Mixtures Research in the 21st Century: Using High-Throughput Screening to Evaluate Simple and Complex Combinations

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National Toxicology Program
National Institute of Environmental Health Sciences
What is NTP?

- Interagency program
  - Headquartered at NIEHS
- Research on nominated test articles
  - Thousands of agents evaluated in comprehensive toxicology studies
  - GLP compliant testing through government contracts
- Analysis activities
  - Report on Carcinogens (RoC)
  - Office of Health Assessment and Translation (OHAT)
  - NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) – administers ICCVAM
What do I mean by mixtures?

Complete reductionism
Binary combinations
Specific question in mind

Defined mixtures
2-50ish components
All components are identified and quantified

Source emissions
Source is known and some components are characterized
Large unidentified fraction

Environmental contaminant mixtures
Multiple sources

Commercial mixtures
Active ingredients known
Some unidentified fraction

Mind-boggling complexity
Real-world scenario
Includes chemical and nonchemical stressors
Different routes of exposure
...Exposome
Is Data Quality Adequate?

- No quantitative assessment; only qualitative assessment
- Yes

**Whole Mixture Data**
- Mixture of concern
- Sufficiently similar mixture
- Group of similar mixtures

**Component Data**

- Do components interact?
  - No
    - Components have similar MOAs
      - Mixture RfD/C; Slope Factor
      - Comparative Potency
    - Components have different MOAs
      - Dose Addition
      - Response Addition
      - Interactions Based Hazard Index
  - Yes

Concepts of additivity

**Dose Addition**

\[ \sum_{i=1}^{n} \frac{D_i}{EDX_i} = 1 \]

**Response Addition**

\[ R_{mix} = 1 - \prod_{i=1}^{n} (1 - R_i) \]
DNTP mixtures research

- Whole Mixtures Testing
  - Herbals program (aloe vera, ginkgo, green tea, etc.)
  - Flame retardants (Firemaster FF-1)
  - Marine diesel fuel and jet fuel (JP-5)

- Defined Mixtures
  - 25 groundwater contaminants
  - Pesticide mixtures
  - AIDS drugs used in combination therapies

- Component Based Approaches
  - Dioxin toxic equivalency factor studies
The Big Challenge…

- 50,000 chemicals in commerce
- Combinations are practically infinite

*Using traditional in vivo approaches to study one mixture at a time is not going to cut it!*
• Goal: Identify and focus on key issues that present challenges in mixtures research
  – Use to inform the development of an intramural and extramural mixtures research strategy

• Multidisciplinary participation
  – Mixtures experts from statistics, biology/toxicology, epidemiology, exposure science, and risk assessment

• Format
  – Background presentation from invited speakers
  – Breakout sessions

• Comprehensive workshop report
  – Available on website

Key issues

• Improved exposure assessment (monitoring, modeling, and unbiased approaches)
  – Develop exposure technologies
  – Evaluate novel methods (e.g., EWAS, exposome)

• Tools and methods for prioritization of chemicals/mixtures
  – More use of exposure data (e.g., NHANES database)
  – High-throughput screening methods to assess interactions and mixtures

• Cross-disciplinary effort is required
  – Relative potency factors generated in toxicology studies to epidemiological assessments
  – Epidemiological findings for identification of important combinations for toxicological studies

• Bridging *in vitro* and *in vivo* approaches
  – Link *in vitro* responses to biologically-meaningful endpoints, which should be validated *in vivo*
Key issues (continued)

- Development and validation of statistical methods
  - Predictive mixture toxicity models (e.g., component-based and sufficient similarity)
  - Assessment of multiple chemical associations in epidemiology
- Systems-based approaches for studying mixtures
  - Predict interactions of chemicals that target a common pathway or system without testing all potential chemical combinations
- Development/refinement of both “bottom-up” (component-based) and “top-down” (whole mixtures) approaches for predicting toxicity of mixtures
- Data collection and management (e.g., federated databases)
  - Raw data on both single chemicals and mixtures
  - Standardization and integration across datasets
  - Significant planning to establish the scope and implementation strategy
How combined environmental exposures affect disease pathogenesis

a) Assess joint action of multiple environmental insults (e.g., chemicals, nonchemical stressors, and nutritional components), on toxicity and disease, and identify interactions resulting from combined exposures

b) Study role of the human microbiome and its influence on environmental health, and explore role of microbiome in responses to environmental exposures

c) Study interactions of infectious agents with environmental exposures

d) Understand how nonchemical stressors, including socioeconomic, behavioral factors, etc., interact with other environmental exposures to impact human health outcomes, and identify preventive measures
Key issues

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High-throughput screening

  - *In vitro* toxicity testing in human cells or cell lines
  - Perturbations of cellular responses in a suite of toxicity pathway assays
  - Using high-throughput robotic assisted methodologies

- 2008 Formation of Tox21 Community
  - Participating groups: National Human Genome Research Institute (NHGRI), NIEHS/NTP, EPA, FDA
  - Goals:
    - Prioritize compounds for more extensive evaluation
    - Identify mechanisms of compound-induced biological activity
    - Develop predictive models for biological response *in vivo*
Tox21

• Phase 1 (2005-2010)
  – EPA via ToxCast™ screened 320 compounds (309 unique, primarily pesticide actives and some endocrine active compounds) in ~550 assays
  – National Chemical Genomics Center screened 1408 compounds (1353 unique) from NTP and 1462 compounds (1384 unique) from EPA in 140 quantitative high-throughput screening (qHTS) assays representing 77 predominantly cell-based reporter gene endpoints

• Phase 2 (2011-2014)
  – Approximately 11,000 chemicals (a.k.a. 10K library)
  – Stage I of Phase II focuses on induction of stress response pathways and nuclear receptor activation or inhibition
  – Stage II of Phase II increased focus on disease-associated pathways
Tox21 (continued)

• Phase 3 (future)
  – Increased focus on tools for *in vitro* to *in vivo* extrapolation
  – Different cell systems
    • Incorporating xenobiotic metabolism (primary hepatocytes, HepaRG, HepG2 3D)
    • ES/iPSC derived differentiated cell populations
  – Expanded utilization of lower organisms (zebrafish, *C. elegans*)
    • High-content screening
  – High-throughput transcriptomics project
    • Selection of 1500 “sentinel” genes
    • Genes included to ensure maximal biological pathway coverage
qHTS and Mixtures

- Pilot studies
  - Goals
    - Evaluate the qHTS platform for mixture assessment
    - Identify technical problems specific to mixtures
  - Mixture types
    - Estrogen receptor agonists
    - Androgen receptor agonists/antagonists
    - Cytotoxic chemicals
• Proof of Principle Study
  – We are confident that the toxicity of estrogenic chemical mixtures can be predicted using a dose addition model
  – Does this hold up using the qHTS platform?

• Design
  – Include chemicals that were positive in the ER agonist assay in Phase 1
  – Individual chemicals and mixtures on the same plate
  – Include multiple ratios of mixtures
  – 15-point dose-response

• ERα Assays (run in both agonist and antagonist mode)
  – Endogenous full length ERα (ER-luc; BG1 cell line)
  – Transfected partial receptor consisting of the ligand binding domain (ER-bla; ERα β-lactamase cell line)
  – For more information on the ER assays see: Huang et al. (2014) Sci. Rep. 4:5664
Analysis of ER mixtures

- Predictions based on components only versus best fit of model to all data
- Predictions using dose addition versus response addition
  - Dose addition predictions calculated using the “Generalized Concentration Addition” developed by Howard and Webster (2009) to accommodate partial agonists
Components versus all data (dose addition)

Luciferase

- Component data only: $r^2 = 0.72$
- All data: $r^2 = 0.90$

β-lactamase

- Component data only: $r^2 = -0.066$
- All data: $r^2 = 0.62$
Response versus dose addition (all data)

Luciferase

$\beta$-lactamase

Response addition

$r^2 = 0.82$

$r^2 = -0.88$

Dose addition

$r^2 = 0.90$

$r^2 = 0.62$
Conclusions

• Assay comparison: The full-length receptor assay was more sensitive than the partial β-lactamase assay in detecting estrogenic activity

• Data: Using all data (including mixtures data) improves predictions (predictably)
  – Poor single chemical data for some chemicals (e.g., zearalenone) is likely responsible for this difference

• At the lower concentrations, the models (both dose and response addition) tend to over-predict, while at the higher concentrations dose addition tends to under-predict and response addition continues to over-predict
  – Possibly due to the inclusion of partial agonists
  – Saturation?

• Dose addition generally provides a better fit to the data than response addition
Now that we know that dose addition does an adequate job of describing the joint action of like-acting chemicals (in HTS), how can we mess things up?

- Androgen Receptor (AR) active mixtures
  - Agonists and antagonists
  - Mixtures containing both ER and AR active compounds
- Cytotoxicant mixtures
  - Previous work classified cytotoxic chemicals into groups based on dose-response shape and kinetics of cytotoxicity
  - Mixtures include chemicals from each group
  - Approximately 100 mixtures containing up to 62 chemicals

Next steps
Next steps (continued)

• Use of additional modeling approaches:
  – Other dose addition models
  – Integrated addition (combination of dose and response addition)
  – Models that are non-linear at low doses (reflecting dimerization of the ligand-receptor complex)
  – Model development and refinement

• Development of recommendations for future HTS mixtures work
  – Inclusion of low doses is critical (capturing responses below maximum)
  – Making mixtures that have significant contributions for more than one component
Key issues (continued)

• Development and validation of statistical methods
  – Predictive mixture toxicity models (e.g., component-based and sufficient similarity)
  – Assessment of multiple chemical associations in epidemiology

• Systems-based approaches for studying mixtures
  – Predict interactions of chemicals that target a common pathway or system without testing all potential chemical combinations

• Development/refinement of both “bottom-up” (component-based) and “top-down” (whole mixtures) approaches for predicting toxicity of mixtures

• Data collection and management (e.g., federated databases)
  – Raw data on both single chemicals and mixtures
  – Standardization and integration across datasets
  – Significant planning to establish the scope and implementation strategy
Complex mixtures at NTP

- **Botanical dietary supplements**
  - *Ginkgo biloba extract*
  - Green tea extract
  - Goldenseal root powder
  - Black cohosh
  - Echinacea
  - Ginseng
  - Kava kava
  - Senna
  - Milk thistle
  - Valerian
  - Aloe vera

- **Mixed entities**
  - Asbestos
  - Nanoparticles

- **Commercial mixtures**
  - Firemaster FF-1
  - Firemaster 550
  - Marine diesel fuel
  - JP-5 Navy Fuel
  - Metalworking fluids
  - Stoddard solvent
  - Technical grade pentachlorophenol

- **Environmental mixtures**
  - Chlorinated water
  - Chloraminated water
  - Groundwater contaminant mixture
Critical issue…

- Did we pick the “right” test article?
- How does the test article compare to related products/formulations?
- What does it mean for the relevance of our studies when a commercial formulation changes (e.g., different ratio of active ingredients, addition of a different excipient/inert ingredient)?

Implication of being unable to answer these questions: If [industry] says that our test article is different from their product, there is no scientifically-based counter-argument.
Ginkgo biloba extract case study: A Tale of 2 Ginkgos

From Nature. For Health.
Dr. Willmar Schwabe Pharmaceuticals

Ginkgo biloba extract (Shanghai Xing Ling)
Extracted tablet (Nature’s Way)
Ginkgo biloba extract (GBE)

• Nominated by NCI for testing – 9/30/98
• Test article selection issues
  – Preferred: Schwabe EGb761® standardized GBE
  – Used: Shanghai Xing Ling GBE
• Assessed in 3-month and 2-year gavage studies in F344/N rats and B6C3F1/N mice
• Results and conclusions:
  – Major toxicity targets of liver, nose, and thyroid gland generally consistent across sex, species, and exposure period
  – Some **evidence of carcinogenicity** in male and female rats based on thyroid tumors; **clear evidence of carcinogenicity** in male and female mice based on liver tumors (**among highest levels of hepatoblastomas ever induced in NTP studies**)

What is sufficient similarity?

<table>
<thead>
<tr>
<th>Class</th>
<th>Identified Chemical Constituents</th>
<th>Target Specification in EGb 761®</th>
<th>Range in other preparations</th>
<th>NTP Test Article</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terpene trilactones</td>
<td>Total</td>
<td>6%</td>
<td>0.07 - 14.23</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>Bilobalide (sesquiterpene)</td>
<td></td>
<td>0.00 – 8.64</td>
<td>6.94</td>
</tr>
<tr>
<td></td>
<td>Ginkgolide A</td>
<td></td>
<td>0.01 – 3.82</td>
<td>3.74</td>
</tr>
<tr>
<td></td>
<td>Ginkgolide B</td>
<td>ND – 2.00</td>
<td>1.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ginkgolide C</td>
<td>ND – 2.35</td>
<td>3.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ginkgolide J</td>
<td>0.03 – 0.78</td>
<td>NM</td>
<td></td>
</tr>
<tr>
<td>Flavonol glycosides</td>
<td>Total</td>
<td>24%</td>
<td>24 – 36</td>
<td>31.2</td>
</tr>
<tr>
<td></td>
<td>Quercetin,</td>
<td>ND – 20.93</td>
<td>16.71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kaempferol</td>
<td>0.05 – 13.98</td>
<td>12.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Isorhamnetin</td>
<td>0.13 – 2.84</td>
<td>2.37</td>
<td></td>
</tr>
<tr>
<td>Alkylphenols</td>
<td>Ginkgolic acids, cardanolns</td>
<td>≤ 5 ppm</td>
<td>&lt; 500 – 90,000 ppm</td>
<td>10.45 ppm</td>
</tr>
</tbody>
</table>

- Claims of herbal industry representatives:
  - NTP selected an inappropriate GBE for testing, which does not resemble EGb761® or other GBEs in the marketplace
  - No two GBEs are alike and all GBEs must be assessed individually
Chemical similarity is meaningless without an understanding of how chemistry relates to biology!
Relating test article findings to the universe

3-month studies in mice and rats
**Findings:** Nonneoplastic histological changes in target tissues (nose, thyroid, liver)

2-year studies in mice and rats
**Findings:** Cancer in target tissues (nose, thyroid, liver)

**Required:** Short-term assays that can generate a biological pattern associated with downstream effects

Too costly and time-consuming to test multiple products
Candidate short-term assays

- High-throughput screening
- 5-Day assay to evaluate the gene expression pattern in a target tissue
High-throughput screening botanicals project

- Annato extract (10)
- Bixin (2)
- Black walnut/Juglone (5)
- Cedarwood oil (2)
- Citral (5)
- Comfrey root (2)
- Corn oil (2)
- Echinacea purpurea (1)
- Emodin (6)
- Eugenol (1)
- Ginkgo biloba extract (4)
- Goldenseal root powder (9)
- Grape seed extract (4)
- Gugulipid (3)
- Gum guggal extract (5)
- Kaempferol (1)
- Kava Kava extract (6)
- Methyleugenol (4)
- Milk thistle extract (6)
- Olive oil (1)
- Pine bark extract (2)
- Pulegone (3)
- Pyrogallol (3)
- Quercetin (3)
- Resorcinol (3)
- Resveratrol (3)
- Safflower oil (2)
- Silybin (3)
- Staurosporine (1)
- Tricaprylin (3)
- Turmeric (4)
Assays

- Estrogen receptor alpha
- Aromatase
- Androgen receptor
- Aryl hydrocarbon receptor
- Peroxisome proliferator-activated receptor gamma
- Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (Nrf2/ARE)
- Heat shock factor response element
- ATAD5
- Mitochondrial membrane potential (MMP)
- P53
- Cytotoxicity
- Cell viability
Goals and expected outcomes

• Evaluate the HTS platform for assessing botanical dietary supplements
  – Plant material dissolved in DMSO – what could go wrong?

• Gain information about the patterns of activity of botanicals
  – Range across botanicals
  – Within botanical variation

• Insight into mechanisms of toxicity and active constituents

• Use informative assay results to move forward on sufficient similarity project
5-day gene expression studies

- Animals: F344 male rats (males more sensitive)
- Exposure: 5-day oral gavage
- Endpoints:
  - Weight
  - Micronucleus assay
  - Clinical chemistry and hematology
  - Organ weights
  - Gene expression in liver tissue
How would this work?

**Step 1: Generate data**
Assess multiple related products with different chemical profiles and select unrelated products at various doses
- GBE1 (reference)
- GBE2
- GBE3
- GBE4
- GBE5
- Goldenseal
- Green tea

**Step 2: Data analysis**
- a) Unsupervised clustering, principle component and network-based nearest neighbor analyses
- b) Leverage databases to link pathway perturbations to toxicological pathology/disease

**Step 3: Expert judgment**
Determine what constitutes a biologically-meaningful difference and use that to define the “similarity region”

**Step 4: Equivalence testing**
Statistical test to determine whether or not the effects of the other GBE samples fall within the similarity region of the reference GBE
Goals of the program

• Develop methods for assessing similarity across nominally related, complex mixtures
  – Novel melding of high-content data with statistical methods to evaluate sufficient similarity

• Contribute to limited field of case studies on determining sufficient similarity of complex mixtures

• Compare data from HTS, short-term gene expression study, 3-month, and 2-year to evaluate predictivity of earlier biomarkers of activity for adverse outcomes
Key issues (continued)

- Development and validation of statistical methods
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NIEHS Workshop - July 2015, Research Triangle Park, NC

Structure of workshop

- Pre-workshop: Epidemiologist/statistician teams will analyze two datasets using their preferred method to assess effects of multiple chemicals in epidemiological studies
  - Synthesized dataset (Chris Gennings and Tom Webster)
  - Real-world dataset
- During workshop: Present results of analyses, discuss pros and cons of different approaches
- Post workshop: Describe results in manuscripts for special issue with recommendations for data analysis

Statistics for Mixtures Data in Epidemiology
Want to learn more about mixtures?

- SOT 2015 CE Course AM03 - “Demystifying Mixtures: From Study Design Selection to Risk Assessment Application”
  - Regulatory Drivers and Available Resources. Moiz Mumtaz (ATSDR/CDC)
  - Berenbaum and Beyond: Concepts and Theories Underlying Mixtures Research and Cumulative Risk Assessment. Cynthia V. Rider (NTP/NIEHS)
  - Designing the Good, Eliminating the Bad and the Ugly. Jane Ellen Simmons (US EPA - NHEERL)
  - Data Quality Assessment and Whole Mixture Assessments (Mixture of Concern, Sufficiently Similar Mixture, Group of Similar Mixtures). Glenn Rice (US EPA - NCEA)
  - Component-Based Additivity Approaches: Benefits and Uncertainties. Richard Hertzberg (Emory University)
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Mix Workshop
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Thank you!
Questions?