Strategies for Studying Combined Exposures and Mixtures

Cynthia Rider, PhD, DABT
Toxicology Branch
Division of National Toxicology Program
National Institute of Environmental Health Sciences

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Mixtures at NTP

Outline

• Background

• Mixtures research areas
  – Component-based approaches
  – Whole mixture approaches
  – Systems biology approaches
- 135 biomarkers measured in blood, serum, or urine
- 91 biomarkers detected on average (range of 60-108)
- Some biomarkers detected in 100% of women (phytoestrogens, PCBs, hexachlorobenzene, perchlorate, PFAS, metals, and PAHs)
External exposure

Personal passive sampling devices

- Kim Anderson (OSU) – NIEHS Superfund grantee
- Methods for measuring ~1400 chemicals in extracts from passive sampling devices
Personal care and consumer products

Project lead: Kyla Taylor (OHAT)
Combined Exposures/Mixtures

- Defined (aka simple) mixture – All components are identified and quantified (e.g., pharmaceutical combination)

- Complex mixture – Many constituents with some unidentified fraction (e.g., source emission like diesel exhaust)

- Whole mixture – Often used interchangeably with complex mixture, but is defined here as consideration of an entire mixture without regard to component data (regardless of complexity)

- Exposome – Totality of exposures over a lifetime
Defined mixture

- Aggregate – includes consideration of different routes
- Cumulative – addition of multiple components
  - Dose addition – Adding chemicals at the dose level; typically applied to chemicals with similar mechanisms of action
  - Independent action or response addition – Adding chemicals at the response level; typically applied to chemicals with different mechanisms of action
Concepts of additivity

**Dose Addition**

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

**Mixture Response**

**Independent Action**

\[ R_{\text{mix}} = 1 - \prod_{i=1}^{n} \left(1 - R_i\right) \]

**Response A** + **Response B**

**Mixture Response**

Background
Implications of model selection

- Independent action – As long as all chemicals in the mixture are below their individual No Observed Adverse Effect Level (NOAEL), the mixture is not expected to have an adverse effect.

- Dose addition – Chemicals below their NOAEL can contribute to a total dose that elicits an adverse effect.

- Therefore, *if* chemicals are **dose additive**, a cumulative risk assessment should be performed to protect human health.
Defined mixture

- Aggregate – includes consideration of different routes
- Cumulative – addition of multiple components
  - Dose addition – Adding chemicals at the dose level; typically applied to chemicals with similar mechanisms of action
  - Independent action or response addition – Adding chemicals at the response level; typically applied to chemicals with different mechanisms of action
- Interaction – deviation from additivity
  - Greater than additivity (formerly synergy) – the combination produces an effect at a lower dose than predicted from the appropriate additivity model
  - Less than additivity (formerly antagonism) – the combination produces an effect at a higher dose than predicted from the appropriate additivity model
Characterizing interaction

Response

Greater than additive

Less than additive

[Mixture]

Prediction based on additivity model

Background
History of mixtures research at NTP

1970s
- 1978: NTP Established
- Mixture of Aspirin, phenacetin, and caffeine (TR 67)

1980s
- 1983-1986: Firemaster FF1 (TR244)
- Marine Diesel Fuel and JP-5 Navy Fuel (TR 310)

1990s
- 1997: NIEHS/NTP IAG with NIOSH to characterize and evaluate complex occupational exposures
- Bucher and Lucier Manuscript on Mixtures at NIEHS and NTP
- NTP Botanical Workshop

2000s
- 2004-2006: Mixtures of dioxin-like chemicals (TR 520, 521, 525, 526, 529, 530, 531)
- Polycyclic aromatic compound Mixtures Assessment Program

2010s
- 2011: NIEHS Mixtures Workshop
- Polycyclic aromatic compound Mixtures Assessment Program

2010-2016
- Ephedra + caffeine
- Aloe vera, Ginkgo biloba, Ginseng, Goldenseal, Green tea, Kava kava, Milk thistle
- Metal working fluids (TR 586 and 591)

1993
- 25 Groundwater Contaminants (TOX 35)
- Pesticide/Fertilizer Mixtures (TOX 36)

1998-present
- Combo AIDS drugs (AIDS 02-09)

2000
- 2010: Mixtures included in NIEHS Strategic Plan

2012
- 2012: Complex Mixture Nominations

2016
- 2016: NTP Botanical Workshop

Background
Understanding health effects of mixtures

Problem formulation

Is Data Quality Adequate?

No quantitative assessment; only qualitative assessment

Whole Mixture Data

- Mixture of concern
- Sufficiently similar mixture
- Group of similar mixtures

- Mixtures RfD/C; Slope Factor
- Comparative Potency

Component Data

- Do components interact?

Components have similar MOAs
- Dose Addition

Components have different MOAs
- Response Addition

Interactions Based Hazard Index

Problem formulation: Choose your own adventure

1. Are you starting from an exposure (e.g., occupational, specific class of chemicals) or a disease or a population?
   - If exposure, use monitoring or modeling data to define the exposure profile
   - If disease, determine which stressors impact disease development
   - If population, characterize exposures within the population

2. Regardless of starting place, at some point, decisions regarding which exposures to include in the health evaluation are required
Deciding which stressors to include

Considering biological similarity

<table>
<thead>
<tr>
<th>Most similar</th>
<th>Chemicals share a...</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common active metabolite</td>
<td>Benzyl butyl phthalate and dibutyl phthalate share the active metabolite monobutyl phthalate</td>
<td></td>
</tr>
<tr>
<td>Molecular initiating event</td>
<td>Parathion and chlorpyrifos both inhibit acetylcholinesterase and elicit the same downstream key events</td>
<td></td>
</tr>
<tr>
<td>Adverse outcome pathway</td>
<td>Perchlorates decreases synthesis of thyroid hormone, while dioxin increases elimination of thyroid hormone</td>
<td></td>
</tr>
<tr>
<td>Target tissue</td>
<td>Ephedrine and caffeine are both cardiotoxic</td>
<td></td>
</tr>
<tr>
<td>Disease</td>
<td>DES and tobacco smoke cause cancer in different tissues</td>
<td></td>
</tr>
</tbody>
</table>

Least similar
Understanding health effects of mixtures

Problem formulation

Is Data Quality Adequate?

Whole Mixture Data
- Real-world: Assess the whole mixture (or sufficiently similar mixture) and proceed as with a single chemical
- Collected or process-generated mixtures
- Fewer assumptions required
- Data rarely available; methods for determining sufficient similarity needed

Component Data
- Simplified/reductionist: Use individual chemical data and additivity models to estimate the toxicity of the mixture
  - Defined (artificial) mixtures
  - Single chemical data available
- Many assumptions required to extrapolate from tested mixture to real world mixture

General description

Mixtures

Advantages

Disadvantages
Mixture of concern

Paradox

• Strong preference for data on the “mixture of concern”
  – Decreases assumptions involved in estimating health effects and risk from exposure to the mixture

• Defining and testing the mixture of concern is not actually possible in the case of complex mixtures
Mixture of concern

Botanical dietary supplement example

Test article

Raw plant material → Extract → Finished product

Sources of variation

- Plant part (aerial, root, whole plant, leaf, seed)
- Climate
- Soil conditions
- Season
- Plant maturity
- Contaminants (mold, pesticides, metals)
- Co-harvested materials (other plants, soil)
- Adulteration

- Extraction process
- Solvents
- Adulteration
- Contamination
- Storage/shipping conditions
- Manufacturing process
- Excipients
- Combination with other botanicals
- Adulteration
- Contamination
- Storage/shipping conditions

Exposure

Background
Whole mixture testing

Mixture of concern

- Botanical dietary supplements
  - Aloe vera, bitter orange, ephedra and ephedra with caffeine, *Echinacea purpurea* extract, *Ginkgo biloba* extract, ginseng, goldenseal root powder, green tea extract, kava kava, milk thistle, senna

- Commercial mixtures
  - Flame retardants (e.g., Firemaster FF-1), metal working fluids (e.g., TRIM® VX)

- Fuels
  - Marine diesel fuel, Jet fuel (JP-5)

- Simulated environmental mixtures
  - Groundwater contaminants (25 chemicals)
  - Pesticide/fertilizer mixture
Whole mixture testing

Key issues

- Chemical analysis
  - Targeted
  - Untargeted
- Test article selection
- Extrapolating findings to related whole mixtures
- Understanding which constituent(s) are driving observed effects
- Deciding which metabolite(s) to measure in TK/ADME studies

Known (identified) fraction: targeted chemistry to quantify constituents; methods and analytical standards required for each constituent

Unknown (unidentified) fraction: untargeted chemistry required
NTP Mixtures Research

Problem formulation → Is Data Quality Adequate?

- Whole Mixture Data
  - Mixture of concern
  - Sufficiently similar mixture
  - Group of similar mixtures
    - Mixture RfD/C; Slope Factor
    - Comparative Potency

- Component Data
  - Do components interact?
    - Components have similar MOAs
      - Dose Addition
    - Components have different MOAs
      - Response Addition
      - Interactions Based Hazard Index

No quantitative assessment; only qualitative assessment

Dioxin mixture studies

Hypothesis: The carcinogenic effects of a mixture of dioxin-like chemicals can be predicted using individual dose-response data in a dose additive model.

Reference chemical

\[
RPF = \frac{ED50_{\text{TCDD}}}{ED50_{\text{TCDD}}} = 1
\]

33 ng x 1 = 33 ng

RPF = \frac{ED50_{\text{TCDD}}}{ED50_{\text{PCB-126}}} = 0.1

333 ng x 0.1 = 33 ng

RPF = \frac{ED50_{\text{TCDD}}}{ED50_{\text{PeCDF}}} = 0.1

66 ng x 0.1 = 6.6 ng

Total TCDD (73 ng)

Walker et al., 2005. Environ Health Persp 113:43-48
- Dose additivity is an appropriate model for this group of chemicals – supporting the current risk assessment framework
- Component-based mixture predictions could be applied to chronic endpoints
- Notable interactions were not observed
Lessons from past NTP mixtures work

- Assessing whole mixtures without a strategy for comparing across related mixtures invites criticism of test article selection by industry and prevents partner agencies from effectively utilizing resulting data.

- Hypothesis driven mixtures research (e.g., dioxin project) yields more interpretable and useful data than exploratory research (e.g., 25 chemical mixture low dose project)
  
  - A: The carcinogenic effects of a mixture of dioxin-like chemicals can be predicted using individual dose-response data in a dose additive model.
  
  - B: If we build a mixture of 25 chemicals based on common exposure ratios and test at human-relevant doses will we see unexpected/impressive toxicity?
Evolution in mixtures toxicology

- **20th Century**
  - Dose addition versus independent action – which model is more appropriate?
  - Looking for the unexpected – which chemicals might have (much) greater effects in combination than alone?

- **21st Century**
  - Dose addition often viewed as a default approach – focus on defining the boundaries of its application
  - Prioritizing which chemicals to evaluate for cumulative effects viewed as more important than possible interactions
  - Developing whole mixture approaches
  - Adapting high throughput tools for use with mixtures
NIEHS Mixtures Workshop

Sept 26-27, 2011

• 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
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
Coordinating across NIEHS

- Meets quarterly to discuss mixtures projects throughout NIEHS
- Updates on mixtures-related activities (e.g., workshops, seminars)
- Development of a logic model to guide prioritization of NIEHS mixtures efforts

Danielle Carlin (DERT)
Outline

• Background

• Mixtures research areas
  – Component-based approaches
  – Whole mixture approaches
  – Systems biology approaches

Clarifying questions
Understanding health effects of mixtures

Problem formulation

Is Data Quality Adequate?

Whole Mixture Data
- Mixture of concern
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  - Comparative Potency

Component Data
- Do components interact?
  - No
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      - Dose Addition
    - Components have different MOAs
      - Response Addition
  - Yes
    - Interactions Based Hazard Index

Component-based approaches

Considerations

- Require dose-response data on all individual components and the defined mixture of interest
  - Uncertainty in individual component data will feed into uncertainty in cumulative assessment
- Relatively few examples of application, particularly with higher order mixtures (>10 components)
- Assumptions required
  - Chemicals adhere to a specific model of additivity (dose addition, independent action)
  - Chemicals do not interact
- Requires appropriate statistical methods for determining deviation from additivity
Risk assessment

- Quantitative cumulative risk assessments of pesticides with a common mechanism of action
  - Organophosphates, N-methyl carbamates, triazines, choroacetanilides, pyrethrins/pyrethroids
- Guidance documents providing potency information on chemicals with similar mechanisms of action
  - Dioxin-like chemicals
  - Polycyclic aromatic hydrocarbons
- Screening level analysis of Superfund site chemicals (regardless of mechanisms) using a hazard index approach loosely based on dose addition concept
Polycyclic aromatic hydrocarbons (PAHs)

Defining the problem

- Multiple diverse sources of exposure to complex, dynamic mixtures
  - Very limited whole mixture cancer data available (e.g., coal tar), with significantly more data available for individual parent PAHs
  - Methods to compare across complex mixtures are still under development

- Multiple routes of exposure possible

- Many known toxicities associated with some chemicals in the class (cancer, neurotoxicity, immunotoxicity, developmental and reproductive toxicity)

Component-based approaches
Cancer risk from PAH exposure

EPA IRIS

- 1993 Provisional Guidance for Quantitative Risk Assessment of PAHs
  - Provided relative potency information for 7 PAHs
  - Only addressed parent PAHs and cancer risk
  - Used extensively for risk assessment by EPA and other groups (e.g., seafood safety following Gulf Oil Spill)

Reference compound (benzo[a]pyrene)

Component-based approaches
Cancer risk from PAH exposure

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• 2010 Draft Development of a Relative Potency Factor (RPF) Approach for Polycyclic Aromatic Hydrocarbon (PAH) Mixtures
  - Update to 1993 guidance – providing relative potency information for 27 PAHs
  - 2001 Workshop to inform development of document
  - Reviewed by a Scientific Advisory Board in 2011

Component-based approaches
Uncertainties and limitations

2001 Workshop on Approaches to PAH Health Assessment

• The RPF approach only considers a small subset of PAHs (i.e., unsubstituted PAHs only, no heterocyclic compounds or nitro- or alkyl- substituted PAHs)

• There are no human toxicity data for any individual PAH

• The assumption of additivity may not be valid, and there may be interactions among PAHs or between PAHs and other components of a mixture (e.g., metals)

• PAHs may generally have a common mode of action (i.e., mutagenicity), but multiple modes of action for carcinogenesis are possible

• The approach is limited to the oral exposure route (i.e., a recommendation was made not to apply the factors to dermal and inhalation exposures)

From 2010 Draft document
The SAB recognized “the pragmatic need for the RPF approach” based on currently available data; however, the SAB recommends:

- Strengthening the rationale for the RPF approach
- Strengthening the rationale for the assumption that there are no interactions among PAHs at environmentally relevant doses
- Generating RPF data only for PAHs with available cancer bioassay data (cancer endpoint data is not a substitute)
- Developing a whole mixture approach
- Working with the NTP to develop whole mixture datasets on relevant complex PAH mixtures
NTP and PAHs

Nominations and scoping

• Motivation
  – 1984-2005 Multiple nominations for PAHs
  – 2010 Deepwater Horizon Oil Spill and SAB review of EPA RPF approach

• Considerations
  – Compared to many other chemical classes, there is a great deal of data available for PAHs, particularly benzo[a]pyrene
  – Responding directly to the SAB proposal for NTP to test multiple (10-12) complex mixtures in 2-year cancer studies would take too long and be prohibitively expensive

• Stakeholder input
  – Discussions with EPA colleagues
  – 2012 SOT Workshop: Sufficient similarity of whole representative mixtures or a relative potency factor approach: PAHs as a Case Study

Component-based approaches
NTP PAC* Mixtures Assessment Program

Addressing uncertainties

- "The RPF approach only considers a small subset of PAHs (i.e., unsubstituted PAHs only, no heterocyclic compounds or nitro- or alkyl- substituted PAHs)" – 2001 EPA Workshop
  
  - Evaluate a broader range of compounds, *hence Polycyclic Aromatic Compounds (PACs) instead of PAHs
  
  - Develop high throughput approaches that can be used to rapidly assess a large number of PACs

- "The assumption of additivity may not be valid, and there may be interactions among PAHs or between PAHs and other components of a mixture (e.g., metals)" – 2001 EPA Workshop

  - Challenge the assumption that PAHs do not interact

- "PAHs may generally have a common mode of action (i.e., mutagenicity), but multiple modes of action for carcinogenesis are possible" – 2001 EPA Workshop

  - Explore other "icities" and adverse outcome pathways

Component-based approaches
PAC Mixtures Assessment Program

Addressing uncertainties (continued)

- "Strengthening the rationale for the RPF approach" – EPA SAB
  - Apply the RPF approach to PACs
  - Investigate the limitations of the RPF approach and how broadly it can be applied to the class
- "Develop a whole mixture approach" – EPA SAB
  - Generate whole mixture chemistry and biological data to contribute to the development of a whole mixture approach
  - Compare results from whole mixture evaluation to component-based evaluation of PAC mixtures
- "Bridging in vitro and in vivo responses" and developing "high-throughput screening methods to assess interactions and mixtures" – 2011 NIEHS Workshop
  - Develop and evaluate in vitro approaches for PAC mixtures
PAC Mixtures Assessment Program

Summary of approach

**Screening Goals**

1. Screen a wide array of structurally-diverse PACs in order to develop QSARs, prioritize PACs for *in vivo* testing, and develop an effective *in vitro* screening battery for PACs
2. Assess multiple complex PAC mixtures

**Assays/tools:**
- Tox21 high throughput screening (HTS) assays
- Pluripotent stem cells (*neurite outgrowth, cardiomyocytes*)
- Cytotoxicity and gene expression in diverse cell lines
- HepaRG cytotoxicity and enzyme induction assays
- Zebrafish developmental assay

**Testing Goals**

1. Generate dose-response data for individual PACs and defined PAC mixtures to systematically evaluate the RPF approach
2. Assess a complex PAC mixture to compare across approaches

**Assay:** 28-Day Immunotoxicity Studies (oral gavage) in B6C3F1/N female mice (*n* = 8)

**Endpoints:**
- Organ weights
- Antigen-specific Ab formation
- Histology of immune tissues
- Immune cell populations from spleen or whole blood
- Hematology
## In vitro summary

### Comparison to cancer data

<table>
<thead>
<tr>
<th>PAC</th>
<th>RPF</th>
<th>HTS</th>
<th>Cyto</th>
<th>HepaRG</th>
<th>Neur</th>
<th>Cardio</th>
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<tbody>
<tr>
<td>Dibenz[a,c]anthracene</td>
<td>4</td>
<td>+++</td>
<td>++</td>
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<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*2010 EPA draft Development of a Relative Potency Factor (RPF) Approach for Polycyclic Aromatic Hydrocarbon (PAH) Mixtures*

Component-based approaches
In vitro next steps

Assessing a broad range of PACs

- Cytotoxicity in 13 cell lines
  - Assay selected based on preliminary data from in vitro/alternative animal assays demonstrating good correlation with in vivo cancer potency data
  - Cell lines included with the goal of covering maximum biological space (based on tissue of origin and gene expression)

- Assessing > 80 individual PACs and ~10 complex PAC mixtures

Component-based approaches
In vivo RPF assessment

Component-based approaches
In vivo RPF assessment

Predicted versus observed

1. Generate dose-response data for individual PACs (in progress)
2. Input data into available component-based models (RPF, other dose addition models, independent action model)
3. Assess the toxicity of mixtures made up of individual chemicals (i.e., generate “observed” mixture data)
4. Compare observed mixture toxicity to predicted mixture response
   - Observed data matches RPF predictions → supports assumptions
   - Observed data deviates from predictions → potential interaction among constituents

Component-based approaches
In vivo RPF assessment

Hypothesis-driven research

1. PAC mixtures will be dose additive and the Relative Potency Factor approach will provide a good approximation of mixture toxicity
   - Individual PACs will not interact when present in mixtures

2. Relative potency factors generated from immunotoxicity studies will be similar to those from carcinogenicity studies

3. Accounting for the toxicity of a subset of known components will adequately approximate the toxicity of a complex mixture
   - Other components (i.e., the unidentified fraction) will not meaningfully contribute to mixture toxicity

Component-based approaches
Relevance to other complex classes

Ongoing NTP class studies

- Phthalates
- Bisphenols
- Flame retardants
- Perfluorinated compounds
- Water disinfection byproducts
Health effects generally assessed on a chemical-by-chemical basis...

Better understanding of the application and limitations of component-based approaches will help to move from single chemical to cumulative assessments, bringing us closer to real-world exposures.

Relative potency data from toxicology studies can be used in epidemiology studies to assess associations between a total class value (e.g., total PAC) and the health endpoint of interest.
Outline

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• Mixtures research areas
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  – Whole mixture approaches
  – Systems biology approaches

Clarifying questions
Assessing risk from mixtures

Is Data Quality Adequate?
- yes
  - Whole Mixture Data
    - Mixture of concern
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  - Component Data
    - Do components interact?
      - no
        - Components have similar MOAs
          - Dose Addition
        - Components have different MOAs
          - Response Addition
      - yes
        - Interactions Based Hazard Index
- no
  - No quantitative assessment; only qualitative assessment
Whole mixture approaches

Sufficient similarity

- There is no single “mixture of interest” for complex mixtures
- What are the options for evaluating the health effects of whole, complex mixtures?
  1. Assume the tested mixture is representative of the mixtures of interest (i.e., all complex mixtures tested at NTP)
  2. Develop an approach for determining whether or not a tested mixture is sufficiently similar to mixtures of interest
Sufficient similarity

Definition

• Refers to a “mixture that is very close in composition to the mixture of concern, such that differences in their components and their proportions are small”

• “The toxicologic consequences of exposure to the two mixtures (i.e., the mixture of concern and the mixture on which data are available) will be identical or at least indistinguishable from one another”
Mixture of concern

Botanical dietary supplement example

Sources of variation

- Plant part (aerial, root, whole plant, leaf, seed)
- Climate
- Soil conditions
- Season
- Plant maturity
- Contaminants (mold, pesticides, metals)
- Co-harvested materials (other plants, soil)
- Adulteration
- Extraction process
- Solvents
- Adulteration
- Contamination
- Storage/shipping conditions
- Manufacturing process
- Excipients
- Combination with other botanicals
- Adulteration
- Contamination
- Storage/shipping conditions

Exposure

Whole mixture approaches
“The unique Ginkgo biloba leaf extract discussed in TR-578 is not representative of other Ginkgo biloba leaf extracts marketed in the United States, and is almost certainly not sold in the United States. It is incorrect to represent it as similar to other Ginkgo biloba leaf extracts based on the dissimilarity of its chemical composition to that of other commercially available Ginkgo biloba leaf extracts.” American Herbal Products Association (AHPA) public comments on TR-578 (slides), February 8, 2012

“The title of NTP TR 585 should be changed to accurately reflect that the green tea extract used in these studies is a unique ingredient that may or may not be similar to other green tea leaf extracts marketed in the United States...All statements in NTP TR 585 that claim or infer that the tested green tea extract is similar to other green tea extracts should be removed.” AHPA written comments on TR 585, May 8, 2014

“...we are concerned that NTP researchers may be erroneously basing its oral consumption toxicity analysis on an Aloe Vera product sample that is not reflective of the products currently marketed in the US and exported in large quantities.” Congressional Inquiry, June 18, 2010

“The Committee urges NTP to be highly precise when describing the results of its studies on particular extracts of an herbal species to avoid any possible confusion about the relevance of such studies to other extracts of the species.” The United States Senate Appropriations Committee in report accompanying the fiscal year 2014 Labor, Health and Human Services and Education Appropriations spending bill

Whole mixture approaches
NTP selected an inappropriate test article that is not representative of anything else in the marketplace.
Exploring sufficient similarity

Ginkgo biloba extract

- Exposure: Popular botanical dietary supplement with an estimated 1.6 million Americans taking it in 2012*

- Toxicity: Major toxicity targets of liver, nose, and thyroid gland generally consistent across sex, species, and exposure period
  - Conclusions from 2-year studies: Some evidence of carcinogenicity in male and female rats based on thyroid tumors and clear evidence of carcinogenicity in male and female mice based on liver tumors

- Doubt: The test article had high levels of some constituents (15% terpene lactones compared with 6% recommended in standardized extract)
Exploring sufficient similarity

Ginkgo biloba extract

- How should we go about determining sufficient similarity?
- Does chemical similarity reflect biological similarity?
- Is there a clear point of divergence from similarity?
- How should scientific judgment be applied?
- Can unsupervised approaches be used to determine similarity?
- Can we develop similarity criteria?
- Are there methods that can be applied across different mixtures or is a case by case approach needed?
How similar is similar enough?

Case studies

- **Ginkgo biloba** extract
  - Chemistry: Relatively large identified fraction; known marker constituents
  - Biology (NTP): Noted *in vivo* effects – hepatotoxicity, pathways identified

- **Black cohosh** extract
  - Chemistry: Large unidentified fraction; low confidence that marker constituents are associated with toxicity
  - Biology (NTP): Genotoxicity

- **Echinacea purpurea** extract
  - Chemistry: Large unidentified fraction
  - Biology (NTP): Weak activity – Enhanced immune response

Whole mixture approaches
Chemical similarity

~ 60 mg/mL GBE in 80:20 Ethanol:Water (v/v)
Ginkgolide B
Suspected Flavonol Glycosides
Peak Used for RRT
**System stopped after this injection. System was restarted the following day and a slight shift in retention times was noted.

Constituent concentration (% of total mass)

Whole mixture approaches
Chemical similarity

 Constituent concentration (% of total mass)

Whole mixture approaches
Primary human hepatocytes

NTP Laboratories

- *In vitro* liver model used to predict drug metabolism and drug-drug interactions

- Endpoints of interest:
  - AhR
  - CAR
  - PXR
  - FXR
  - PPARα
  - ARNT

  CYP1A2
  CYP2B6
  CYP3A4
  ABCB11
  HMGCS2

Whole mixture approaches
5-Day Rat Studies

Evaluation of a subset of *Ginkgo biloba* extracts

- Study design
  - 5 chemically-diverse lots
    - NTP (1), G, N, P, T
  - 5-Day oral gavage
    - Doses: 0, 3, 30, 100, 300, 1000 mg/kg/day
  - F344 rats
- Endpoints
  - Organ weights
  - Clinical chemistry and hematology
  - Gene expression in liver

Whole mixture approaches
Determining sufficient similarity

Chemistry

In vitro

Liver weight

Average lipid accumulation score

In vivo

Biological process sensitivity

Similarity intersect

Whole mixture approaches
Conclusions

- The NTP test article was most similar to NIST standards and EGb761® samples (gold standard in the marketplace)

- Based on chemistry alone, NTP selected an appropriate test article
  - A combination of chemistry and targeted *in vitro* analysis is recommended for future comparisons

- There were *Ginkgo biloba* extract bulk samples that did not resemble standardized extract, and others that were obviously adulterated with flavonol aglycones or unknown material

- In at least one case, it appears that a non-*Ginkgo biloba* extract constituent(s) is responsible for some biological activity (e.g., sample containing Gotu kola)

- Terpene lactones appear to be driving the hepatotoxicity observed in the *in vivo* studies
Relevance to other complex mixtures

NTP projects

- PAC-MAP
- Crumb rubber
- Glyphosate formulations
- Flame retardant formulations
- Metal working fluids
- All other botanical dietary supplements
- Personal care products
- Water disinfection byproducts

Whole mixture approaches
Developing whole mixtures approaches that can be broadly applied will have a huge impact on our ability to estimate health effects from exposure to complex mixtures.

Whole mixture approaches provide a bridge between toxicology and epidemiology.

Methods are also relevant to read-across efforts for single chemicals.
Outline

- Background

- Mixtures research areas
  - Component-based approaches
  - Whole mixture approaches
  - Systems biology approaches

Clarifying questions
Assessing risk from mixtures

Problem formulation

Is Data Quality Adequate?

- no
  - No quantitative assessment; only qualitative assessment

- yes
  - Whole Mixture Data
    - Mixture of concern
    - Sufficiently similar mixture
    - Group of similar mixtures
      - Mixture RfD/C; Slope Factor
      - Comparative Potency

  - Component Data
    - Do components interact?
      - no
        - Components have different MOAs
          - Components have different MOAs
            - Dose Addition
            - Response Addition
            - Interactions Based Hazard Index
      - yes

Deciding which stressors to include

Considering biological similarity

<table>
<thead>
<tr>
<th>Most similar</th>
<th>Chemicals share an...</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common active</td>
<td></td>
<td>Benzyl butyl phthalate and dibutyl phthalate share the active metabolite</td>
</tr>
<tr>
<td>metabolite</td>
<td></td>
<td>monobutyl phthalate</td>
</tr>
<tr>
<td>Molecular</td>
<td></td>
<td>Parathion and chlorpyrifos both inhibit acetylcholinesterase and...</td>
</tr>
<tr>
<td>initiating event</td>
<td></td>
<td>elicit the same downstream key events</td>
</tr>
<tr>
<td>Adverse outcome</td>
<td></td>
<td>Perchlorates decreases synthesis of thyroid hormone, while dioxin...</td>
</tr>
<tr>
<td>pathway</td>
<td></td>
<td>increases elimination of thyroid hormone</td>
</tr>
<tr>
<td>Target tissue</td>
<td></td>
<td>Ephedrine and caffeine are both cardiotoxic</td>
</tr>
<tr>
<td>Disease</td>
<td></td>
<td>DES and tobacco smoke cause cancer in different tissues</td>
</tr>
</tbody>
</table>

Systems biology approaches
Background

- Standard practice: only perform cumulative risk assessments on chemicals that share molecular initiating events (e.g., organophosphates)

- In 2008, EPA asked the National Research Council to review the data on phthalates and determine whether or not a cumulative risk assessment should be performed

- NRC reviewed the data on phthalate mixtures and recommended including not only phthalates but other chemicals that disrupt androgen signaling

Systems biology approaches
Data supporting NRC conclusions

Phthalates and other antiandrogens

• Combinations of phthalates
  – Binary combinations: DBP+BBP, DBP+DEHP
  – 5 phthalate mixture: DBP+BBP+DEHP+DiBP+DPeP
  – 9 phthalate mixture:
    DBP+BBP+DEHP+DiBP+DPeP+DHP+DHeP+DiHeP+DCHP

• Combinations of phthalates and other antiandrogens
  – Binary: DBP+linuron, DBP+procymidone
  – 7 chemical mixture: DBP+BBP+DEHP+linuron+prochlorz+
    procymidone+vinclozolin
  – 10 chemical mixture:
    DBP+BBP+DEHP+DiBP+DiHeP+linuron+prochlorz+procymidone+
    vinclozolin

Conclusion from body of work: Dose addition generally provides a better fit than independent action, supporting inclusion of phthalates and other antiandrogenic chemicals in a cumulative risk assessment

From Howdeshell et al., 2017. IJHEH 220:179-188
Network of AOPs

Dibutyl phthalate

MIE: Unknown → ↓ Steroidogenic enzyme expression → ↓ Testosterone production [by Leydig cells] → ↓ Activation of Androgen receptor at target tissues → Disruption of target tissue development

↓ Cholesterol (precursor to steroid hormones) → MIE: Competitive Inhibition of HMG-CoA reductase

Simvastatin

Vinclozolin

MIE: Binding to androgen receptor

Unknown series of key events

MIE: Binding to Aryl hydrocarbon receptor

Dioxin

MIE = Molecular Initiating Event

Systems biology approaches
Systems-based approaches

Relevant projects

- Evaluating mixtures of chemicals that disrupt lipid signaling and steroid hormone production
- Biological Mechanisms/Pathways of the Combined Effects of Chemical and Non-chemical Stressors Associated with Atherosclerosis
- CNVERGE: Cancer Network and enVironmental Exposure Research aGEnda
Targeting lipid signaling

- Fatty acid catabolism
  - Acetyl-CoA
  - Cholesterol/Fatty Acid Pool
  - Isoprenes
- Statins
- Cholesterol
  - Bile Salts
  - Imidazole antifungals
  - Steroids
  - Phthalates
- Fibrates
  - Fatty Acids
  - Modified Fatty Acids
    - (prostaglandins and leukotrienes)
- Progestins
- Estrogens
- Androgens
- AR antagonists

Target tissue

Systems biology approaches
Biological Mechanisms/Pathways of the Combined Effects of Chemical and Non-chemical Stressors Associated with Atherosclerosis

Organizers: Danielle Carlin (DERT) and Michelle Olive (NHLBI)
Inflammation and atherosclerosis

Analytical Framework

Example Exposures
- Major environmental compounds
  - particulate matter
  - diesel exhaust particles
  - POPs
  - metals
  - tobacco
- Infectious agents
  - CMV
  - Chlamydia pneumonia
  - Helicobacter pylori
  - HCV
  - influenza A virus
  - Porphyromonas gingivalis

Example Indicators
- Indicators of inflammation linked to atherosclerosis
  - C-reactive protein
  - chemokines
  - fibrinogen
  - IFN-γ
  - integrins
  - NF-kB
  - selectins
  - TNF-α
  - Von Willebrand factor
  - interleukins (IL-1, IL-2, IL-6, IL-8)

Health Effect
- Established role for inflammation

Examine support for temporal sequence (i.e., exposure → inflammation → atherosclerosis)

Systems biology approaches
Cancer Pathways and Mixtures

CNVERGE background

  - Goal: Bring together cancer researchers and environmental scientists to identify mixtures that target the hallmarks of cancer


Mark Miller (OD)

Nicole Kleinstreuer (NICEATM)

Systems biology approaches
Hypothesis

Chemicals present at low levels that would not be expected to elicit cancer, can contribute to the development of cancer by acting on different pathways.

Environmental Working Group Nomination to NTP: Experimentally evaluate the hypothesis proposed by the Halifax Project
Path forward

- Focus on a specific cancer type (e.g., breast cancer)
  - Considerations for identification of animal model: human relevance, short timeframe to cancer development, incorporation of genetic instability hallmark

- Identify key molecular targets associated with each hallmark and develop in vitro assays for each target

- Build a list of chemicals that interact with each of the identified targets
  - Considerations: specificity, environmental relevance

Systems biology approaches
## Identifying targets and candidate chemicals

<table>
<thead>
<tr>
<th>Hallmark</th>
<th>Molecular target</th>
<th>Candidate chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiogenesis</td>
<td>↑ VEGF</td>
<td>Nicotine</td>
</tr>
<tr>
<td>Evading immune destruction</td>
<td>Complement system</td>
<td>PAHs</td>
</tr>
<tr>
<td>Sustaining proliferative signaling</td>
<td>AhR or ER activation</td>
<td>Bisphenol A, phytoestrogens</td>
</tr>
<tr>
<td>Evading growth suppression</td>
<td>p53 or Rb inhibition</td>
<td>Arsenite</td>
</tr>
<tr>
<td>Invasion and metastasis</td>
<td>↑ EMT pathway</td>
<td>NNK, hexachlorobenzene</td>
</tr>
<tr>
<td>Enabling replicative immortality</td>
<td>↑ hTERT</td>
<td>Nickel, acetaminophen</td>
</tr>
<tr>
<td>Resistance to cell death</td>
<td>↓ pro-apoptotic signaling</td>
<td>Sulfonamides</td>
</tr>
<tr>
<td>Inflammation</td>
<td>↑ cytokine signaling</td>
<td>Infectious agents, asbestos, silica</td>
</tr>
<tr>
<td>Reprogramming energy metabolism</td>
<td>Mitochondrial electron transport chain disruption</td>
<td>Organophosphates, pyrethroids</td>
</tr>
<tr>
<td>Genome instability</td>
<td>Oncogene mutation</td>
<td>Genotoxic agents or genetic predisposition</td>
</tr>
</tbody>
</table>

Systems biology approaches
Conclusions

• NTP is tackling big mixtures questions using the latest toxicology tools
• Efforts are concentrated in areas that will provide data to inform
  1. Application and refinement of component-based risk assessments
  2. Development and application of whole mixtures approaches
  3. Prioritization of chemicals for inclusion in cumulative risk assessment based on knowledge of biological systems
All of NTP has been involved in mixtures work!

Scott Auerbach
Linda Birnbaum
John Bucher
Danielle Carlin (DERT)
Mike DeVito
Gregg Dinse (DIR)
Paul Foster
Kembra Howdeshell (OHAT)
Scott Masten
David Umbach (DIR)
Suramya Waidyanatha
Nigel Walker
Outline

• Background

• Mixtures research areas
  – Component-based approaches
  – Whole mixture approaches
  – Systems biology approaches

Clarifying questions
Questions for BSC

• Please comment on whether NTP is addressing the highest priority questions in mixtures toxicology to inform risk assessment.

• How would you rank the importance and tractability of the three areas discussed?

• What do you anticipate will likely be NTP’s most challenging obstacles in achieving meaningful contributions to understanding mixtures toxicity?

• Are there additional areas in mixtures toxicology on which NTP should focus effort?

• Please comment on whether the selected test articles (noted below) are appropriate for exploring the stated challenge:
  
  – Uncertainties in the relative potency factor approach – PACs
  
  – Sufficient similarity of whole mixtures – botanicals
  
  – Systems-based prioritization of chemicals for cumulative risk assessment – chemicals that target the hallmarks of cancer