Assessment of Chemical Effects on Neurite Outgrowth in PC12 cells Using High Content Screening

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Identification of chemicals that pose a hazard to the developing nervous system is the first step in reducing human exposure and preventing health risks to infants and children. In response to the need for more efficient methods to identify potential developmental neurotoxicants, the present study evaluated the utility of an automated high content screening system to detect chemical effects on neurite outgrowth in Neuroscreen-1 cells (NS-1), a subclone of PC12 cells. Plating 2000 NS-1 cells per well with 100 ng/ml nerve growth factor for 96 h produced optimal neurite growth in a 96-well format. Using this protocol, five chemicals that had been previously shown to inhibit neurite outgrowth in PC12 cells were examined. Inhibition of neurite outgrowth (assessed as total neurite length per cell) was observed for all five chemicals. For three of the chemicals, inhibition was associated with decreased cell viability. To demonstrate the utility of this approach for screening, a further set of chemicals (eight known in vivo developmental neurotoxicants and eight chemicals with little evidence of in vivo neurotoxicity) were tested over a wide concentration range (1nM–100μM). Retinoic acid, dexamethasone, cadmium, and methylmercury inhibited neurite outgrowth, although dexamethasone and cadmium only affected neurite outgrowth at concentrations that decreased viability. Amphetamine facilitated neurite outgrowth, whereas valproic acid, diphenylhydantoin, and lead had no effect. Of the chemicals that were not neurotoxic, there were no effects on cell viability, but two (dimethyl phthalate and omeprazole) increased neurite outgrowth at the highest concentration tested. These results demonstrate that a high content screening system can rapidly quantify chemical effects on neurite outgrowth in vitro. Concentration-response data for both neurite outgrowth and cell viability allowed for the determination of the specificity of chemical effects on a neurodevelopmental endpoint. Further studies will examine the utility of other in vitro preparations for cell-based assays of neurite outgrowth.

Key Words: alternatives to animal testing; cell culture; neurotoxicity.

A major challenge in characterizing the potential developmental neurotoxic risk of environmental chemicals is the paucity of available hazard data. There are thousands of chemicals in commerce, but relatively few have been adequately characterized for their potential effects on human health. For example, of the 3000 high production volume chemicals (chemicals produced or imported into the United States at or above 1 million pounds per year), nearly half have no basic toxicity data available (U.S. EPA, 1998a). Only 7% have a complete set of toxicity data, including developmental toxicity. In the absence of data, the risk of developmental neurotoxicity for these chemicals is unknown, but it is estimated to be high (Grandjean and Landrigan, 2006; OECD, 2007). Accordingly, there is increased public concern that exposure to chemicals in the environment may be partially responsible for the increased number of cases of neurological disorders in children, including autism and attention deficit hyperactivity disorder (Colborn, 2004; May, 2000; Rauh et al., 2006).

In vivo animal studies provide useful data for hazard assessment, but the extensive resources and time needed to complete single chemical evaluation do not make these studies amenable to hazard identification in the face of thousands of untested chemicals. For developmental neurotoxicity testing, the U.S. EPA guidelines specify in utero and lactational chemical exposure with subsequent morphological, behavioral, and biochemical analysis in the offspring (U.S. EPA, 1998b). This protocol requires hundreds of animals and 1–2 years to complete and is not used as a first tier screen. In its recent report on toxicity testing in the 21st century, the National Research Council of the National Academy of Sciences proposes a shift away from chemical testing in animals and toward in vitro approaches that can reduce the cost and time of testing (NRC, 2007). Rapid and cost-efficient approaches for evaluating developmental neurotoxicity, including the use of in vitro neural cell cultures and nonmammalian species, have been proposed (Coecke et al., 2007; Lein et al., 2007). Although routinely used to investigate mechanisms underlying developmental neurotoxicity, in vitro systems would also facilitate chemical screening and initial hazard identification. The
resulting data could be used to prioritize chemicals for further in vitro and in vivo testing (Coecke et al., 2007; Costa et al., 2007).

Development of the nervous system involves the coordinated expression of specific cellular events including proliferation, differentiation, migration, neurite outgrowth, synaptogenesis, myelination, and programmed cell death (Cowan et al., 1997; Sanes et al., 2005). Chemical-mediated disruption of one or more of these processes can potentially impair nervous system development (Barone et al., 2000). The use of neural cell cultures for in vitro screening assays is based on the premise that these fundamental processes underlying nervous system development can be examined at the cellular level. Many of these neurodevelopmental processes can be observed in neural cells in culture, and a variety of in vitro preparations derived from nervous system tissue have been employed to study specific aspects on neuronal development (Boulton et al., 1999; Cestelli et al., 1992; Federoff and Richardson, 1997). Thus, it has been suggested that a battery of tests based on in vitro assessment of these discrete neurodevelopmental endpoints may be useful as a screen to identify potential developmental neurotoxicants (Lein et al., 2005).

One aspect of neurodevelopment that has been studied extensively in vitro is neurite outgrowth. The growth of axonal and dendritic processes (collectively called neurites) during brain development is a critical determinant of neuronal connectivity, and disruption of this process can lead to cognitive deficits (Berger-Sweeney and Hohmann, 1997; Ramakers, 2002; Webb et al., 2001). There are a number of in vitro models that are well characterized and have been used to examine chemical effects on neurite outgrowth (Radio and Mundy, 2008). PC12 cells are a cell line that have been widely used in neurobiological investigations (Fujita et al., 1989; Vaudry et al., 2002) and to evaluate chemical effects on neurite outgrowth (Radio and Mundy, 2008). Following exposure to nerve growth factor (NGF), PC12 cells differentiate into a sympathetic-like neuron and develop extensive neuritic outgrowth (Radio and Mundy, 2008). Many studies have used PC12 cells to evaluate the effect of environmental chemicals on neurite outgrowth (Chan and Quik, 1993; Crumpton et al., 2000; Das and Barone, 1999; Lein et al., 2000; Parran et al., 2001). Although these studies demonstrate the ability of an in vitro model to detect changes in this endpoint, in most cases neurite outgrowth was assessed on a single chemical using manual or semiautomated methods to acquire microscopic images and quantify neurite development. For chemical screening, fully automated techniques amenable to high-throughput analysis are needed in order to facilitate the rapid assessment of large numbers of chemicals for their effects on neurite outgrowth.

Recent advances in automated microscopy have led to a cell-based screening approach called high content screening. High content screening integrates quantitative fluorescent microscopy with automated technology for image acquisition, image analysis, and data collection (Abraham et al., 2004; Smith and Eisenstein, 2005). High content screening platforms are designed to track phenotypic changes of individual cells in a multiwell format using separate fluorescent labels. The data are considered “high content” because multiple parameters are derived from measurements of hundreds of individual cells within each image. This approach has been widely adopted by the pharmaceutical industry for the drug discovery and safety pharmacology phases of drug development. High content screening systems are available for the assessment of neuronal morphology, including neurite outgrowth (Simpson et al., 2001). Images are analyzed using algorithms to quantify several measures pertinent to neurite outgrowth including neurite number, length, and the extent of branching for each cell in the image. Using this technology, neurite outgrowth can be quantified in each well derived from hundreds of cell measurements in a 96-well microplate in approximately 30 min.

The goal of the present study was to evaluate the utility of an automated high content screening system, the Cellomics ArrayScan, to detect chemical effects on neurite outgrowth. A PC12 cell clone, NS-1, was used as the in vitro model. In comparison to the parent PC12 cell line, NS-1 cells are less prone to cellular aggregation, allowing for the evaluation of neurite outgrowth in individual cells (Ramer et al., 2003). Following optimization of the conditions for NGF-induced neurite outgrowth in NS-1 cells in a 96-well microplate format, the ability of the ArrayScan to detect chemical-induced changes in neurite outgrowth was evaluated using several compounds known to inhibit this process in PC12 cells. In order to demonstrate the utility of this approach for screening chemicals over a wide concentration range, a protocol for 96-well plates was used to examine the effects of a set of test compounds (including known developmental neurotoxicants and chemicals considered not to be neurotoxic) on neurite outgrowth.

MATERIALS AND METHODS

Cell culture. Neuroscreen-1 cells (NS-1), a PC12 subclone, were obtained from Thermo Fisher Scientific (Pittsburgh, PA) and maintained at 37°C in a 95% humidified incubator containing 5% CO2. Cells were cultured in RPMI media (BioWhittaker, Walkersville, MD) containing 10% equine serum (HyClone, Logan, UT), 5% heat-inactivated fetal bovine serum (HyClone), 1% t-glutamine (BioWhittaker), and 1% penicillin/streptomycin (BioWhittaker). A single passage of NS-1 cells (passage 10 after receipt of initial vial) were exclusively used for neurite outgrowth and viability analysis to avoid potential interpassage phenotypic variability (Heumann et al., 1977). Cells were plated onto either transparent 96-well microplates precoated with collagen IV or opaque 96-well microplates precoated with collagen I (Becton, Dickinson and Company, Bedford, MA) for neurite outgrowth or cellular viability, respectively. Preliminary studies indicated that the form of collagen used did not influence neurite growth or viability. Cells were plated at a density of 2000 cells per well (6500 cells/cm2) unless stated otherwise. NGF (1–500 ng/mL, Sigma-Aldrich, St. Louis, MO) was included in the culture media at the time of plating to induce neuronal differentiation. Cells were cultured for 24–96 h prior to neurite outgrowth evaluation.

Chemicals. Compounds previously shown to inhibit neurite outgrowth in PC12 cells were used to evaluate this process in NS-1 cells (Table 1). A set of 16 commercially available, toxicologically diverse, test chemicals was chosen to demonstrate the utility of the high content analysis system for screening chemicals over a wide concentration range (Table 2). Eight chemicals were selected based on the availability of data demonstrating adverse effects on the
developing nervous system. For these eight developmental neurotoxicants, a literature review confirmed evidence of neurotoxicity after developmental exposure in mammals, including studies in experimental animals and in humans. A second set of eight chemicals was selected based on the presumed absence of data indicating effects on the developing nervous system and/or approval for their use during pregnancy. Using PubMed, each chemical was searched along with the following terms: neurotoxicity, developmental neurotoxicity, neurite, axon, and dendrite to find any peer-reviewed publications relevant to developmental neurotoxicity or the endpoint of neurite outgrowth in neuronal preparations. For one compound, diphenhydramine, a report of developmental neurotoxicity in rats was found (Chiavegetto et al., 1997); otherwise no relevant peer-reviewed publications for the second set of eight compounds were located in PubMed.

**Chemical exposure.** Chemicals were prepared in dimethyl sulfoxide (DMSO) (amoxicillin, d-sorbitol, saccharin, acetaminophen, dimethyl phthalate, diphenhydramine HCl, omeprazole, cadmium, methylmercury, diphenylhydantoin, trans-retinoic acid, valproic acid, dexamethasone, Bisindolemaleamide I [Bis-I], U0126, okadaic acid, K252a) or distilled H2O (lead, glyphosate, amphetamine, vincristine). The vehicle was selected on the basis of chemical solubility. Stock solutions were prepared at a concentration range of 1 μM–100 mM (except for lead, trans-retinoic acid, and glyphosate, for which the

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### TABLE 1

**Known Inhibitors of Neurite Outgrowth in PC-12 Cells**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Vehicle</th>
<th>Source</th>
<th>Purity</th>
<th>DNT in vivo&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Neurite outgrowth in vitro&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bis-I</td>
<td>DMSO</td>
<td>EMD</td>
<td>98%</td>
<td>NR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Das et al., 2004</td>
</tr>
<tr>
<td>K252a</td>
<td>DMSO</td>
<td>EMD</td>
<td>&gt; 95%</td>
<td>Kawamurs et al., 2007</td>
<td>Koizumi et al., 1988</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>DMSO</td>
<td>EMD</td>
<td>&gt; 95%</td>
<td>NR</td>
<td>Chiu and Westhead, 1992</td>
</tr>
<tr>
<td>U0126</td>
<td>DMSO</td>
<td>Promega</td>
<td>NA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NR</td>
<td>Das et al., 2004</td>
</tr>
<tr>
<td>Vincristine</td>
<td>H2O</td>
<td>Sigma</td>
<td>98%</td>
<td></td>
<td>Geldoff et al., 1998</td>
</tr>
</tbody>
</table>

<sup>Note.</sup> DNT, developmental neurotoxicity.
<sup>a</sup>Reference for developmental neurotoxicity of compound in vivo.
<sup>b</sup>Reference for inhibition of neurite outgrowth in vitro in PC12 cells.
<sup>c</sup>Not reported in literature.
<sup>d</sup>Not available.

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### TABLE 2

**Test Compounds Evaluated for Effects on Neurite Outgrowth and Viability in NS-1 Cells**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Vehicle</th>
<th>Source</th>
<th>Purity</th>
<th>DNT in vivo&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Neurite outgrowth in vitro&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-Amphetamine</td>
<td>H2O</td>
<td>Sigma</td>
<td>NA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Golub et al., 2005</td>
<td>Park et al., 2002</td>
</tr>
<tr>
<td>Cadmium chloride</td>
<td>DMSO</td>
<td>Aldrich</td>
<td>98%</td>
<td>Desi et al., 1998</td>
<td>NR&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>DMSO</td>
<td>Sigma</td>
<td>&gt; 97%</td>
<td>Baud, 2004</td>
<td>Jap Tjoen San et al., 1992</td>
</tr>
<tr>
<td>5,5-Diphenylhydantoin</td>
<td>DMSO</td>
<td>Sigma</td>
<td>99%</td>
<td>Adams et al., 1990</td>
<td>NR</td>
</tr>
<tr>
<td>Lead acetate</td>
<td>H2O</td>
<td>Aldrich</td>
<td>98%</td>
<td>Davis et al., 1990</td>
<td>Crumpton et al., 2000</td>
</tr>
<tr>
<td>Methylmercury chloride</td>
<td>DMSO</td>
<td>Aldrich</td>
<td>93%</td>
<td>Burbachet et al., 1990</td>
<td>Parran et al., 2001</td>
</tr>
<tr>
<td>trans-Retinoic acid</td>
<td>DMSO</td>
<td>Sigma</td>
<td>&gt; 98%</td>
<td>Adams and Lammer, 1991</td>
<td>Simpson et al., 2001</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>DMSO</td>
<td>Alfa Aesar</td>
<td>&gt; 98%</td>
<td>Iqbal et al., 2001</td>
<td>van Bergeijk et al., 2006</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>DMSO</td>
<td>Sigma</td>
<td>&gt; 99%</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>DMSO</td>
<td>Sigma</td>
<td>NA</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Dimethyl phthalate</td>
<td>DMSO</td>
<td>Aldrich</td>
<td>&gt; 99%</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td>DMSO</td>
<td>Sigma</td>
<td>&gt; 98%</td>
<td>Moraes et al., 2004</td>
<td>NR</td>
</tr>
<tr>
<td>Hydrochloride</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glyphosate</td>
<td>H2O</td>
<td>Chem Service</td>
<td>99%</td>
<td>Axelrad et al., 2003&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Omeprazole</td>
<td>DMSO</td>
<td>Sigma</td>
<td>98%</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Saccharin sodium salt</td>
<td>DMSO</td>
<td>Sigma</td>
<td>&gt; 99%</td>
<td>NR</td>
<td>Ishi, 1982&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hydrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Sorbitol</td>
<td>DMSO</td>
<td>Sigma</td>
<td>&gt; 98%</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

<sup>Note.</sup> DNT, developmental neurotoxicity.
<sup>a</sup>Reference for developmental neurotoxicity of compound in vivo.
<sup>b</sup>Reference for effect of compound on neurite outgrowth in vitro.
<sup>c</sup>Not available.
<sup>d</sup>Not reported in literature.
<sup>e</sup>Inhibition at concentrations > 1 mM.
<sup>f</sup>Inhibition at concentrations > 10 mM.
highest concentration was 30mM) and stored at the temperature recommended by the manufacturer. On the day of use, chemicals were diluted 1/1000 into the culture media containing 100 ng/ml NGF to yield a final chemical concentration range of 1nM–30 (or 100) μM. Except where noted, the final DMSO concentration in the culture media was 0.1%. Chemicals were added to the cells 2 h after plating to ensure the cells adhered to the collagen substrate.

Neurite outgrowth evaluation. Immunocytochemical evaluation of neurite outgrowth was performed using the supplies and procedures provided in the Neurite Outgrowth Hitkit (Thermo Fisher Scientific). At the stated time following chemical exposure, cells in transparent collagen IV–coated plates were fixed for 20 min using 4% paraformaldehyde in PBS at 37°C. Cell bodies and processes were labeled using an anti-βIII tubulin primary antibody, followed by an Alexa Fluor 488–conjugated secondary antibody. Hoechst 33258 dye was included in the fixative to label cell nuclei. Plates were then loaded into a Cellomics ArrayScan VTI high content imaging platform for automated image capture and analysis. The system is based on an inverted epifluorescence microscope that automatically focuses and scans fields in individual wells using a motorized stage. Fluorescence images were produced using a multiple bandpass emission filter and matched excitation filters for the blue channel (nuclei) and green channel (cell body and processes) and acquired with a high-resolution charge-coupled device camera. Objects were identified as cells if they had valid nuclei and cell bodies measurements based on size, shape, and fluorescence intensity. Acceptable ranges for these parameters were determined in preliminary studies to ensure that aggregated cells and noncellular particles were excluded from analysis. The acquired images were analyzed by the ArrayScan software, using the Neuronal Profiling bioapplication (Thermo Fisher Scientific) to measure a number of morphological parameters including total neurite length per cell, neurites per cell, branch points and cell body area for each valid cell in the image. A cell was defined as differentiated when it had a total neurite length > 20 μm (twice the total neurite length in cells in the absence of NGF). Using a 10× objective, a sufficient number of fields were acquired for the analysis of at least 200 cells per well.

Viability. Cell viability was quantified in cells grown in opaque collagen I–coated 96-well microplates using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). This assay estimates the number of viable cells in culture based on the quantification of ATP present, which is an indication of metabolically active cells. On the day of assay, cells were lysed and ATP determined by the addition of 100 μl of the reagent directly to the media in each well. ATP was measured through the luciferase-catalyzed reaction of ATP with luciferin to generate a luminescent signal proportional to the amount of ATP present. Luminescence in each well was measured thirty minutes after reagent addition using a FLUOstar Optima plate reader (BMG LABTECH, Durham, NC). Preliminary experiments verified that luminescent signal for ATP content was proportional to the number of live cells in a well ($r^2 = 0.996$).

Statistics. Effects on neurite outgrowth and/or cellular viability were determined by using a one-way ANOVA, with the level of significance set at $p < 0.05$. For studies examining the effect of time of NGF exposure and cell density, post hoc comparisons between all groups were performed using the Student-Newman-Keuls test. For studies examining NGF and test chemical concentration-response curves, Dunnett’s post hoc test was used to identify which chemical concentrations were significantly different from vehicle control. Each well was considered as an $N$ of 1 and experiments were repeated in separate plates on at least two different culture days.

RESULTS

Morphology of NS-1 Cells following NGF-Stimulated Differentiation

Initial studies qualitatively assessed the morphology of the PC12 cell subclone, NS-1. Morphological changes of NS-1 cells following 96-h incubation in either basal medium or 100 ng/ml NGF are shown in Figure 1. Cells cultured in media alone remained in an undifferentiated state, and exhibited short processes that were less than one diameter of the cell soma (Fig. 1A). Conversely, cells incubated in media containing NGF exhibited extensive neurite outgrowth, and resembled the parent PC12 cell line after differentiation (Fig. 1B). For quantitative assessment of neurite outgrowth using automated image acquisition and analysis, NS-1 cells were immunostained using the Hoechst 33258 dye and anti-βIII tubulin primary antibody and Alexa 488–conjugated secondary antibody (C). The resulting image is analyzed using Cellomics Neuronal Profiling software for morphological endpoints including cell body area, neurite number, neurite length, and branch points for each identified cell (D).
following a 96-h incubation period. NGF was added to the media at the time of plating, with no further addition over time. As observed previously in PC12 cells (Shaughnessy and Barone, 1997), exposure to NGF produced a concentration-dependent increase in neurite outgrowth as assessed by total neurite length (Fig. 2A). An increase in neurite outgrowth was observed at 1 ng/ml NGF, with a maximal effect observed at 10 ng/ml. No further increases were noted at concentrations up to 500 ng/ml. A similar concentration-response to NGF was observed for other endpoints (number of neurites, percentage of differentiated cells, and cell body area) indicative of PC12 cell differentiation (Figs. 2B–D). Based on these results, a saturating concentration of 100 ng/ml NGF was selected to induce neurite outgrowth in subsequent experiments. Results are presented for total neurite length because this parameter had the greatest dynamic range and concentration-response curves were similar for all endpoints.

Time course studies were performed to evaluate the effect of NGF exposure duration on neurite outgrowth. As described above, neurite outgrowth is dependent upon addition of NGF to the media (Fig. 2E). In the absence of NGF (undifferentiated cells), total neurite length was approximately 10 μm and did not change over time. Addition of 100 ng/ml NGF at the time of plating induced a time-dependent increase in neurite outgrowth that continued up to 96 h.
The influence of plating density on neurite outgrowth was also examined (Fig. 2F). Cells were plated at 1000, 1500, and 2000 cells per well (approximately 3250–6500 cells/cm²) and exposed to 100 ng/ml NGF for 96 h. Neurite outgrowth increased with increasing cell density. Plating cells at densities greater than 2000 cells per well resulted in overly confluent cells at 96 h that were not optimal for automated analysis of neurite outgrowth (data not shown).

Evaluating Vehicle and Edge Effects on Neurite Outgrowth

A concentration curve was performed to evaluate potential effects of the DMSO solvent vehicle on NGF-induced neurite outgrowth (Fig. 3A). Concentrations of DMSO between 0.1 and 0.5% (vol/vol) did not affect total neurite length after 96 h of exposure. A concentration of 1.0% DMSO significantly reduced total neurite length without affecting viability (data not shown). A final DMSO concentration of 0.1% was used as the vehicle in all subsequent studies.

To determine whether total neurite length was influenced by the location of wells within the 96-well plate, NGF-induced neurite outgrowth was compared between cells plated along exterior and interior of a 96-well plate. No significant difference in total neurite length was observed following a 96-h exposure to NGF (Fig. 3B). There was also no significant difference in a comparison of row and column values (data not shown). Neurite length measurements were reproducible between separate cultures (mean total neurite length = 63.3 μm with a standard deviation of 11.4 μm, N = 22 plates over 6 months).

Effect of Neurite Outgrowth Inhibitors on Neurite Outgrowth and Cell Viability

The ability of the ArrayScan to detect chemical-induced changes in neurite outgrowth was evaluated using several compounds previously shown to inhibit neurite outgrowth. The protein kinase C inhibitor Bis-I (2.5–50 μM; Das et al., 2004), MAP kinase inhibitor U0126 (10–30 μM; Das et al., 2004), phosphatase inhibitor okadaic acid (5–10 nM; Chiou and Westhead, 1992), microtubule-depolymerizing agent vincristine (0.55–11.0 nM; Geldof et al., 1998), and the tyrosine kinase inhibitor K252a (100–300 nM; Koizumi et al., 1988) can significantly reduce neurite outgrowth in PC12 cells. NS-1 cells were exposed to the effective concentration range of these chemicals 2 h after plating, and total neurite length evaluated 96 h later. Parallel plates were exposed to the same concentrations and evaluated for viability. The effect of the PC12 neurite outgrowth inhibitors on neurite outgrowth and viability in NS-1 cells is shown in Figure 4. All five compounds decreased neurite outgrowth. The effects of Bis-I and K252a were relatively selective in that there were several concentrations that decreased neurite outgrowth in the absence of changes in cell viability. Exposure to Bis-I caused a significant concentration-dependent decrease in neurite outgrowth at 0.1, 0.3, 1, 3, and 10 μM; viability was decreased only at 10 μM (Fig. 4A). For K252a, decreases in neurite outgrowth occurred in the absence of a change in cell viability at 10 and 30 μM. Higher concentrations of K252a also resulted in reduced cell viability (Fig. 4E). Although U0126, vincristine, and okadaic acid all decreased neurite outgrowth, this effect was accompanied by decreased cell viability at 3, 10, and 30 μM, accompanied by significant reductions in cell viability for most of the concentrations tested (Figs. 4B–D).

Screening for Chemical Effects on Neurite Outgrowth and Cell Viability

In order to demonstrate the utility of the ArrayScan high content screening system as an efficient screening tool, a protocol was developed to examine the effects of a wide range of chemical concentrations on neurite outgrowth and viability. Eight different chemicals were analyzed per plate at a concentration range of 1 nM–100 μM. Internal plate controls were included for undifferentiated cells in the absence of NGF, control neurite outgrowth induced by 100 ng/ml NGF, and chemical inhibition of NGF-induced neurite outgrowth 3 μM.
Bis-I. All wells contained 0.1% final DMSO. NS-1 cells were exposed to a set of eight chemicals with minimal data indicating neurotoxicity and eight chemicals known to induce developmental neurotoxicity \textit{in vivo}. The effects of these chemicals on neurite outgrowth and cell viability are shown in Figures 5 and 6. There was no decrease in neurite outgrowth or viability observed for any of the eight chemicals regarded as not neurotoxic (Fig. 5). However, for two of these chemicals, dimethyl phthalate (Fig. 5E) and omeprazole (Fig. 5H), a significant increase in neurite outgrowth was observed at the highest concentration tested (100 μM).

Several patterns of effect were noted for the chemicals known to induce developmental neurotoxicity \textit{in vivo} (Fig. 6). Diphenylhydantoin, valproic acid, and lead had no effect on neurite outgrowth or cell viability. Amphetamine significantly increased neurite outgrowth at a high concentration in the absence of effects on viability. Dexamethasone and cadmium significantly inhibited neurite outgrowth and decreased viability at similar concentrations. \textit{Trans}-retinoic acid and methylmercury caused significant reductions in total neurite length at concentrations that did not affect viability. For \textit{trans}-retinoic acid, the first significant decrease in neurite outgrowth was observed at 30 nM, with no effect on viability at any concentration tested. For methylmercury, the first significant decrease in neurite outgrowth was observed at 1 nM, with cell viability unaffected until 1 μM (1000-fold higher).

**FIG. 4.** Effects of neurite outgrowth inhibitors on NS-1 cells. NS-1 cells were plated at 2000 cells per well in 100 ng/ml NGF and evaluated for either total neurite length (closed circle) or viability (open square) following 96 h exposure to Bis-I (A), U0126 (B), vincristine (C), okadaic acid (D), or K252a (E). Figure A insert shows representative images of either control (0.1% DMSO) or 3 μM Bis-I treated cells following the 96-h exposure. Data are expressed as percent of the 0.1% DMSO control, and are presented as means ± standard deviation from six total wells analyzed across two independent experiments. Significantly different from control for total neurite length (*) or viability (x) (one-way ANOVA followed by Dunnett’s test, \( p < 0.05 \)).

Effect of Time of Exposure on Neurite Outgrowth

To determine if a shorter exposure would affect the relationship between chemical-induced changes in neurite outgrowth and viability, NS-1 cells were exposed to cadmium (which decreased both endpoints at similar concentrations), and \textit{trans}-retinoic acid (which decreased neurite outgrowth only)
The reduced time of exposure paradigm did not reduce cytotoxicity for cadmium compared with the 96-h exposure (Fig. 7A). Moreover, exposure to trans-retinoic acid for 48 h did not produce the neurite outgrowth inhibition evident during the 96-h exposure (Fig. 7B). These results suggest that a 48-h chemical exposure may not be appropriate for evaluating a chemical’s ability to disturb neurite outgrowth.

FIG. 5. Effects of nontoxic test chemical exposure on neurite outgrowth and viability. NS-1 cells were plated at 2000 cells per well in 100 ng/ml NGF and evaluated for either total neurite length (open circle) or viability (closed square) following 96 h exposure to 1nM–100µM concentrations of the nontoxic chemicals amoxicillin (A), sorbitol (B), saccharin (C), acetaminophen (D), diphenhydramine (E), dimethyl phthalate (F), omeprazole (G), or glyphosate (H). Data are expressed as percent of the 0.1% DMSO control, and are presented as means ± standard deviation from six total wells analyzed across two independent experiments. Significantly different from control for total neurite length (*) or viability (x) (one-way ANOVA followed by Dunnett’s test, *p < 0.05).
DISCUSSION

The current study evaluated the utility of an automated image acquisition and analysis system to quantify chemical-induced changes in neuronal morphology using an *in vitro* model of neurite outgrowth. We found that NS-1 cells responded to NGF in a concentration- and time-dependent manner consistent with the parent PC12 cell line. Using NGF concentration (100 ng/ml) and time of exposure (96 h) values that produced robust neurite outgrowth, the effect of chemicals...
known to inhibit neurite outgrowth in PC12 cells was examined. A concentration-dependent inhibition of neurite outgrowth was detected for every neurite outgrowth inhibitor tested. In some cases inhibition of neurite outgrowth was accompanied by decreases in cell viability. To demonstrate the utility of this approach for screening, a set of chemicals (eight known in vivo developmental neurotoxicants and eight chemicals with little evidence of in vivo neurotoxicity) were tested over a wide concentration range. A protocol was developed that allowed for the testing of eight chemicals at 11 concentrations in one 96-well plate. The results showed several patterns of effects: (1) no alterations in neurite outgrowth or cell viability, (2) decreases in both neurite outgrowth and cell viability at similar concentrations, (3) a selective decrease in neurite outgrowth at concentrations that did not affect viability, and (4) a selective increase in neurite outgrowth. Taken together, these data demonstrated that an automated high content screening system can provide a rapid and sensitive method for the analysis of chemical effects on NS-1 neurite outgrowth, and that chemical effects on this neurodevelopmental endpoint could be separated from alterations in cell viability.

The NS-1 cell line was used in this study because it is less prone to aggregate than PC12 cells (Ramer et al., personal communication). This facilitated the quantification of neurite outgrowth from individual cells. Our data showed that NS-1 cells respond to NGF in a manner similar to PC12 cells. NGF treatment resulted in concentration-dependent increases in several measures of differentiation in the NS-1 cells including neurite length, neurites per cell, and cell body area. Total neurite length increased in a manner consistent with that observed in the parent PC12 cell line (Shaughnessy and Barone, 1997). Neurite outgrowth in NS-1 cells also increased over time similar to PC12 cells (Das and Barone, 1999; Das et al., 2004). These data indicated that the NS-1 cell subclone retain many of the properties of the parent PC12 cell line that make them a useful model for the study of differentiation and neurite growth.

The Cellomics ArrayScan high content screening technology quantified changes in neurite outgrowth in a high-throughput manner. Using this system, image acquisition and analysis of neuronal morphology required approximately 30 min per 96-well plate. Within each well, over 200 individual cells were analyzed. In addition to total neurite length, other relevant measures of neuronal differentiation, including number of neurites per cell, cell body area, and the extent of branching were obtained simultaneously. The data obtained using the automated screening technology was reproducible across wells, plates, and experiments. The reproducibility may be due to both the consistent use of a single passage of NS-1 cells and the large number of individual cells analyzed in each well. Thus, the well-based numbers, which constitute a single data point, consist of an average measurement of at least 200 cells. Investigating a similar number of cells using manual or semiautomated methods would clearly require greater resources in terms of time and personnel.

The ability of this screening system to detect changes in neurite outgrowth was evaluated using several chemicals previously shown to inhibit neurite outgrowth in PC12 cells. Using manual and semiautomated image acquisition and neurite tracing methods, we have previously demonstrated that Bis-I (2.5–5μM), U0126 (10–30μM), and K252a (30nM) can inhibit neurite outgrowth in PC12 cells (Das et al., 2004; Parran et al., 2003). The current results confirm this effect in the NS-1 cell line, and in addition, show that the high content screening system detected significant effects on neurite outgrowth at lower concentrations than previously reported. Furthermore, inhibition was also observed with vincristine and okadaic acid, which have been shown by others to inhibit neurite outgrowth in PC12 cells at 0.55 and 5.0nM, respectively (Chiou and Westhead, 1992; Geldof et al., 1998). Again, the high content screening system detected effects at the same or lower concentrations. The response of other measures of neurite outgrowth, as well as chemical effects on viability were also examined. Decreases in the number of neurites per cell and branch points were similar to that observed for total

FIG. 7. Effects of 48 h exposure to representative developmental neurotoxicant test chemicals on neurite outgrowth and viability. NS-1 cells were plated at 2000 cells per well in 100 ng/ml NGF and evaluated for either total neurite length (closed circle) or viability (open square) following 48 h exposure to 1nM–100μM concentrations of trans-retinoic acid (A), or cadmium (B). Data are expressed as percent of the 0.1% DMSO control, and are presented as means ± standard deviation from six total wells analyzed across two independent experiments. Significantly different from control for total neurite length (*) or viability (x) (one-way ANOVA followed by Dunnett’s test, p < 0.05).
neurite length (data not shown). Effects on cell viability were chemical-specific. Bis-I, U0126, and K252a inhibited neurite outgrowth in the absence of cytotoxicity. For okadaic acid and vincristine, inhibition of neurite outgrowth occurred at the same concentrations that reduced cell viability. Viability data was not reported in the previous studies on neurite outgrowth for these compounds. Based on these findings, we used Bis-I coadministered with 100 ng/ml NGF as an internal plate control to monitor chemical-induced inhibition of neurite outgrowth.

The use of the 96-well format allowed for chemical screening over a wide concentration range. This is useful because in most cases, the active concentrations of environmental compounds will be unknown. It also allows for a more complete assessment of biological activity compared with single concentration screening typical in the pharmaceutical industry (Xia et al., 2008). We demonstrated this approach using 16 compounds of diverse structure that included chemicals with reported developmental neurotoxicity in vivo and chemicals with little evidence of neurotoxicity. Four general patterns of effects were discerned from the data. First, a number of chemicals (including the majority of those lacking previous data for neurotoxicity) had no effect on neurite outgrowth or viability. A second set of chemicals (including cadmium and dexamethasone) inhibited neurite outgrowth, but only at concentrations that decreased cell viability. A third set of compounds (trans-retinoic acid, methylmercury) selectively inhibited neurite outgrowth at several concentrations in the absence of changes in viability. Finally, several chemicals (dimethyl phthalate, omeprazole, amphetamine) selectively increased neurite outgrowth. These patterns of effects may be useful for chemical prioritization. Chemicals with selective effects on neurite outgrowth (including inhibition and facilitation) could be considered as having a relatively high potential to result in developmental neurotoxicity in vivo. These chemicals would be designated for further testing. Likewise, chemicals with no effect on neurite outgrowth or cell viability could be considered as having a relatively low potential to result in developmental neurotoxicity in vivo, and would receive a low priority for further testing. Interpreting the data from chemicals for which effects on neurite outgrowth and cell viability occurred at similar concentrations is more problematic. The screening data alone cannot discern between chemicals that alter neurite outgrowth as a result of a general impairment of cell health, and chemicals acting via multiple mechanisms to affect both neurite outgrowth and cell viability at overlapping concentrations. Thus, chemicals which affect both specific neurodevelopmental endpoints and more general measures of cell health should not be dismissed. Further research is warranted to define the relationship between chemical-induced alterations in neurite outgrowth (and other neural-specific endpoints) and cytotoxicity. One approach that has been proposed is to include a non-neuronal cell as part of the screening battery (Gartlon et al., 2006).

In general, the present results are consistent with previous reports of effects of these chemicals on neurite outgrowth where available (see Table 2). Methylmercury, trans-retinoic acid, and dexamethasone inhibited, whereas amphetamine facilitated, neurite outgrowth in agreement with reported findings. In contrast, lead and valproic acid have been shown to facilitate neurite outgrowth in PC12 cells, which was not observed in the present study. Differences in exposure paradigms may explain the lack of effect observed for lead and valproic acid. For example, in Crompton et al. (2001) neurite outgrowth facilitation induced by lead was most evident in the absence of NGF as well as with 50 ng/ml NGF. The use of lower NGF concentrations may increase the sensitivity of the assay for detection of increased neurite outgrowth. Separate facilitation assays are being evaluated using submaximal NGF concentrations to characterize these effects.

Although the current studies were not designed to calculate a prediction rate, the high content screening system performed well in detecting changes in neurite outgrowth. Automated analysis detected five out of five of the known inhibitors of neurite outgrowth in PC12 cells (Table 1). Of the eight chemicals reported to result in developmental neurotoxicity in vivo, three had previous studies indicating inhibition of neurite outgrowth in vitro, three had previous studies indicating enhancement of neurite outgrowth in vitro, and two had no reported data (Table 2). The automated system detected three out of three that inhibited neurite outgrowth, and one out of three that enhanced neurite outgrowth. Thus, the overall detection of chemicals known to affect neurite outgrowth was 9 out of 11 (82%). For the chemicals with no reported evidence of developmental neurotoxicity (none of which had previous evidence of effects on neurite outgrowth at concentrations below 10mM), the automated system detected an increase in neurite outgrowth at the highest concentration tested for two out of eight (dimethyl phthalate and omeprazole). The lack of in vitro neurotoxicity data for these compounds makes it difficult to interpret these results, and highlights the difficulty in selecting “negative” compounds. However, there are in vivo studies for both dimethyl phthalate (Field et al., 1993) and omeprazole (Lalkin et al., 1998) concluding that these chemicals are not potent developmental toxicants. Thus, they could be considered as false positives in the present study.

The current study demonstrates the utility of high content screening technology to assess chemical effects on cell-based endpoints such as neurite outgrowth in a rapid and efficient manner. This approach can be successfully applied to other neurodevelopmental endpoints including proliferation, as shown in the work by Breier et al. (2008). In vitro models of neurodevelopmental endpoints could serve as part of a test battery for hazard identification and chemical prioritization. Confirmation of the neurotoxic potential of identified chemicals would require further assessment using in vivo protocols. In the development of in vitro systems for chemical screening of neurite outgrowth, other cell culture models should be
considered. Primary cells provide a model of outgrowth that closely corresponds to neurons in situ, but the need to continually prepare new cultures and the potential for variability make it unlikely that they will be widely used for screening. Neuronal cell lines are widely available and can provide a homogenous population of cells in large numbers. Thus, they are likely to be examined as a model for screening. The recent report on toxicity testing in the 21st century by the National Research Council of the National Academy of Sciences recommends the use of cell lines of human origin (NRC, 2007). The increasing availability of neural stem cells (including immortalized neural stem cell lines) of human origin will provide the opportunity to examine neurite outgrowth in cell that is almost identical to that found in the human nervous system.

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