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

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Research Triangle Park, NC
25 September 2008
In Vitro Screening for Developmental Neurotoxicity

- Central nervous system development is complex

- Research focus on processes of development rather than specific targets (e.g. proliferation, migration, neurite growth, synaptogenesis)

- Possible cell-based models:
  - Rodent models (primary cell culture, PC12 cells)
  - Human-derived models (primary neural cells, SH-SY5Y)
  - Embryonic stem cells

- Limitations
  - Need for fresh tissue
  - System of interest
  - Phenotypic/genotypic stability over multiple passages

- Goal is to develop in vitro models of human origin
  - Human neural progenitor cells
ReNcell CX Cells

- Immortalized neural progenitor cells derived from a 14-week sample of human cortex
- Express intermediate filament protein nestin
- Proliferate in the presence of growth factors EGF and FGF-2
- Differentiate into neuronal, astrocytic, and oligodendrocytic cell populations with growth factor removal

Donato et al., 2007
ReNcell CX Cells Are Neural Progenitor Cells

Breier et al., 2008
Cell Proliferation as a Screening Endpoint for Developmental Neurotoxicity

- Cell proliferation is a critical developmental process

- Proliferation is inhibited by chemicals for which evidence of developmental neurotoxicity exists
  - MeHg, Pb, EtOH

- Proliferation has been used as a screening endpoint

- Screening for effects on proliferation
  - BrdU incorporation is one of the most well-established methods
  - Amenable to high-throughput screening

- Cell viability was assessed to evaluate any overt toxicity associated with the chemicals of interest
  - Propidium iodide exclusion
High-Content Microscopy to Assess Cell Proliferation and Viability

*Cellomics ArrayScan VTI*:
- Fully automated image acquisition and analysis that is time-efficient
- High-content and high-throughput capacity
- Accompanying software (bioapplications) allows automated image analysis and provide data for individual cells

Potential to examine chemical effects on cell proliferation and viability using a 96-well format
Detection of BrdU Incorporation Using a High-Content Screening System

- 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 were determined in channel 2 based on objects detected in channel 1

Propidium iodide staining was evaluated using a similar approach.
Known Anti-Proliferative Compounds Inhibit ReNcell CX Cell Proliferation

Others tested: Aphidicolin, 5-fluorouracil, hydroxyurea
Protocol for Chemical Screening Using ReNcell CX cells

Cell expansion with EGF and FGF-2

Subcultured at 10,000 cells per well

Cells were exposed 16 hours later to chemicals from stock plate in the final concentration range of 1 nM – 100 μM

Chemicals dissolved in DMSO vehicle diluted in growth media

Proliferation (BrdU incorporation) or cell viability (propidium iodide exclusion) were determined 24 hours later
## Plate Layout for Chemical Screening Using ReNcell CX Cells

### 11 Chemical Concentrations (Molar)

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<th>9</th>
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<tbody>
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<td>-8</td>
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<td>-7</td>
<td>UNT</td>
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<td>-6</td>
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<td>-4.5</td>
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<tr>
<td>B</td>
<td>-9</td>
<td>-8.5</td>
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<td>-4.5</td>
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<tr>
<td>E</td>
<td>-9</td>
<td>-8.5</td>
<td>-8</td>
<td>-7.5</td>
<td>-7</td>
<td>DMSG</td>
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<td>-5.5</td>
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<td>-4.5</td>
<td>-4</td>
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<td>F</td>
<td>-9</td>
<td>-8.5</td>
<td>-8</td>
<td>-7.5</td>
<td>-7</td>
<td>DMSG</td>
<td>-6.5</td>
<td>-6</td>
<td>-5.5</td>
<td>-5</td>
<td>-4.5</td>
<td>-4</td>
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<tr>
<td>G</td>
<td>-9</td>
<td>-8.5</td>
<td>-8</td>
<td>-7.5</td>
<td>-7</td>
<td>-GFs</td>
<td>-6.5</td>
<td>-6</td>
<td>-5.5</td>
<td>-5</td>
<td>-4.5</td>
<td>-4</td>
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<td>-9</td>
<td>-8.5</td>
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<td>-GFs</td>
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<td>-6</td>
<td>-5.5</td>
<td>-5</td>
<td>-4.5</td>
<td>-4</td>
</tr>
</tbody>
</table>

### 8 Different Chemicals

- A
- B
- C
- D
- E
- F
- G
- H

- UNT: Untreated
- APH: APH
- DMSG: DMSG
- -GFs: -GFs
## Known Anti-Proliferative Compounds *In Vitro*

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Proliferation</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration Range:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>(1 nM – 100 μM)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Amphetamine Sulfate</td>
<td>.01 μM</td>
<td>20</td>
</tr>
<tr>
<td>Methylmercury (II) chloride</td>
<td>3 μM</td>
<td>75</td>
</tr>
<tr>
<td>Cadmium chloride, hydrate</td>
<td>3 μM</td>
<td>30</td>
</tr>
<tr>
<td>Lead (II) chloride</td>
<td>10 μM</td>
<td>20</td>
</tr>
<tr>
<td>Trans-Retinoic Acid</td>
<td>30 μM</td>
<td>80</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>100 μM</td>
<td>50</td>
</tr>
<tr>
<td>5,5-Diphenylhydantoin</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Valproic Acid</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>
# Chemical Proliferation Viability

Concentration Range: (1 nM – 100 μM)

## Lowest Effective Concentration

### Percent Inhibition

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Proliferation</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lowest Effective Concentration</td>
<td>Percent Inhibition</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>30 μM</td>
<td>30</td>
</tr>
<tr>
<td>Diphenhydramine hydrochloride</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Saccharin sodium salt hydrate</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>D-Sorbitol</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Dimethyl Phthalate</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>
NCCT 320: Screening for Effects on ReNcell CX Cell Proliferation and Viability

• National Center for Computational Toxicology (NCCT) – launched ToxCast in 2007

• Using methodology described above, 320 chemicals provided by the NCCT were screened for effects on ReNcell CX cell proliferation and viability

• Initial Screen: ReNcell CX cells exposed to every chemical at highest concentration only (40 µM)
NCCT 320: Screening for Effects on ReNcell CX Cell Proliferation and Viability

Proliferation

Viability

“Hit” – chemical effects $\geq 3$ standard deviations from control
NCCT 320: Hits for Effects on ReNcell CX Cell Proliferation and Viability

“Hit” – chemical effects $\geq 3$ standard deviations from control

- **No Effect**: 61% (195 chemicals)
- **Proliferation Only**: 20% (63 chemicals)
- **Viability Only**: 15% (49 chemicals)
- **Proliferation and Viability**: 4% (13 chemicals)
High Content Screening - Neurite Outgrowth

seed, treat, grow PC12 cells in 96-well plate (4 days)

stain cells to visualize neurites (4 hrs)

analyze 96-well plate (30 min) using ArrayScan
Patterns of Effects - Neurite Growth and Cytotoxicity
96hr exposure

1) No effect
Diphenhydramine

2) Outgrowth inhibition at cytotoxic concentrations
Dexamethasone

3) Outgrowth inhibition at concentrations that are not cytotoxic
trans-Retinoic Acid

- Total Neurite Length
- Cell Titer Glo Viability
# Training Set Results

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Neurite Growth</th>
<th>DNT in vivo</th>
<th>Chemical</th>
<th>in vitro/in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>K252a</td>
<td>+</td>
<td>nd</td>
<td>*Dimethyl phthalate</td>
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<td>U0126</td>
<td>+</td>
<td>nd</td>
<td>d-Sorbitol</td>
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<tr>
<td>Okadaic Acid</td>
<td>+</td>
<td>nd</td>
<td>Acetaminophen</td>
<td>-</td>
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<tr>
<td>Vincristine</td>
<td>+</td>
<td>+</td>
<td>*Omeprazole</td>
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<tr>
<td>Lead Acetate</td>
<td>+</td>
<td>+</td>
<td>Amoxicillin</td>
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<tr>
<td>Valproic Acid</td>
<td>+</td>
<td>+</td>
<td>Diphenhydramine</td>
<td>-</td>
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<tr>
<td>Dexamethasone</td>
<td>+</td>
<td>+</td>
<td>Saccharin</td>
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<tr>
<td>Methylmercury</td>
<td>+</td>
<td>+</td>
<td>Glyphosate</td>
<td>-</td>
</tr>
<tr>
<td>Trans-Retinoic Acid</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Amphetamine</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

* Increase at highest concentration tested
NCCT 320: Screening for Effects on NS-1 Neurite Outgrowth and Viability

“Hit” – chemical effects ≥ 3 standard deviations from control
NCCT 320: Hits for Effects on NS-1 Neurite Outgrowth and Viability

“Hit” – chemical effects $\geq$ 3 standard deviations from control

- No Effect: 85% (273 chemicals)
- Neurite Outgrowth Only: 9% (29 chemicals)
- Viability Only: 4% (14 chemicals)
- Neurite Outgrowth and Viability: 1% (4 chemicals)
Summary of Screening Effects on Cell Proliferation, Neurite Outgrowth, and Viability

- **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)

- 20 chemicals were hits on all endpoints
- Proliferation most sensitive endpoint
- Neurite outgrowth was not uniquely affected as proliferation was with regard to effects on viability
Summary / Conclusions

• ReNcell CX cells are a useful hNPC model for screening for developmental neurotoxicity

• Screening for chemical effects on cell proliferation, neurite outgrowth and viability can be achieved in a high-throughput format

• Protocols were developed for screening and prioritization of chemicals for further testing that may reduce the demands associated with toxicity testing *in vivo*

• These data will be incorporated into the larger ToxCast dataset and evaluated for their ability to predict *in vivo* toxicities
Acknowledgements

♦ Tim Shafer, Ph.D.
♦ William Mundy, Ph.D.
♦ Nicholas Radio, Ph.D.
♦ Theresa Freudenrich

♦ Kevin Crofton, Ph.D.
♦ Stephanie Padilla, Ph.D.
♦ Keith Houck, Ph.D.

♦ David Holbrook, Ph.D.
  ♦ University of North Carolina, Curriculum in Toxicology

♦ Developmental Neurotoxicity Team
  ♦ Neurotoxicology Division, US EPA