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Neuronal models for evaluation of proliferation in vitro using high content screening☆

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ABSTRACT

In vitro test methods can provide a rapid approach for the screening of large numbers of chemicals for their potential to produce toxicity (*hazard identification*). In order to identify potential developmental neurotoxicants, a battery of in vitro tests for neurodevelopmental processes such as cell proliferation, differentiation, growth, and synaptogenesis has been proposed. The development of in vitro approaches for toxicity testing will require choosing a model system that is appropriate to the endpoint of concern. This study compared several cell lines as models for neuronal proliferation. The sensitivities of neuronal cell lines derived from three species (PC12, rat; N1E-115, mouse; SH-SY5Y, human) to chemicals known to affect cell proliferation were assessed using a high content screening system. After optimizing conditions for cell growth in 96-well plates, proliferation was measured as the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into replicating DNA during S phase. BrdU-labeled cells were detected by immunocytochemistry and cell counts were obtained using automated image acquisition and analysis. The three cell lines showed approximately 30–40% of the population in S phase after a 4 h pulse of BrdU. Exposure to the DNA polymerase inhibitor aphidicolin for 20 h prior to the 4 h pulse of BrdU significantly decreased proliferation in all three cell lines. The sensitivities of the cell lines were compared by exposure to eight chemicals known to affect proliferation (positive controls) and determination of the concentration inhibiting proliferation by 50% of control (I_{50}). PC12 cells were the most sensitive to chemicals; 6 out of 8 chemicals (aphidicolin, cadmium, cytosine arabinoside, dexamethasone, 5-fluorouracil, and methylmercury) inhibited proliferation at the concentrations tested. SH-SY5Y cells were somewhat less sensitive to chemical effects, with five out of eight chemicals inhibiting proliferation; dexamethasone had no effect, and cadmium inhibited proliferation only at concentrations that decreased cell viability. Data from the N1E-115 cell line was extremely variable between experiments, and only 4 out of 8 chemicals resulted in inhibition of proliferation. Chemicals that had not been previously shown to alter proliferation (negative controls) did not affect proliferation or cell viability in any cell line. The results show that high content screening can be used to rapidly assess chemical effects on proliferation. Three neuronal cell lines exhibited differential sensitivity to the effect of chemicals on this endpoint, with PC12 cells being the most sensitive to inhibition of proliferation.

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1. Introduction

There are approximately 30,000 chemicals in wide commercial use in the U.S., Canada, and Europe (Judson et al., 2009). However, relatively few of these chemicals have sufficient data to define their potential toxicity to humans. In particular, less than 10% have been evaluated for neurotoxicity or developmental neurotoxicity (Landrigan, 1994), and there is increasing concern that environmental exposure to chemicals may play a role in a broad spectrum of learning and neurodevelopmental disorders including autism and attention deficit and hyperactivity disorder (Grandjean and Landrigan, 2006). Current approaches to toxicity testing (including developmental neurotoxicity testing) rely heavily on the use of animals, can cost millions of dollars and can take years to complete

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for a single chemical. In light of these considerations, new screening assays are needed that can rapidly and efficiently identify chemicals of potential concern (*hazard identification*) and provide information on mechanisms and pathways that mediate toxicity (Houck and Kavlock, 2008; NRC, 2007). This approach would target further testing to the most relevant chemicals and endpoints. For developmental neurotoxicity testing, in vitro cell cultures may be useful as model systems for chemical screening. While in vitro systems can not fully replicate the complex interactions in the developing brain, neuronal cultures can recapitulate many key neurodevelopmental processes such as cell proliferation, differentiation, growth, and synaptogenesis (Lein et al., 2005). A battery of in vitro, cell-based assays for these neurodevelopmental processes have been proposed as one approach for the development of high-throughput methods for screening chemicals for their potential to produce developmental neurotoxicity (Coecke et al., 2007; Radio and Mundy, 2008).

Neurogenesis and neuronal proliferation are dependent upon the orderly division of neural progenitor cells that ultimately supply the full complement of cells in the nervous system (Zhong and Chia, 2008). Alterations in neuronal proliferation during development can arise from genetic and environmental causes, and chemical-induced changes in proliferation have been shown to result in neurotoxicity and developmental disorders (reviewed in Barone et al., 2000; Acosta et al., 2002). Thus, cell proliferation is a potential target for chemicals that could affect the developing nervous system and should be incorporated as a component of an in vitro screening battery.

Several studies have shown that proliferation is a sensitive endpoint for detecting the neurotoxic actions of chemicals in vitro (Costa et al., 2007; Jacobs and Miller, 2001). Neuronal proliferation is controlled by multiple factors that regulate the cell cycle (Ohnuma and Harris, 2003; Ulloa and Brisco, 2007) and represent potential sites of action for chemicals. Because the mechanism(s) of action for chemical effects on proliferation are in many cases unknown, a cell-based approach that can detect effects on proliferation regardless of the site of action would be suitable for chemical screening. In a previous study, we developed a high-throughput, in vitro assay for cell proliferation based on the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into replicating DNA during S phase (Breier et al., 2008). Using a high content screening platform which integrates fluorescent microscopy with automated image acquisition and analysis, we demonstrated the rapid quantification of concentration-dependent effects of chemicals on proliferation in a neural progenitor cell line. The use of a concurrent assay for viability allowed for the discrimination between selective chemical effects on proliferation and non-specific effects on cell health. Evaluation of a small set of chemicals known to affect proliferation was used to make an initial evaluation of the sensitivity of this progenitor cell line (Breier et al., 2008).

Before screening large numbers of chemicals, a sensitive and reproducible cell model should be selected. The purpose of the present study was to compare directly several neuronal cell lines for their ability to detect chemical-induced changes in proliferation. There are a number of desirable attributes for models used in screening of neurodevelopmental processes. These include a model that recapitulates the process in vitro, is widely available, and provides reproducible data between laboratories. As primary neuronal cultures prepared from fresh nervous system tissue are for the most part post-mitotic and can vary substantially from culture to culture, neuronal cell lines that are tumor-derived or virally transformed have been extensively used in studies of proliferation and differentiation (De Laat and Van der Saag, 1982; Shastry et al., 2001). Both human and rodent neuronal cell lines are widely available, can provide a uniform population of cells, and maintain cell division in culture. Thus, the current study compared tumor-derived cell lines from three different species that have been used previously as models of neuronal proliferation: PC12 (rat pheochromocytoma; Greene and Tischler, 1982), N1E-115 (mouse neuroblastoma; Kimhi et al., 1976), and SH-SY5Y (human neuroblastoma; Påhlman et al., 1990). To examine their sensitivity, the cell lines were grown under proliferating conditions in 96-well plates and exposed to a set of chemicals over a wide concentration range. The set included chemicals known to affect proliferation in vitro, and chemicals without known affects on proliferation. Cell proliferation was measured using a format amenable to high-throughput screening based on automated imaging of BrdU incorporation as described previously (Breier et al., 2008). Cellular ATP level was examined under identical conditions in order to discriminate between selective effects of chemicals on the process of proliferation and general effects on cell health and viability.

2. Materials and methods

2.1. Cell cultures

Cells were cultured under conditions that were optimal for proliferation of the particular cell line used. A subclone of PC12 cells, Neuroscreen-1 (NS-1), was obtained from Thermo Fisher Scientific (Pittsburgh, PA). We have found that upon plating this PC12 cell clone shows minimal clumping and responds well to NGF (Radio et al., 2008). Cells were cultured in RPMI media (BioWhittaker, Walkersville, MD) containing 10% equine serum (HyClone, Logan, UT), 5% heat-inactivated fetal bovine serum (HyClone), 1% L-glutamate (BioWhittaker) and 1% penicillin/streptomycin (BioWhittaker). N1E-115 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and cultured in DMEM (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). SH-SY5Y cells were obtained from the ATCC and cultured in DMEM/F12 (GIBCO), 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Bio Whittaker). The genotype and phenotype of cell lines can vary over multiple passages (Heumann et al., 1977). Thus, for each experiment cells from the same passage number (passage 8 after receipt of the initial vial) were thawed and expanded in 75 cm^2 flasks (Becton Dickinson coated with Collagen I for NS1 cells or Corning with no substrate for SH-SY5Y and N1E 115) using the appropriate media until they reached approximately 80% confluence. Cells were then subcultured on clear 96-well plates (Costar, Corning NY,) for use in studies of proliferation or on opaque 96-well plates (Perkin Elmer, Shelton, CT) for viability. Cells were maintained at 37 °C in a humidified incubator under a 95% air/5% CO_2 atmosphere.

2.2. Doubling time

Cells were subcultured in clear 96-well plates at initial densities (2000 cells/well for PC12 and N1E-115 cells, and 10,000 cells/well for SH-SY5Y cells) that were optimized to provide sufficient cell numbers over the 96 h assessment period. Cell counts were performed every 24 h after plating by staining nuclei with the DNA-binding dye Hoechst 33342. Briefly, 3 μM Hoechst 33342 (Calbiochem, La Jolla, CA) was added directly to the media in each well and incubated at room temperature for 20 min. Cells were imaged using Cellomics ArrayScan V^{TI} HCS system (Thermo Fisher Scientific, Pittsburgh, PA). The system uses an automated inverted epifluorescence microscope to focus and record images from multiple fields in each individual well. Fluorescence images of the stained nuclei were obtained using an excitation filter of 365 ± 25 nm and an emission filter of 535 ± 20 nm with a 10× objective. The number of nuclei per field was determined using the Cellomics Target Activation Bioapplication. This software identifies objects (nuclei) based on size, shape, and fluorescence intensity. In order to assure that only nuclei from intact (live) cells were counted, objects that did not match preset criteria for intact cell nuclei (based on preliminary studies of control cells in each cell type) were not counted and excluded from analysis. This typically excludes fluorescent artifacts such as dust, cellular debris, and nuclei from dead or dying apoptotic cells. In each well a sufficient number of fields were imaged in order to count at least 400 intact cells.

2.3. Chemical treatment

A set of 16 commercially available chemicals was used to assess the sensitivity of the cell lines to alterations in proliferation (Table 1). Eight chemicals were selected based on prior evidence obtained from peer-reviewed, published literature of statistically significant effects on neuronal proliferation in vitro in any model. A second set of eight chemicals was selected due to the absence of evidence for effects on proliferation in the published literature. Chemicals were dissolved in either 100% DMSO (Sigma–Aldrich, St. Louis, MO) or double distilled H_2O based on solubility (Table 1). Stock solutions were prepared at a concentration range of 1 μM to 100 mM, except for aphidicolin (highest concentration 10 mM), glyphosate (highest concentration 30 mM), ochratoxin-A (highest concentration 10 mM), and