Applying In vitro Approaches to Understand Complex Mixtures in Assessing Botanical Safety

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Mixtures risk assessment framework

Problem formulation

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
  - Dose Addition
  - Response Addition

Yes

- Components have different MOAs
  - Interactions Based Hazard Index

Current research at NTP

- Bisphenol A & Analogues
- Botanical Dietary Supplements
- Cell Phones
- Glyphosate & Formulations
- Medicines & Therapeutics
- Mold
- Nanomaterials
- Polycyclic Aromatic Compounds
- Synthetic Turf/ Crumb Rubber
Botanical dietary supplements

Widespread exposure + relatively high doses

- Approximately 18% of adults in the U.S. (~40 million people) used nonvitamin, nonmineral dietary supplements in the past 12 months according to the 2012 National Health Interview Survey.
- Recommended doses can be in the range of 100s - 1000s mg per day.

NTP interest in botanicals

- NTP evaluates substances that are of public health concern
- There is little safety data on most botanicals
- Public concern about the quality and integrity of botanicals available in the marketplace
- NTP has received a number of nominations to study botanical dietary supplements
  - National Cancer Institute (9), NIEHS (5), Private Individuals (3), FDA (2)

Botanical Supplements Come From Nature, But That Doesn't Mean They're Safe.

Manufacturers of supplements aren't required to demonstrate to the government their products are effective or safe.

Experts: Oversight needed for safety, efficacy of nutritional supplements

By Brian P. Dunleavy
1994 Dietary Supplement Health and Education Act

- Amends the FD&C – created a regulatory framework for dietary supplements
  - Intent: Balance consumer access and consumer protection
  - Defines dietary supplements as foods and excludes them from consideration as food additives
  - Puts the burden of proof for risk on FDA (i.e., FDA has to prove that a dietary supplement is not safe)
  - Clarifies labeling requirements
  - Requires new dietary supplement ingredients to be registered with the FDA
  - Specifies Good Manufacturing Practices for dietary supplements
  - Created the Office of Dietary Supplements at NIH

• Consensus statements on history of safe use:
  
  – The safety of a botanical cannot be judged based solely on a history of food use unless it can be demonstrated that a comparable composition is ingested on a regular basis across broad geographic and demographic populations.
  
  – In the assessment of a botanical, it is misleading to assume that a history of human use addresses all aspects of safety.
Current NTP botanical portfolio

**Completed**
- *Aloe vera* nondecolorized whole leaf extract
- Bitter orange extract
- *Ephedra* (ma huang)
- Ginseng root extract
- *Ginkgo biloba* extract
- Goldenseal root powder
- Green tea extract
- Gum guggul extract
- Kava kava extract
- Milk thistle extract
- *Senna*

**Ongoing**
- Black cohosh extract
- Dong quai (root powder or extract)
- *Echinacea purpurea* extract
- Garcinia cambogia
- *Usnea lichen*
- Valerian root extract
Testing approach

• Identify knowledge gaps
  – Specific concern: Ephedra and cardiotoxicity
  – General: Lack of toxicity and carcinogenicity data

• Test article selection

• Study design (general)
  – Animals: Male and female B6C3F1/N mice and Sprague Dawley rats (previously F344)
  – Exposure duration: 2-week, 3-month, 2-year
  – Dosing paradigm: typically oral gavage for botanical dietary supplements
  – Endpoints: clinical chemistry, hematology, genotoxicity, sperm motility and vaginal cytology, histopathology
<table>
<thead>
<tr>
<th>Botanical</th>
<th>Male Rats</th>
<th>Female Rats</th>
<th>Male Mice</th>
<th>Female Mice</th>
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<tbody>
<tr>
<td>Aloe vera</td>
<td>Clear</td>
<td>Clear</td>
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<tr>
<td>Ginkgo biloba</td>
<td>Some</td>
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<tr>
<td>Ginseng</td>
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<td>Goldenseal</td>
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<tr>
<td>Green tea</td>
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<tr>
<td>Kava Kava</td>
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<tr>
<td>Milk thistle</td>
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<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Senna</td>
<td>Not tested</td>
<td>Not tested</td>
<td>No</td>
<td>No</td>
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<tr>
<td>Bitter orange</td>
<td></td>
<td></td>
<td></td>
<td>Increased heart rate and blood pressure</td>
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<tr>
<td>Ephedra</td>
<td></td>
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<td>Cardiototoxicity</td>
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</table>

Green tea *Camellia sinensis*
### Sources of variation

<table>
<thead>
<tr>
<th>Source material</th>
<th>Processing</th>
<th>Finished product</th>
<th>Exposure</th>
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<tbody>
<tr>
<td><strong>Plant part</strong> (aerial, root, whole plant, leaf, seed)</td>
<td>Extraction process*</td>
<td>Manufacturing process*</td>
<td>Dose (use pattern)</td>
</tr>
<tr>
<td>Climate</td>
<td>Solvents</td>
<td>Excipients</td>
<td>Length of dosing</td>
</tr>
<tr>
<td>Soil conditions</td>
<td>Adulteration</td>
<td>Combination with other botanicals</td>
<td>Life-stage</td>
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<tr>
<td>Season</td>
<td>Contamination</td>
<td>Adulteration</td>
<td>Disease-state</td>
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<tr>
<td>Plant maturity</td>
<td>Storage/shipping conditions</td>
<td>Contamination</td>
<td>Nutritional status</td>
</tr>
<tr>
<td>Contaminants (mold, pesticides, metals)</td>
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<td>Storage/shipping conditions</td>
<td>Background genetics</td>
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<tr>
<td>Co-harvested materials (other plants, soil)</td>
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<td></td>
<td>Co-exposures</td>
</tr>
<tr>
<td>Adulteration</td>
<td></td>
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</table>

*Proprietary
April 26-27, 2016, NIH Campus, Bethesda, MD

Workshop: Addressing Challenges in the Assessment of Botanical Dietary Supplement Safety

April 26-27, 2016
9 a.m. - 5 p.m. EDT
Location: Lister Hill Auditorium
National Institutes of Health (NIH), Bethesda, Maryland

http://ntp.niehs.nih.gov/about/presscenter/events/2016/index.html
Key challenges in assessing safety

- Identifying active constituents
- Comparing across botanicals
- Understanding ADME of botanicals

Hazard characterization
Product development
Regulation
Comparing across botanicals

Sufficient similarity

Sufficient similarity = phytoequivalence

Two mixtures are similar enough that data from one of the mixtures (reference mixture) is transferable to the other (mixture of interest).

Why is this important?

There are thousands of products in the marketplace and we are not going to test all of them.
Sufficient similarity framework

Phase 1: Comparing reference to mixtures of interest within each datastream
Phase 2: Integrating across datastreams and making an overall similarity call for each mixture of interest
Comparing the reference to the mixture(s) of interest

Simple rules

1. Generate data (any kind of data – chemistry, *in vitro*, *in vivo*) on the reference and mixtures of interest

2. Multivariate statistical approaches to analyze large datasets (PCA, hierarchical clustering)

3. Similarity judgment
   a) Mixtures in the same group as the reference are considered “similar”
   b) Mixtures in the most different group are considered “different”
   c) Mixtures in neither the most similar or the most different groups are considered “maybe similar”
Figure 2. Non-Targeted Fingerprint Chromatograms of First Set of GBE Samples (Not Hydrolyzed), HPLC-ELSD

Black cohosh (Actaea racemosa)

Natural variation, contamination, and adulteration

Black cohosh
Actaea racemosa

Yellow cohosh
Actaea podocarpa

Red cohosh
Actaea rubra

Chinese cohosh
Sheng ma
Actaea dahurica

http://bonap.net/Napa/TaxonMaps/Genus/County/Actaea
In vitro assessment

Evidence for an Anuogenic Mechanism of Action for Micronucleus Induction by Black Cohosh Extract

Black Cohosh Extracts and Powders Induce Micronuclei, a Biomarker of Genetic Damage, in Human Cells

Stephanie L. Smith-Roe,¹  Carol D. Swartz,²  Kim G. Shepard,²  Steven M. Bryce,²  Stephen D. DerTinger,³  Suranya Waidyanatha,¹  Grace E. Kissling,¹  Scott S. Auerbach,¹  and Kristine L. Witt¹

Environmental and Molecular Mutagenesis 59:416–426 (2018)

Environmental and Molecular Mutagenesis 60:845–856 (2019)
Black cohosh (Actaea racemosa)

• What are we comparing?
  – Reference black cohosh extract – assessed in 90-day
  – Black cohosh extract unfinished samples
  – Black cohosh extract Standard Reference Material
  – Other cohosh extract Standard Reference Materials
  – Formulated black cohosh extract products

• How are we comparing?
  – Chemical comparison
    • Non-targeted chemistry – chromatographic profiles
  – Biological comparison
    • In vitro assay
      – Human hepatocyte assay (AhR, CAR, PXR, FXR, PPARα)
      – Genotoxicity – micronucleus assay
    – Combining chemical and biological information
Black cohosh (*Actaea racemosa*)

### Strength of evidence

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<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
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<td>1</td>
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</table>

### Visual interval inspection
Black cohosh (*Actaea racemosa*)

Key points

• Micronucleus induction and megaloblastic anemia are the critical endpoints identified in animal studies

• This finding was replicated in human cells (not a rodent-specific finding)

• An aneugenic mechanism was identified, which indicates there is likely a threshold effect

• All cohoshes induced micronucleus formation (not specific to subset of black cohosh samples and active constituent has not been identified)

• The next step is to identify the constituent(s) responsible for the genotoxic effect
Identifying active constituents

1. Extraction
2. Bioassay
3. Separation

Black cohosh

Chemical Structure

Isolation/Identification

Bioassay

Active fraction

Roberts et al., 2019. Food and Chemical Toxicology. 124: 431-438.
Toxicology in the 21st Century (Tox21) is a federal collaboration between EPA, NIH (National Center for Advancing Translational Sciences and the National Toxicology Program) and the Food and Drug Administration.

Phase 2 involved evaluating the 10k chemical library (8193 unique chemicals) in over 75 quantitative high throughput assays measuring stress response and nuclear receptor activity.

Mostly focused on single chemicals, some defined mixtures included.

Can the Tox21 platform be used to evaluate botanical dietary supplements and other complex mixtures?
<table>
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<th>Rank</th>
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<td>hse-bla-agonist</td>
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<tr>
<td>mmp-antagonist</td>
<td>25.72</td>
<td>3</td>
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<tr>
<td>aromatase/er-er-agonist</td>
<td>23.55</td>
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<td>ahr-agonist</td>
<td>21.79</td>
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<td>er-luc-bg1-4e2-agonist</td>
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<td>rt-viability-hepg2-flor</td>
<td>10.57</td>
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</table>

“...as with other commodities that the agency regulates, it’s critical that FDA continue to work closely with our partners in industry to achieve our primary goal of protecting public health and safety. As the dietary supplement industry develops new products and ingredients, advances new delivery systems and innovates in other ways, the FDA must do more to leverage its existing resources and authorities to evaluate these products. This requires collaborative research and a shared understanding. I’m pleased to announce that we’ve recently created the Botanical Safety Consortium, a public-private partnership that will gather leading scientific minds from industry, academia and government to promote scientific advances in evaluating the safety of botanical ingredients and mixtures in dietary supplements. This group will look at novel ways to use cutting-edge toxicology tools, including alternatives to animal testing, to promote the goals of safety and effectiveness we share with consumers and other stakeholders.”
A public-private partnership aimed at developing a toolbox of *in vitro* and *in silico* assays and approaches for evaluating botanical safety.
Objective 2.3: Botanical Safety Consortium

**Objectives**

- Engage with a broad group of global stakeholders to leverage the best scientific approaches.
- Establish the appropriate levels of chemical characterization for complex botanical ingredients.
- Identify pragmatic, fit-for-purpose *in vitro* & *in silico* assays to evaluate botanical safety.
- Evaluate the application of these tools via comparison to the currently available safety information.
- Integrate these tools and approaches into a framework that will facilitate robust evaluation of botanical ingredients.
• Better understanding the transition from adaptive to adverse responses in sensitive *in vitro* systems to identify real safety concerns

• Developing recommendations for chemical analysis of complex botanical ingredients and products

• Achieving an appropriate level of biological coverage to identify likely toxicity targets while maintaining a manageable testing platform

• Identifying active constituents and measuring concentrations in *in vitro* assessments to aid in translating findings to humans and comparing across products

• Refining complex mixture read-across methods
Conclusions

- In vitro assays combined with non-targeted chemical analysis were useful in evaluating sufficient similarity of complex mixtures.

- In vitro assays can be incorporated into bioassay-guided fractionation approaches to identify active constituents in complex mixtures.

- Botanicals evaluated in Tox21 assays point to both challenges and opportunities for complex mixtures.

- The Botanical Safety Consortium is actively working to develop a toolkit of in vitro assays and recommended framework for assessing botanical safety.

Turmeric
Curcuma longa
Acknowledgements

• Chemistry
  – Suramya Waidyanatha
  – Brad Collins
  – Esra Mutlu
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  – Stephanie Smith-Roe
  – ILS
• Echinacea
  – Kristen Ryan
  – Mimi Huang
• Ginkgo biloba extract case study
  – Stephen Ferguson
  – Scott Auerbach
  – Sreenivasa Ramaiahgari
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  – Paul Dunlap
  – Arun Pandiri
• Botanical Safety Consortium
  – Michelle Embry
  – Connie Mitchell
• High throughput screening of botanicals
  – Troy Hubbard
  – Jui-Hua Hsieh
  – NCATS

Garcinia cambogia
Garcinia gummi-gutta