Challenges and Approaches for Using Animal and Human Data to Evaluate *In vitro* Systems in Botanical Safety Assessment

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National Institute of Environmental Health Sciences

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• Current paradigm for safety evaluation of botanicals
• Case studies comparing across data streams
  – Black cohosh
  – Green tea extract
  – *Ginkgo biloba* extract
• Challenges and next steps
History of safe use

• 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.
Evidence used in safety evaluation

**Human**
- **Data streams**
  - Clinical trials
  - Adverse event reporting
- **Considerations**
  - Genetic diversity
  - Product variability
  - Under-reporting
    - Latency
    - Difficulty proving causality

**Animal**
- **Data streams**
  - Toxicity studies in rodents
  - Preclinical safety studies
- **Considerations**
  - Lack of genetic diversity
  - Single test article
  - Resource intensive

**In vitro**
- **Data streams**
  - Untargeted (Tox21)
  - Targeted/mechanistic
- **Considerations**
  - Isolated systems
  - Human-relevant
  - IVIVE required
  - Can test multiple samples
Botanical safety case studies
Black cohosh (Actaea racemosa)

Background

• Use: menopausal symptoms, menstrual cramps, premenopausal symptoms

• Recommended dose: 20-40 mg twice daily

• Background on safety:
  – Long history of use (included in the first USP monographs in 1820!)
  – No serious side effects noted in clinical trials (NCCIH)
  – Case reports indicate potential hepatotoxicity
  – Limited animal safety data
  – Commonly adulterated with other cohoshes and should not be confused with blue cohosh (used for stimulating labor and not considered to be safe)
Natural variation, contamination, and adulteration

Black cohosh (Actaea racemosa)

- 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
Animal studies

- 90-day oral gavage toxicity study with ♀ Wistar Han rats: 0, 15, 125, 250, 500, 1000 mg/kg
- 90-day oral gavage toxicity study with ♀ B6C3F1/N mice: 0, 62.5, 125, 250, 500, 1000 mg/kg
- 3-day subcutaneous injection immature ♀ CD-1 mouse uterotrophic assay with ♀ B6C3F1/N mice: 0, 0.001, 0.1, 1, 10, 100, 500 mg/kg
- 90-day oral gavage with ♀ B6C3F1/N mice to evaluate mechanism of megaloblastic anemia: 0, 1000 mg/kg


**In vitro assessment**

Black cohosh (Actaea racemosa)

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

Derek T. Bernacki,¹ Steven M. Bryce,¹ Jeffrey C. Bemis,¹ Stephen D. Dertinger,¹ Kristine L. Witt,² and Stephanie L. Smith-Roe²*

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

*Environmental and Molecular Mutagenesis 60:845–856 (2019)*

- Clastogen positive control
- Aneugen positive control
Black cohosh (Actaea racemosa)

### Strength of evidence

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### Visual interval inspection

![Visual interval inspection graph]

### Evaluating Sufficient Similarity of Botanical Dietary Supplements: Combining Chemical and In Vitro Biological Data

Kristen R. Ryan,*1 Madelyn C. Huang,*1 Stephen S. Ferguson,*, Suramy Waidyanatha,* Sreenivasa Ramaiahgari,* Julie R. Rice,* Paul E. Dunlap,* Scott S. Auerbach,* Esra Mutlu,* Tim Cristy,† Jessica Peirfelice,† Michael J. DeVito,* Stephanie L. Smith-Roe,* and Cynthia V. Rider*2

* Corresponding author.

**Present address.

†Present address.

TOXICOLOGICAL SCIENCES, 172(2), 2019, 316–329
Comparing across data streams

Recommended dose = 40-80 mg/day
40-80 mg / 60 kg (female weight) = 0.67-1.33 mg/kg

1 black cohosh extract tested

LOAEL = 250 mg/kg (mice)
HED = 20 mg/kg

15-30X higher dose in animals than humans

Test article | Supplier
---|---
Black cohosh extract XRM | Chromadex
Black cohosh extract | Supplier 1*
Black cohosh extract | Supplier 2
Black cohosh extract | Supplier 3
Black cohosh extract | Supplier 3
Black cohosh extract | Supplier 3
Black cohosh extract | Supplier 4
Black cohosh extract | Supplier 4
Black cohosh extract | Supplier 5
Black cohosh extract | Supplier 6
Black cohosh extract | Supplier 7
Black cohosh root powder | Supplier 8
Chinese cohosh VBRM | Chromadex
Red cohosh VBRM | Chromadex
Yellow cohosh VBRM | Chromadex
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).

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

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

Rodent doses were 15-30x above human doses.

Cost-benefit should be considered: some risk, no convincing efficacy data.
Green tea (*Camellia sinensis*) extract

**Background**

- **Use:** weight loss
- **Recommended dose:** 400-800 mg daily
- **Background on safety:**
  - Purported active constituent(s): catechins (Epigallocatechin-gallate)
  - Long history of use of green tea, not green tea extract
  - Liver toxicity noted in clinical trials and adverse event reports
  - Liver identified as a target in animal safety data
Human data

- Liver injury characteristics
  - Typically arises within 3 months; latency of 10 days – 7 months
  - Acute hepatitis-like syndrome
  - Hepatocellular pattern of serum enzyme elevations
  - Liver biopsy findings of necrosis, inflammation, and eosinophils resembling acute hepatitis

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<th>CAUSALITY ASSESSMENT RESULTS FOR CASES RELATED TO SIDS</th>
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<td>DILIN causality Scale</td>
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<td>1 = definite</td>
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<td>2 = highly likely</td>
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<td>3 = probable</td>
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<td>4 = possible</td>
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<td>5 = unlikely</td>
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<td>6 = insufficient data</td>
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SIDS: single-ingredient dietary supplement, containing only green tea extract as ingredient
DILIN: Drug-Induced Liver Injury Network
HDS: herbal dietary supplement
GTE: green tea extract
Drug name: different drugs were involved, some used alone or concurrently with others
RUCAM: Roussel-Uclaf Causality Assessment Method [28, 29].


Animal data

• Fasted versus fed beagle studies
  – Finding: Liver toxicity in fasted animals more severe and occurs at lower doses than in fed animals

• Multiple rodent subchronic studies and chronic studies
  – Convert all exposures to EGCG units to compare across studies
  – Confirm the key toxicity target is the liver


Church et al., (2015). Sensitivity to hepatotoxicity due to epigallocatechin gallate is affected by genetic background in diversity outbred mice. Food and Chemical Toxicology. 76:19-26.
In vitro data

- Model system: rat primary hepatocytes

### Table: In vitro Toxicity of Epigallocatechin Gallate in Rat Liver Mitochondria and Hepatocytes

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<td>Albumin</td>
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<td>TNFα</td>
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Comparing across data streams

Recommended dose = 400-800 mg/day
400-800 mg / 60 kg (female weight) =
6.67-13.3 mg/kg

Liver effects observed in
animal studies at doses that
are relevant to humans

525 mg EGCG dose in
humans led to plasma levels
of 4.41 µmol/L
(Nakagawa et al. 1997)

LOAEL = 120 mg/kg
EGCG (fasted dog)
HED = 66.7 mg/kg

LOAEL = 50 mg/kg
EGCG (DO mice)
HED = 4.07

Mechanistic insight:
• Pro-apoptotic pathway
• Mitochondrial membrane
damage

LOAEL = 10 µmol/L EGCG
EGCG is useful in comparing across studies and doses
  
  – Studies with pure EGCG support that it is the active constituent for liver toxicity
  
  – Many issues associated with complex mixtures are not relevant to this case study

Liver toxicity is evident across testing platforms at doses that are relevant to recommended green tea extract doses

Pharmacokinetics and accompanying changes in internal dose based on fed/fasted state is important in understanding liver toxicity

There seems to be a genetic (and/or underlying condition) component to green tea extract liver toxicity susceptibility
Ginkgo biloba extract

Background

• Use: mental acuity
• Recommended dose: 120 – 240 mg/day
• Background on safety:
  – Long history of use
  – No serious side effects noted in clinical trials
  – Some case studies involving excessive bleeding and inconsistent signals regarding drug-botanical interactions
  – Commonly adulterated with cheaper plants (*Sophora japonica*) or pure flavonols (rutin)
Animal data

- 90-day oral gavage toxicity study with Fisher 344 rats: 0, 62.5, 125, 250, 500, 1000 mg/kg
- 90-day oral gavage toxicity study with B6C3F1/N mice: 0, 125, 250, 500, 1000, 2000 mg/kg
- 2-year oral gavage toxicity study with Fisher 344 rats: 0, 100, 300, 1000 mg/kg
- 2-year oral gavage toxicity study with B6C3F1/N rats: 0, 200, 600, 2000 mg/kg
- Mechanistic studies in gpt Delta and CAR-knockout mice

Carcinogenicity across species and sex

Targets:
- Liver
- Nose
- Thyroid

NTP, (2013). Toxicity and carcinogenesis studies of Ginkgo biloba extract in F344/N rats and B6C3F1/N mice.

Maeda et al. (2015). Essential role of constitutive androstane receptor in Ginkgo biloba extract induced liver hypertrophy and hepatocarcinogenesis. Food and Chemical Toxicology 83:201-209
**In vitro data**

**Ginkgo biloba extract**

**CYP2B6 (CAR)**

**CYP 3A4 (PXR)**

- Log$_{10}$ Concentration (% v/v)
- Log$_{10}$ Relative Fold mRNA Content

**GBE A**
**GBE B**
**GBE C**
**GBE D**
**GBE E**
**GBE F**
**GBE G**
**GBE H**
**GBE I**
**GBE J**
**GBE K**
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**GBE W**
**GBE X**
**GBE Y**
**GBE Z**
**GBE 1**
**GBE 1A**
**GBE 2**
Moderate doses of commercial preparations of *Ginkgo biloba* do not alter markers of liver function but moderate alcohol intake does: A new approach to identify and quantify biomarkers of ‘adverse effects’ of dietary supplements 

Harris R. Lierberman, Mark D. Kellogg, Victor L. Fulgoni III, Sanjiv Agarwal 

Regulatory Toxicology and Pharmacology (2017) 84: 45-53
Comparing across data streams

Recommended dose = 120-240 mg/day
120-240 mg / 60 kg (female weight) = 2-4 mg/kg

4-8X higher dose in rat than human

LOAEL = 100 mg/kg (rat) HED = 16 mg/kg

CAR and PXR were induced in human cells

32% of samples tested did not resemble standardized GBE

Molecular pathways adapted from Maeda 2015
• The CAR/PXR mechanism of action does not result in the same suite of effects in humans as it does in rodents, therefore, the observed carcinogenesis is not directly translatable.

• However, this does not mean that gingko is without safety concerns – the changes in drug metabolizing enzymes indicate potential for drug-herb interactions.

• Terpene trilactones are the likely active constituents in GBE and there is a wide range of phytochemical compositions among samples.
• There is value in building case studies for evaluating botanical safety that incorporate human, animal, and *in vitro* data

• The current paradigm involves human adverse event signals or animal toxicity studies preceding and triggering *in vitro* research that is typically used to:
  – provide a bridge between human and animal findings (black cohosh and GBE)
  – provide mechanistic insights (green tea extract)

• What considerations are needed to move toward a future where *in vitro* and *in silico* approaches provide a preliminary approach for evaluating botanical safety?
  – Coverage of adequate biological space
  – Decreasing the likelihood of false negatives due to deficiencies in the model system or conditions
  – Note that for most botanicals, we have human use data to identify potential toxicity targets, for completely new ingredients, animal evaluation is likely warranted
**Botanicals in Tox21**

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• Better understanding the transition from adaptive to adverse responses in sensitive *in vitro* systems to identify real safety concerns

• 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 aids in translating findings to humans and comparing across products
• Continue to evaluate case studies that contain all three data streams

• Botanical Safety Consortium work to identify a suite of recommended assays in target areas (hepatotoxicity, genetic toxicity) and evaluate a library of botanicals in those assays
  – Identify areas that require additional assay coverage (DART, cardiotox, systems toxicity)
  – Characterize the domain of applicability for the assays
  – Compare results to existing data from animal and human studies
- Data synthesis and evaluation
  - Katelyn Lavrich
  - Nisha Sipes

- Chemistry
  - Suramya Waidyanatha
  - Brad Collins
  - Esra Mutlu
  - MRI
  - Battelle

- Green tea extract case study
  - Hellen Oketch-Rabah
  - Amy Roe
  - USP Green Tea Expert Committee
  - DILIN

- Black cohosh case study
  - Stephanie Smith-Roe
  - Kristine Witt
  - Chad Blystone
  - Michelle Cora

- Ginkgo biloba extract case study
  - Stephen Ferguson
  - Scott Auerbach
  - Sreenivasa Ramaiahgari
  - Julie Rice
  - Paul Dunlap