Mixtures Research at the NIEHS

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Outline

• Past and present mixtures research at NIEHS
• 2011 NIEHS Mixtures Workshop
• Current projects
  – High-throughput screening of mixtures
  – Antilipidemic mixtures
  – Polycyclic Aromatic Compounds
  – Herbals
• Future directions
Mission of the NIEHS

Discover how the environment affects people in order to promote healthier lives

- Division of Extramural Research & Training (DERT)
- Division of Intramural Research (DIR)
- Division of the National Toxicology Program (DNTP)
DERT mixtures research

• RFA ES-98-002: Chemical Mixtures In Environmental Health
  – Mechanistic basis for chemical interactions in biological systems and related health effects, development of better mathematical tools for risk assessment, characterization of real-life mixtures based on human exposure and body burden, and conduct exposure assessments, including environmental transport and fate

• Superfund Research Program (SRP) (P42)
  – Funds multidisciplinary research that addresses the broad, complex human and environmental health issues surrounding hazardous waste sites

• Individual mixtures grants via available investigator-initiated mechanisms
**DERT mixtures research**

**Chemical Mixture Types:**
- Air Pollution and PM
- PCBs
- PBDEs
- Metals
- Pesticides
- Organochlorines
- PAHs
- Xenoestrogens

**Research Study Types:**
- Epidemiology
- Mechanistic
- Statistical Analysis
- Developmental
- Exposure
- Mathematical modeling
- Remediation
- Fate and Transport

**Health Outcomes:**
- Cognitive and neurodevelopmental changes
- Reproductive effects
- Mortality and hospital admissions
- Oxidative stress
- Immunological effects
- Genetic and epigenetic alternations
- Disruption of transition metal homeostasis
- Carcinogenesis
DIR mixtures research

Gulf Long-term Follow-up (GuLF) Study

Objectives:

• Assess health effects associated with oil spill clean-up following the Deepwater Horizon disaster
  – Physiologic/biologic effects from oil
  – Effects due to disaster-related stress

• Investigate biomarkers of adverse biological effects

• Create a resource for future collaborative research

www.nihgulfstudy.org

www.niehs.nih.gov
DIR mixtures research

1a. Polymorphism Alters Binding
- p53
- Polymorphism: G>T

1b. DNA Methylation Affects Accessibility and Transcription
- 5-methyl CpG

2a. AHRR cg05575921
- % Methylation
- Smoking Category:
  - NS
  - 1 Pk/day
  - 2 Pk/day

2b. Smoking Altered DNA Methylation
- AHR
- ARNT
- AHRR
What is DNTP?

- **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
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 study
Challenge of current approach

- Previous mixtures testing focused predominantly on characterizing the toxicity of each mixture.
- There are approximately 80,000 chemicals in use in the US and the potential combinations are practically infinite.

We are not going to test our way out of this!
Predicting mixture effects

**Whole Mixtures**
Requires toxicity data on whole mixture
- Data on mixture of interest
- Data on “sufficiently similar” reference mixture

**Component-based**
Requires toxicity data for individual chemicals within the mixture
- Dose addition
- Response addition

Estimating human health risk from exposure to environmental mixtures

• 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 – e.g., exposome); determine “relevant” mixtures
  – Portable monitoring devices, subdermal microchips, GIS data

• 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
NIEHS strategic plan – goal 4

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
Current mixtures projects
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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 (in progress)
  – 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 will increasingly focus on disease-associated pathways
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
Estrogen receptor agonist mixtures

• 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

• Analysis
  – Generalized dose addition model (Howard and Webster 2009)
  – Component data versus all data
Other qHTS mixtures

• 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
Analysis in progress

- Predictions based on components only versus all data
- Predictions using dose addition versus response addition
Key issues (continued)

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Dr. Andreas Kortenkamp – Brunel University
Dr. Chirag Patel – Stanford
Dr. Linda Birnbaum and Dr. Nigel Walker- NIEHS

National Institutes of Health
U.S. Department of Health and Human Services
Lipid signaling and development

- Nominations to NTP:
  - Drinking water disinfection by-products: 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibition and developmental toxicity
  - Drinking water disinfection by-products: interactive effects of antilipidemic agents and drinking water contaminants in producing developmental toxicity
## MoAs and developmental effects

<table>
<thead>
<tr>
<th>Class</th>
<th>Mode(s) of action</th>
<th>Fetal/perinatal findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statins</td>
<td>Inhibit HMG-CoA reductase resulting in lower circulating cholesterol levels</td>
<td>Associated with: ↑ post implantation loss, maternal cardiomyopathy, ↓ pup survival, fetal axial skeleton malformations</td>
</tr>
<tr>
<td>Dichloroacetic acid</td>
<td>PPAR agonist; inhibitor of HMG-CoA reductase</td>
<td>Fetal cardiac malformations, anophthalmia</td>
</tr>
<tr>
<td>Fibrates</td>
<td>Potent PPAR agonists that results in ↑β-oxidation, ↓hepatic triglyceride secretion, ↑lipoprotein lipase activity, ↑VLDL clearance, ↑HDL</td>
<td>Altered lipid profiles are associated with: embryotoxicity, ↑ in skeletal variations and fetal axial skeletal malformations, anophthalmia</td>
</tr>
<tr>
<td>Imidazole anti-fungals</td>
<td>Inhibit cholesterol-side-chain-cleavage enzyme, CYP17A1 (17 alpha-hydroxylase and 17,20-lyase activities); lowers steroid levels (especially T)</td>
<td>Weak affector of androgen-mediated endpoints</td>
</tr>
<tr>
<td>Specific phthalates</td>
<td>Inhibit T synthesis by an unknown mechanism; PPAR agonist at high concentrations</td>
<td>Disruption of T-mediated development; malformations</td>
</tr>
</tbody>
</table>
Lipid signaling disruption

Fatty acid catabolism → Cholesterol/Fatty Acid Pool

- Acetyl-CoA
- Statins

Cholesterol → Isoprenes

- Fibrates

Cholesterol → Bile Salts

- Imidazole antifungals

Cholesterol → Steroids

- Phthalates

Cholesterol → Estrogens

- Progestins

Fatty Acids

- Modified Fatty Acids (prostaglandins and leukotrienes)

- Bile Salts
- Steroids
- Estrogens
- Progestins
- Androgens
Interaction study

- Hypothesis: The addition of a statin (at or below its NOEL) will interact with the phthalate to shift its dose-response curve.
Interaction study

• Hypothesis: The addition of a statin (at or below its NOEL) will interact with the phthalate to shift its dose-response curve

• Studies
  – Preliminary study to characterize the developmental effects of a representative statin
  – Binary mixture study with phthalate alone and phthalate plus a statin
  – Future studies including more chemicals that target other points in the lipid signaling pathway
Key issues

- Improved exposure assessment (monitoring, modeling, and unbiased approaches – e.g., exposome); determine “relevant” mixtures
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Polycyclic aromatic compounds

• Polycyclic aromatic compounds (PACs) occur naturally in petroleum and coal or are created and released into the environment through natural events (e.g., volcanic eruptions, forest fires) and human activity (e.g., combustion processes)

• Over 1500 have been referenced in the literature, though millions of structural configurations are possible

• Exist in complex and dynamic mixtures
  – Profiles of PACs differ by source (e.g., pyrogenic versus petrogenic)
Polycyclic aromatic compounds

Relative Potency Factor approach (dose additivity) to estimate carcinogenic potential of parent PAHs

Reference compound (benzo(a)pyrene)

Convert to BaP equivalents

Add to get total mixture dose

Response vs. BaP dose
Major knowledge gaps

• Exposure
  – Do the 16 commonly monitored PAHs capture the PAC class?

• Hazard characterization
  – The vast majority of PACs have not been characterized
  – Genotoxicity/carcinogenicity have been the focus of characterization, when it is clear that PACs display additional toxicities

• Risk assessment
  – There is a great deal of uncertainty in the application of the RPF approach to PAH risk assessment
  – There is no path forward for developing a sufficient similarity approach to assess the risk associated with complex PAH mixtures
PAC mixtures assessment program

- Short-term *in vitro* and *in vivo* testing battery
  - Assess a broad range of structurally diverse PACs and PAC mixtures
  - Include a robust suite of endpoints
  - Evaluate available models for predicting mixture toxicity

Phase 1: Evaluate as many structurally diverse PACs, defined mixtures, and complex environmental samples as possible in *in vitro* and zebrafish assays

Phase 2: Assess a select subset of individual PACs and defined mixtures in 28-day *in vivo* assay

Phase 3: Evaluate a limited number of priority PACs and/or mixtures in targeted studies (e.g., carcinogenicity, developmental)
In vitro/alternative animal

- HepaRG High Content Screen
  - Metabolically-competent cell line
  - Endpoints: cell loss, nuclear size, oxidative stress, mitochondrial damage, steatosis and phospholipidosis, genotoxicity, DNA damage, apoptosis, antioxidant (GSH) depletion, membrane leakage, metabolism

- Cytotoxicity and gene expression in diverse cell lines
  - Cell lines include PC3, HL60, MCF7, HepG2, and HepaRG
  - Endpoints: cytotoxicity and gene expression (Affymetrix microarray)

- Zebrafish developmental assay
  - Endpoints: Mortality at 24- and 120-hours post fertilization, developmental progress, yolk sac edema, pericardial edema, body axis, snout, jaw, eye, ear, brain, trunk, fin malformations, pigment, circulation, trunk malformations, and notochord
In vitro/alternative animal
Pilot study: Cytotoxicity and gene expression

- **Group 1**: dibenz[a,h]anthracene, benzo(a)pyrene (reference chemical and positive control), and benzo[b]fluoranthene – contain a bay region, require metabolic activation, and display carcinogenicity *in vivo*.

- **Group 2**: phenanthrene (reference chemical and “negative” control), anthracene, pyrene – are relatively inactive *in vivo*.

- **Group 3**:acenaphthenequinone, 5,12-naphthacenequinone – oxy-PACs with similar patterns of effect in the zebrafish assay (high mortality without developmental malformations).

- **Group 4**:aceanthrenequinone, perinaphthenone – oxy-PACs with similar patterns of effect in the zebrafish assay that differ from Group 3 effects (low mortality and developmental malformations).

- **Group 5**: 9-methylnanthracene, 1-methylfluorene – alkylated PACs that are structurally similar and both inhibit gap junctional intercellular communication.
Herbals: Sufficient similarity of whole mixtures

<table>
<thead>
<tr>
<th>Class</th>
<th>Identified Chemical Constituents</th>
<th>NTP Test Article</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terpene trilactones (%)</td>
<td>Total</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>Bilobalide (sesquiterpene)</td>
<td>6.94</td>
</tr>
<tr>
<td></td>
<td>Ginkgolide A</td>
<td>3.74</td>
</tr>
<tr>
<td></td>
<td>Ginkgolide B</td>
<td>1.62</td>
</tr>
<tr>
<td></td>
<td>Ginkgolide C</td>
<td>3.06</td>
</tr>
<tr>
<td>Flavonol glycosides (%)</td>
<td>Total</td>
<td>31.2</td>
</tr>
<tr>
<td></td>
<td>Quercetin,</td>
<td>16.71</td>
</tr>
<tr>
<td></td>
<td>Kaempferol</td>
<td>12.20</td>
</tr>
<tr>
<td></td>
<td>Isorhamnetin</td>
<td>2.37</td>
</tr>
<tr>
<td>Alkylphenols (ppm)</td>
<td>Ginkgolic acids, cardanols</td>
<td>10.45</td>
</tr>
<tr>
<td>Unidentified fraction (%)</td>
<td></td>
<td>53.4</td>
</tr>
</tbody>
</table>
Herbals: Sufficient similarity of whole mixtures
Future directions

• Greater use of exposure data in prioritizing mixtures for study
  – Use NHANES data to design mixtures for \textit{in vitro} / \textit{in vivo} assessment

• Second phase of qHTS and mixtures

• Provide complex mixture data to statistical community to develop methods for determining sufficient similarity of whole mixtures

• Epidemiology mixtures workshop
Statistics for Mixtures Data in Epidemiology

• 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
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Thank you!
Questions?