hermanson, O., Ceccatelli, S., 2008. Methylmercury inhibits differentiation of rat neural stem cells via Notch signaling. Neuroreport 19, 339–343.

Vogel, T., 2013. Chapter 2: Insulin/IGF-signalling in embryonic and adult neural proliferation and differentiation in the mammalian central nervous system. In: Wislet-Gendebien, S. (Ed.), Trends in Cell Signaling Pathways in Neuronal Fate Decision. InTech. https://doi.org/10.5772/34445. (Accessed 1 May 2015). http://www.intechopen.com/books/trends-in-cell-signaling-pathways-in-neuronal-fate-decision.

Wilson, L., Maden, M., 2005. The mechanisms of dorsoventral patterning in the vertebrate neural tube. Dev. Biol. 282, 1–13.

Winer, B.J., 1971. Statistical Principles in Experimental Design, 2nd edition. McGraw-Hill, New York, pp. 201–204 208-209.

Yao, Z.-X., Han, Z., Xu, J., Greeson, J., Lecanu, L., Papadopoulos, V., 2007. 22R-Hydroxycholesterol induces differentiation of human NT2 precursor (Ntera2/D1 teratocarcinoma) cells. Neuroscience 148, 441–453.

Zeller, M., Strauss, W.L., 1995. Retinoic acid induces cholinergic differentiation of NTera 2 human embryonal carcinoma cells. Int. J. Dev. Neurosci. 13, 437–445.

Zhang, Y., Hu, W., 2013. Chapter 8: NFκB signaling directs neuronal fate decision. In: Wislet-Gendebien, S. (Ed.), Trends in Cell Signaling Pathways in Neuronal Fate Decision. InTech. https://doi.org/10.5772/34445. (Accessed 1 May 2015). http://www.intechopen.com/books/trends-in-cell-signaling-pathways-in-neuronal-fate-decision.