IN VITRO SCREENING FOR INTER-INDIVIDUAL AND POPULATION VARIABILITY IN TOXICITY OF PESTICIDE MIXTURES

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The Future Use of *in Vitro* Data in Risk Assessment to Set Human Exposure Standards: Challenging Problems and Familiar Solutions

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**Current paradigm based on *in vivo* data**
- *In vivo* data on apical end points
  - Benchmark or no-effect analysis
  - Animal POD (*in vivo* animal exposure)
  - Pharmacokinetic model
  - Human POD (external exposure)
  - Safety and host susceptibility factors
  - Human RfD

**Future paradigm based on *in vitro* data**
- *In vitro* data on toxic pathways
  - Benchmark or no-effect analysis
  - *In vitro* POD (cellular concentration)
  - Pharmacokinetic model
  - Human POD (external exposure)
  - Safety and host susceptibility factors
  - Human RfD

*In vitro* testing will present new technical challenges:
- Volatile chemicals
- Metabolism
- Feedback signaling
- Long-term effects, including transgenerational

Testing will need to be conducted in cell lines from many human donors, both from representative populations and from potentially sensitive subgroups.

Identifying exposures without “significant biological perturbations” will be problematic. A type of POD-safety factor method will be required.

Complex mechanistic models are unlikely to provide useful quantitative input in the foreseeable future.

A large degree of scientific judgment will be needed in this step.
Chemical Specific Adjustment Factor (CSAF)

100-fold uncertainty factor

Inter-species differences 10-fold

Toxicokinetics
AK
10 E0.6 (4.0)

Toxicodynamics
AD
10 E0.4 (2.5)

Intra-species differences 10-fold

Toxicokinetics
HK
10 E0.5 (3.2)

Toxicodynamics
HD
10 E0.5 (3.2)
Genetically Homogeneous models → Many chemicals → Many Assays → Different Endpoints

- Carcinogen?
- Cell Death?
- Apoptosis?
- DNA Damage?
- Endocrine disruptor?

A population-based model, Genetically-diverse and identified → Many chemicals → One Assay or more → Genetic underpinnings of MOA

www.nature.com

www.redoxis.se

Cell titer Glo Luminiscense cell viability assay (ATP Production)

Genes

SNPs

Pathways
Population genetics effort enables *in vitro* toxicity testing

The 1000 Genomes and HapMap Projects have established thousands of immortalized cell lines LCLs (B-lymphocyte derived) from geographically and genetically diverse human populations.
1000 Genomes Toxicogenetics Project (UNC-NTP-NCATS): Addressing chemical toxicity and population variability in a human \textit{in vitro} model system.
"WHY SHOULD I CARE?" REASON #1: ASSESSING HAZARD AND INTER-INDIVIDUAL VARIABILITY IN TOXICODYNAMICS FOR INDIVIDUAL CHEMICALS

Default uncertainty factor for inter-individual variability in TD
“WHY SHOULD I CARE?” REASON #2: IDENTIFYING SUSCEPTIBLE SUB-POPULATIONS

Subpopulation-specific profiles (all 179 chemicals)

High heritability $\rightarrow$ genetic determinants

Significant population effect (2 of 79 chemicals shown)

Significant population and sex effect
### “WHY SHOULD I CARE?” REASON #3: UNDERSTANDING GENETIC DETERMINANTS OF INTER-INDIVIDUAL VARIABILITY

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>ChrNum</th>
<th>RSID</th>
<th>$-\log_{10}(p)$</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Amino-4-methylphenol</td>
<td>4</td>
<td>rs13120371</td>
<td>9.9</td>
<td>SLC7A11</td>
</tr>
<tr>
<td>o-Aminophenol</td>
<td>16</td>
<td>rs1800566</td>
<td>8.9</td>
<td>NQO1</td>
</tr>
<tr>
<td>13-Dicyclohexylcarbodiimide</td>
<td>8</td>
<td>rs28437300</td>
<td>8.9</td>
<td>SLC39A14</td>
</tr>
<tr>
<td>N-Isopropyl-N-phenyl-p-phenylendiamine</td>
<td>7</td>
<td>rs1159874</td>
<td>8.8</td>
<td>None</td>
</tr>
<tr>
<td>Methylmercuric(I)chloride</td>
<td>4</td>
<td>rs13120371</td>
<td>7.9</td>
<td>SLC7A11</td>
</tr>
<tr>
<td>Aldrin</td>
<td>3</td>
<td>rs340251</td>
<td>7.8</td>
<td>MFSD1</td>
</tr>
<tr>
<td>Titanocenedichloride</td>
<td>15</td>
<td>rs62009303</td>
<td>7.7</td>
<td>SNP not known</td>
</tr>
<tr>
<td>Reserpine</td>
<td>4</td>
<td>rs13143102</td>
<td>7.6</td>
<td>None</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>16</td>
<td>rs8053118</td>
<td>7.5</td>
<td>WWOX</td>
</tr>
<tr>
<td>Dieldrin</td>
<td>1</td>
<td>rs504504</td>
<td>7.5</td>
<td>MCOLN2</td>
</tr>
</tbody>
</table>

#### Diagram

![Genetic Variability Diagram]
"WHY SHOULD I CARE?" REASON #4:
GENERATE TESTABLE HYPOTHESES ABOUT TOXICITY PATHWAYS

GWAS-based Pathway Analysis:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Gene set</th>
<th>Gene Set Name</th>
<th>Num</th>
<th>P (fwer)</th>
<th>P (raw)</th>
<th>P (fdr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Pivalyl-1,3-indandione</td>
<td>GO.BP</td>
<td>Cellular response to dexamethasone stimulus</td>
<td>7</td>
<td>0.323</td>
<td>1.69E-05</td>
<td>0.0705</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>KEGG</td>
<td>Steroid hormone biosynthesis</td>
<td>52</td>
<td>0.02</td>
<td>0.0006</td>
<td>0.0652</td>
</tr>
<tr>
<td>Cadmium chloride</td>
<td>GO.BP</td>
<td>Gonadotropin secretion</td>
<td>8</td>
<td>0.132</td>
<td>2.80E-06</td>
<td>0.0057</td>
</tr>
<tr>
<td>Pentaerythritol triacylate</td>
<td>GO.BP</td>
<td>Cellular response to dexamethasone stimulus</td>
<td>7</td>
<td>0.215</td>
<td>6.10E-06</td>
<td>0.0254</td>
</tr>
<tr>
<td>Triamterene</td>
<td>GO.BP</td>
<td>Negative regulation of sterol transport</td>
<td>8</td>
<td>0.19</td>
<td>5.46E-06</td>
<td>0.0228</td>
</tr>
</tbody>
</table>

Correlation analysis of basal gene expression across 350 cell lines (RNA-sequencing) and chemical-specific cytotoxicity phenotypes:
- Common toxicity pathways
- Similar susceptibility drivers
"WHY SHOULD I CARE?" REASON #5: CAN WE BE PREDICTIVE IN SILICO?

NIEHS-NCATS-UNC DREAM8: Toxicogenetics Challenge

- 232 registered participants
- 99 submissions from 34 teams for SC1
- 91 submissions from 24 teams for SC2
- Nature Biotechnology will consider an overview paper describing the results and insights
Can we expand our *in vitro* population-based model to address environmental chemical mixtures?

Real Chemical Mixtures

Lab Chemical Mixtures

[Links to www.eweb.org]
Background

- Evaluation of the toxicity of mixtures is less structured
- Critiques for current toxicity testing paradigms
  - co-exposures
  - population variability
- Whole animal testing is difficult to employ for testing chemical mixtures.

Pesticides

Widespread use and relatively high exposure

Varied MOA, meant to be toxic to pests

Potential health outcomes

Applied as Mixtures
Experimental design

Surface water universal passive sampling device (Project 4):

*Organochlorine pesticide environmental mixture*

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Human population-based *in vitro* toxicity screening (Project 2)

146 human lymphoblast cell lines
2 mixtures of pesticides (UNC Project 4)
8 concentrations (0.0003 to 330 µM)
All lines screened in 2+ plate replicates
1 assay (CellTiter-Glo®, ATP content)

~5,000 data points

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A mixture of 36 currently used pesticides
### Populations Screened

<table>
<thead>
<tr>
<th>Population</th>
<th># (%)</th>
<th>Screened</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEU: Utah residents with Northern &amp; Western European ancestry</td>
<td>76 (22.9%)</td>
<td>47 (32.2%)</td>
</tr>
<tr>
<td>YRI: Yoruban in Ibadan, Nigeria</td>
<td>77 (23.3%)</td>
<td>40 (27.4%)</td>
</tr>
<tr>
<td>TSI: Tuscan in Italy</td>
<td>87 (26.3%)</td>
<td>32 (21.2%)</td>
</tr>
<tr>
<td>GBR: British from England &amp; Scotland</td>
<td>91 (27.5%)</td>
<td>27 (18.5%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>331</strong></td>
<td><strong>146</strong></td>
</tr>
</tbody>
</table>
Deriving a Quantitative Toxicity Phenotype (EC10)

Deriving quantitative cytotoxicity phenotypes (EC_{10}): Curves were fit using a logistic model with baseline (lowest conc.) responses estimated from the data, and the maximum response value fixed at -100% (positive control). EC_{10} estimate is the concentration for which the estimated response dropped to 90% of the fitted value at the lowest concentration.
Population Variability in Response to Pesticide Mixtures

(a) Chlorinated pesticide mixture
Variability distribution of EC10 values
Population mean
Individual responses
Cytotoxicity (% Change from Control)
Cumulative Concentration, μM

(b) Current use pesticide mixture
Cytotoxicity (% Change from Control)
Cumulative Concentration, μM

Second Replicate EC10, μM
First Replicate EC10, μM

\[ p = 0.56 \]
\[ r = 0.65 \]
\[ p < 0.0001 \]

\[ p = 0.55 \]
\[ r = 0.62 \]
\[ p < 0.0001 \]
Inter-individual variability in cytotoxic response across cell lines

<table>
<thead>
<tr>
<th>Pesticide Mixture</th>
<th>Mean</th>
<th>STD</th>
<th>Range</th>
<th>Median</th>
<th>Q05</th>
<th>Q95</th>
<th>UFk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorinated Pesticide Mixture</td>
<td>11.6</td>
<td>1.96</td>
<td>(0.180-40.6)</td>
<td>13.1</td>
<td>4.36</td>
<td>21.7</td>
<td>3.00</td>
</tr>
<tr>
<td>Current Pesticide Mixture</td>
<td>11.1</td>
<td>1.85</td>
<td>(0.649-39.9)</td>
<td>11.9</td>
<td>3.89</td>
<td>24.7</td>
<td>3.05</td>
</tr>
</tbody>
</table>
What does “LCLs cytotoxicity” mean? How to go from blood toxicity to exposure?
What does “LCLs cytotoxicity” mean?

*In vitro to in vivo* extrapolation (IVIVE)

- Hepatic Clearance
- Non metabolic renal clearance
- Blood binding

Population Simulation: 10,000 (20-50 yrs) males & females

Chemical specific steady-state blood concentration (Css)

Current-use pesticide mixture: 31 out of 36 chemicals

Chlorinated pesticide mixture: 4 out of 10 chemicals

How missing values were handled:

**Scenario 1: “Worst Case Scenario”**
- No hepatic clearance
- Only renal clearance
- High blood binding

**Scenario 2: “Median”**
- Assuming the median or highest Css value within each mixture for chemicals with missing values

- Weighted by % of chemical in 1ml
- Assuming Equal weight for each chemical

- Oral equivalent (OE) doses were calculated for each scenario using reverse dosimetry from the upper 95th % Css value:
  - OE was calculated for each cell line-chemical pair.
  - A cumulative OE was computed for each mixture for each cell line.

Predicted Exposure Limits

1936 chemicals evaluated by far-field mass balance & human exposure models

An indicator for indoor and/or consumer use

Bayesian analysis from urine concentrations for 82 chemicals reported in (NHANES)

Chemical specific predicted exposure

Current-use pesticide mixture:
35 out of 36 chemicals

Chlorinated pesticide mixture:
6 out of 10 chemicals

• Missing values were replaced by the highest predicted exposure within each mixture
• A cumulative predictive exposure was computed for each mixture from the upper 95th %.

**In vitro to in vivo extrapolation**

### Table 5

**Current-Use Pesticide Mixture**

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Cumulative Margin of Exposure</th>
<th>95th percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WCS</td>
<td>Median</td>
</tr>
<tr>
<td>Weighted by chemical %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Worst Case Scenario</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Median</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>Equally Weighted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Worst Case Scenario</td>
<td>2.9</td>
<td>3.1</td>
</tr>
<tr>
<td>Median</td>
<td>3.0</td>
<td>3.1</td>
</tr>
</tbody>
</table>

### Table 6

**Chlorinated Pesticide Mixture**

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Cumulative Margin of Exposure</th>
<th>95th percentile</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WCS</td>
<td>Median</td>
<td>WCS</td>
</tr>
<tr>
<td>Weighted by chemical %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Worst Case Scenario</td>
<td>5.9</td>
<td>6.0</td>
<td>6.7</td>
</tr>
<tr>
<td>Median</td>
<td>6.4</td>
<td>6.6</td>
<td>7.3</td>
</tr>
<tr>
<td>Equally Weighted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Worst Case Scenario</td>
<td>7.1</td>
<td>7.2</td>
<td>7.9</td>
</tr>
<tr>
<td>Median</td>
<td>7.5</td>
<td>7.6</td>
<td>8.4</td>
</tr>
</tbody>
</table>
What does “LCLs cytotoxicity” mean?

*In vitro to in vivo* extrapolation.
How the two pesticide mixtures compare?

\[ \rho = 0.25 \quad p < 0.01 \\
\rho = 0.53 \quad p < 0.0001 \]

- CEU
- GBR
- TSI
- YRI

\[ \text{Chlorinated pesticide mixture EC}_{10}, \mu \text{M} \]

\[ \text{Current use pesticide mixture EC}_{10}, \mu \text{M} \]

b

PC3

Chlorinated pesticide mixture
Current use pesticide mixture
Nematicide

PC1

C

Median \( \rho \)

Pesticide mixtures \( \rho \)
Identifying susceptible sub-populations

Chlorinated Pesticide Mixture

Current Pesticide Mixture

Cumulative Concentration EC10, µM

<table>
<thead>
<tr>
<th></th>
<th>CEU</th>
<th>GBR</th>
<th>TSI</th>
<th>YRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorinated Pesticide Mixture</td>
<td><img src="image1" alt="Boxplot" /></td>
<td><img src="image2" alt="Boxplot" /></td>
<td><img src="image3" alt="Boxplot" /></td>
<td><img src="image4" alt="Boxplot" /></td>
</tr>
<tr>
<td>Current Pesticide Mixture</td>
<td><img src="image5" alt="Boxplot" /></td>
<td><img src="image6" alt="Boxplot" /></td>
<td><img src="image7" alt="Boxplot" /></td>
<td><img src="image8" alt="Boxplot" /></td>
</tr>
</tbody>
</table>

p=0.52

p=0.0586
Genome-wide association with cytotoxicity to current use pesticide mixture (36 pesticides)
<table>
<thead>
<tr>
<th>Term</th>
<th>N Genes</th>
<th>Top 7 genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbate and aldolurate metabolism</td>
<td>22</td>
<td>UGT2B11, UGT2B7, UGT1A3, UGT1A7, UGT1A4, UGT1A5, UGT1A6</td>
</tr>
<tr>
<td>Starch and sucrose metabolism</td>
<td>48</td>
<td>UGT2B11, UGT2B7, UGT1A3, UGT1A7, UGT1A4, UGT1A5, UGT1A6</td>
</tr>
<tr>
<td>Porphyrin and chlorophyll metabolism</td>
<td>39</td>
<td>EARS2, UGT2B11, UGT2B7, BLVRA UGT1A3, UGT1A7, UGT1A4</td>
</tr>
<tr>
<td>Pentose and glucuronate interconversions</td>
<td>28</td>
<td>UGT2B11, UGT2B7, UGT1A3, UGT1A7, UGT1A4, UGT1A5, UGT1A6</td>
</tr>
<tr>
<td>Nitrogen metabolism</td>
<td>23</td>
<td>CA6, GLUL, CA2, CA4, HAL, CTH, CA5A</td>
</tr>
</tbody>
</table>

P FWER<0.1
Why is population-based toxicity screening more powerful than traditional approaches?

- Quantitatively assess hazard and population variability in chemical mixtures \textit{in vitro}.
- Identify susceptible sub-populations.
- Understand genetic underpinning and probe toxicity pathways.
- Extrapolating the in vitro POD to oral equivalent dose.
- Assessing risk by comparing to estimated human exposure.
Acknowledgments

University of North Carolina at Chapel Hill:
Advisors
Ivan Rusyn
Fred Wright

Biostatistics
Paul Gallins

North Carolina State University:
Damian Shea  Alison Motsinger-Reif
Chad Brown  John Jack

University of Liverpool, UK:
Munir Pirmohamed  Philippe Marlot

The Hamner Institutes
Barbara Wetmore

US EPA
Mathew Martin
John Wambaugh

NIH: NIEHS/NTP
Raymond Tice

With additional support from:
CAROLINA CENTER FOR COMPUTATIONAL TOXICOLOGY
http://comptox.unc.edu