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Screening for Chemical Effects on Neuronal Proliferation and Neurite Outgrowth Using High-Content Microscopy

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ABSTRACT

The need to develop screening methods for developmental neurotoxicity to reduce the cost, time, and animals required for in vivo toxicity studies is well recognized. The U.S. EPA ToxCast program aims to develop rapid and cost-effective toxicity testing approaches. Data collected from various high-throughput assays to contributes to computational models designed to forecast potential human toxicity. As part of this effort, we have screened a library of 320 chemicals (primarily pesticide compounds tested in vivo) using previously developed in vitro assays. Using high-content microscopy, chemical effects (40 µM; 24 hr) on ReNcell CX cell proliferation and viability were assessed by BrdU incorporation and propidium iodide exclusion, respectively. NS-1 cells, a PC12 subclone, were assessed for chemical-induced changes (40 µM; 96 hr) in neurite outgrowth (NOG) and viability by βIII-tubulin staining and ATP quantification assays, respectively. Effects on any endpoint were defined as changes beyond 3x the standard deviation of control means. 112 chemicals inhibited ReNcell CX cell proliferation, while 62 decreased viability; of these, 49 chemicals decreased both measures. NOG in NS-1 cells was enhanced by 4 chemicals, but inhibited by 29 chemicals. NS-1 cell viability was decreased by 43 chemicals; all chemicals that inhibited neurite outgrowth also inhibited viability. Viability in both cell types was decreased by 28 chemicals, and 20 chemicals produced effects on all endpoints. These results demonstrate that these cell models can be used to screen large numbers of chemicals for effects on proliferation, neurite outgrowth, and viability using a high-content platform, and will contribute to ongoing data collection in ToxCast for the creation of predictive models for human chemical toxicity. (This abstract does not represent EPA policy)

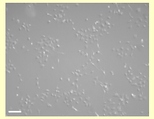
INTRODUCTION

The NAS report on toxicity testing in the 21st century envisions the use of in vitro, high-throughput screening (HTS) approaches based on toxicity pathways that will be predictive in nature. Proliferation of neuroprogenitor cells and outgrowth of neurites are critical processes in nervous system development and disruption of these processes may constitute pathways to neurotoxicity. As part of the ToxCast program, assays for these processes were conducted to evaluate their utility as HTS approaches using a set of chemicals for which there is significant in vivo toxicity data.

CHEMICALS

Phase I of the EPA ToxCast program employs a chemical library containing 320 compounds (ToxCast_320) with physicochemical properties that are compatible with cell-free and in vitro cellular assays (http://www.epa.gov/ncct/toxcast/). The availability of in vivo toxicity information for most of these chemicals provides data for correlating in vitro and in vivo endpoints. The criteria used for selection of the library were: 1) compounds have chronic, cancer, multi-generational reproductive and developmental assay data available; 2) chemicals must be soluble in DMSO (-1<logP<6); 3) chemicals must be in the molecular weight range 250-1000 amu; and 4) chemicals must be available with purity >90%. These criteria were satisfied by a diverse set of pesticidal active chemicals, which have had in vivo toxicology studies conducted according to regulatory guidelines as part of their registration process with the EPA. Several other chemicals of environmental interest and meeting these criteria were also included. In order to assure quality control, chemical names were blinded and included three sets of triplicate and five sets of duplicate samples. There are 309 unique chemical structures, yielding 320 chemical samples for testing when combined with the internal replicates.

CELL MODELS



ReNcell CX cells

- Immortalized neural progenitor cells derived from a sample of human cortex (14-week fetus)
- Express neuroprogenitor markers nestin and SOX2
- Proliferate in the presence of growth factors EGF and FGF-2
- Differentiate into neuronal, astrocytic, and oligodendrocytic cell populations with growth factor removal
- Commercially available

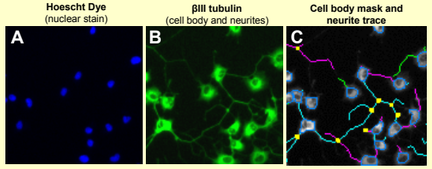
NS-1 Cells

- PC12 cell subclone
- Widely utilized for neurotoxicity and neurodevelopment studies
- Differentiate into neuronal phenotype upon treatment with nerve growth factor
- Signal transduction pathways associated with differentiation have been widely studied
- Have been utilized for assays of neurite outgrowth by several investigators
- Commercially available

METHODS

- All chemicals assessed at 40 µM in 0.1% DMSO
- Effects on Neurite outgrowth were assessed in NS-1 cells after 96 hr; and viability assessed at 96 hr using ATP content (CellTiter-Glo)
- Effects on cell proliferation (BrdU incorporation) and viability (exclusion of propidium iodide) were assessed in ReNcell CX cells after 24 hr exposure.
- Cellomics® ArrayScan V^{TI} high-content screening system used.
- Chemicals samples were provided by BioFocus DPI (South San Francisco, CA), under EPA contract EP-D-07-060. The chemical samples (20 mM stocks in DMSO) were provided in four 96-well plates and stored at -80°C prior to use.
- chemical structures available at http://www.epa.gov/ncct/dsstox/sdf_toxcst.html).

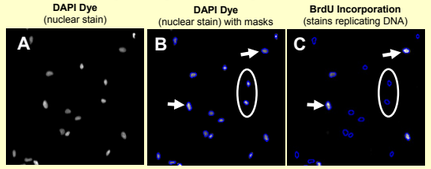
HIGH-CONTENT SCREENING FOR NEURITE OUTGROWTH



NS-1 cells were grown in 96-well plates and treated with NGF (100 ng/ml) for 96 hr. Images in two channels were automatically captured and analyzed using the Cellomics Neuronal Profiler software. A) Nuclei are labeled blue with Hoescht dye and used to identify valid cells (Channel 1). B) Cell bodies and neurites are labeled green with ICC for βIII tubulin (Channel 2). C) The image analysis algorithm identifies the cell body and measures a number of parameters (e.g. cell body size, neurite number, neurite length, branch points).

Viability was determined in sister cultures using the CellTiter-Glo assay to assess ATP levels in live cells.

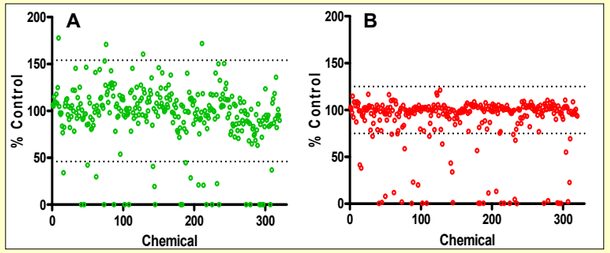
DETECTION OF BrdU INCORPORATION USING HIGH-CONTENT SCREENING



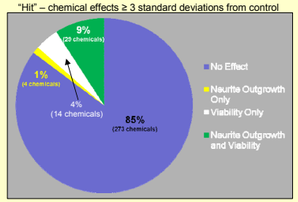
Proliferation of ReNcell CX cells was assessed by measuring BrdU incorporation using the Cellomics ArrayScan system. This series of photographs illustrates the method by which BrdU incorporation was detected. A) Gray-scale image of nuclei stained with DAPI dye (Channel 1). B) Objects were determined by computer algorithm and outlined with a blue mask (Channel 1). C) Nuclei positive for BrdU (arrows) were determined in Channel 2 based on objects detected in channel 1. The oval indicates objects that were negative for BrdU.

Viability was determined in a similar fashion using Propidium Iodide staining of dead cells

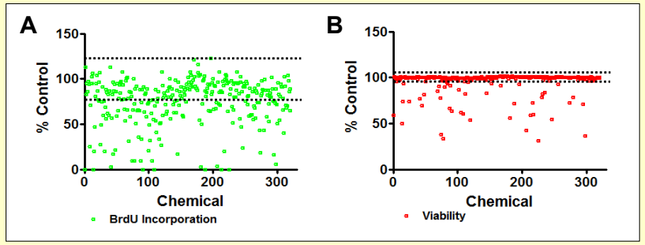
EFFECTS ON NEURITE OUTGROWTH AND VIABILITY



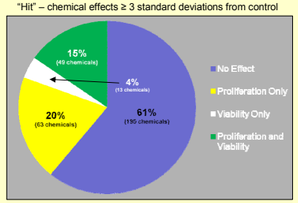
Effects of ToxCast_320 on Neurite Outgrowth and Viability in NS-1 cells. A) Effects of 40 µM of each chemical on neurite outgrowth (A) and viability (B) as a percent of vehicle (DMSO)-treated controls. On each graph, the dotted lines represent the upper and lower bounds of 3x the standard deviation (54% for neurite outgrowth; 25% for viability) for controls. The pie chart on the right illustrates the proportions of the ToxCast_320 that were considered "hits" (>3x s.d) in the assays.



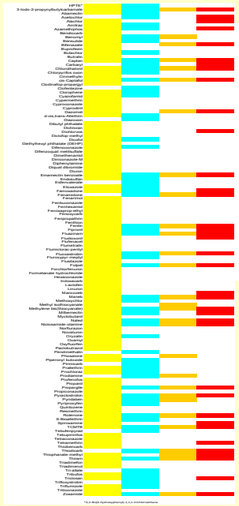
EFFECTS ON PROGENITOR CELL PROLIFERATION AND VIABILITY



Effects of ToxCast_320 on Proliferation and Viability in ReNcell CX cells. A) Effects of 40 µM of each chemical on proliferation (A) and viability (B) as a percent of vehicle (DMSO)-treated controls. On each graph, the dotted lines represent the upper and lower bounds of 3x the standard deviation (26% for proliferation; 1.7% for viability) for controls. The pie chart on the right illustrates the proportions of the ToxCast_320 that were considered "hits" (>3x s.d) in the assays.



SUMMARY OF HITS BY ASSAY



Proliferation Hits (ReNcell CX cells)
(112 hits)

Viability Hits (ReNcell CX cells)
(63 hits)

Neurite Outgrowth Hits (NS-1 cells)
(33 hits)

Viability Hits (NS-1 cells)
(43 hits)

The following chemicals were hits on all four endpoints:

3-Iodo-2-propynylbutylcarbamate	Maneb
Benomyl	Methylene bis(thiocyanate)
Captan	Naled
Chlorothalonil	Niclosamide-olamine
cis-Captafol	Propargite
Emamectin benzoate	Pyraclostrobin
Fentin	Rotenone
Fluazinam	TCMTB
Fluoxastrobin	Thiram
Folpet	Zoxamide

WITHIN-PLATE AND BETWEEN PLATE REPRODUCIBILITY IS HIGH FOR ALL ASSAYS

	Proliferation or Neurite Outgrowth	Viability
ReNcell CX cells	6/8	8/8
NS-1 Cells	8/8	8/8

Eight of the ToxCast_320 chemicals supplied by NCCT were repeated either within the same plate or between two different plates. Three compounds were repeated in triplicate, and five compounds in duplicate. The table indicates, for each assay, how many of times the result ("Hit" or not) was the same for the within or between well repeats of the eight chemicals.

SUMMARY / CONCLUSIONS

- These results demonstrate that high-throughput screens for neurite outgrowth and proliferation can be utilized to test large numbers of chemicals for effects on neurite outgrowth, proliferation and viability in neuronal cells.
- Measurement of proliferation and viability in human ReNcell CX cells detected "hits" for more chemicals than did neurite outgrowth and viability measurements in rat NS-1 cells.
- 20 chemicals were "hits" on all endpoints. This may reflect frank cytotoxicity of those compounds.
- There was a high degree of consistency of results from both within plate and between plate repeated chemicals for all endpoints.
- Neurite outgrowth was not as uniquely affected as proliferation was with regard to effects on viability.
- Concentration-response characterization of hits for all endpoints is underway.

†Currently with Bayer CropScience, RTP, NC; #Currently with Cellumen, Inc, Pittsburg, PA