Can High Throughput Assays/Tox 21 Inform Hazard Assessment?

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Outline

- Introduction
  - Challenges Facing Toxicology and Hazard Assessment
  - Tox21 vs. ToxCast vs. Tox21 approaches
- Case studies
  - Evaluating dose addition in Tox21
  - Evaluating mixtures in Tox21
  - Deep Dive on *Ginkgo biloba* using Tox21 Approaches

Toxicological Challenges in the 21st Century

- Too many chemicals.
  - Thousands of chemicals on the market with significant toxicological data gaps
- Too many commercial mixtures.
  - Botanicals
  - Pesticide formulations
  - PAHs
- Too many co-exposures.
  - We are exposed to mixtures of mixtures
- We cannot use traditional methods to test our way out of this!

Toxicity Testing in the 21st Century

- Early 2000's it became apparent to a number of organizations that our traditional testing approaches were unsustainable.
  - 2004
    - NTP Road Map
  - 2005
    - Tox21 initiated with NTP, NCGC, USEPA
    - USEPA implemented ToxCast
  - 2007
    - NAS Report: Toxicity Testing in the 21st Century: A Vision and a Strategy (2007)
  - 2010
    - US FDA Joins Tox21

Tox21 vs. Tox21 Approaches

- Tox21
  - Focus on human biology/human cells/tissues.
  - Initially focused on the 10K library and HTS methods using robotics.
    - Screening one pathway at a time, but 75-100 different pathways.

- Tox21 Approaches
  - Focus on human biology/human cells/tissues.
  - Smaller libraries–no robots but liquid handling stations using 384 well plates.
    - Hypothesis based screening; limited number of pathway based assays but can do HTS transcriptomics.

Mixtures Risk Assessment

How can we estimate human health risk from exposure to mixtures?

Whole Mixtures

Requires toxicity data on whole mixtures

- Data on mixture of interest
- Data on "sufficiently similar" reference mixture

Component-based

Requires toxicity data for individual chemicals within the mixture

- Dose addition
  - Relative Potency Factor
- Response addition

Definition of Sufficient Similarity*

- 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”
- Goal: *Use toxicity data for one mixture to estimate risk posed by the mixture of concern*

*US EPA 2000 Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures

Case Study 1: Evaluating Dose Addition in Tox21

- Focus on chemicals positive in Phase I of Tox21 in the Estrogen Receptor (10 chemicals) and Androgen Receptor (8 chemicals) assays.
- Made 67 mixtures of these 18 chemicals.
- All individual chemicals and mixtures were in phase II of Tox21 for all assays.
  - Initial analysis of two ER assays (BG1 whole receptor assay; B-Gal partial receptor assay.

Chemicals and Mixtures

ER actives

- Zeralenone
- Bisphenol A
- Ethylenediamine
- Chlordecone
- Acetochlor
- Butylbenzylphtalate
- Dicumyl peroxide
- o,p-DDT
- P,n-nonylphenol
- alachlor

AR actives

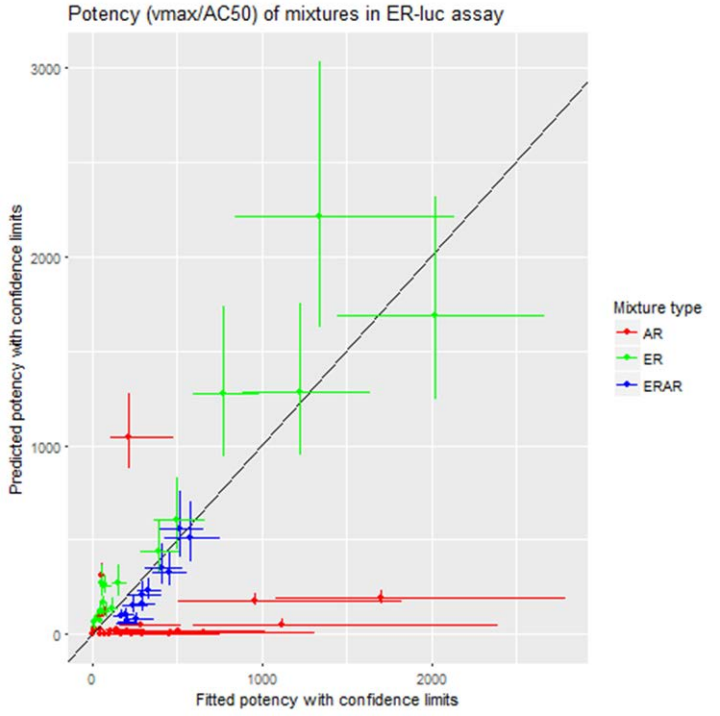
- Oxymetholone
- Fluoxymestrone
- Progesterone
- Dexamethasone
- Medroxyprogesterone acetate
- O-methoxyphenol
- Hydroxyflutamide
- Androstenedione

Challenges in Hypothesis Testing in Tox21

- No going back!
  - Think about the 10K library and HTS as a ship leaving port. You are either on it or you are at the dock. Once you leave port you do not get off the ship until the trip is finished.
- Data inconsistencies between phase I and II data.
  - All chemicals tested were positive in phase I and about half were positive in phase II.
  - All concentrations of zeralenone tested were at maximal responses

Results of Dose Addition Predictions

- Mixtures of ER agonists alone or ER/AR agonists with predicted low responses were well predicted.
- Mixtures of ER agonists with predicted high response were less well predicted due to uncertainty of zeralenone dose response relationship.
- Mixtures of AR agonists were poorly predicted, but predictions were highly uncertain.



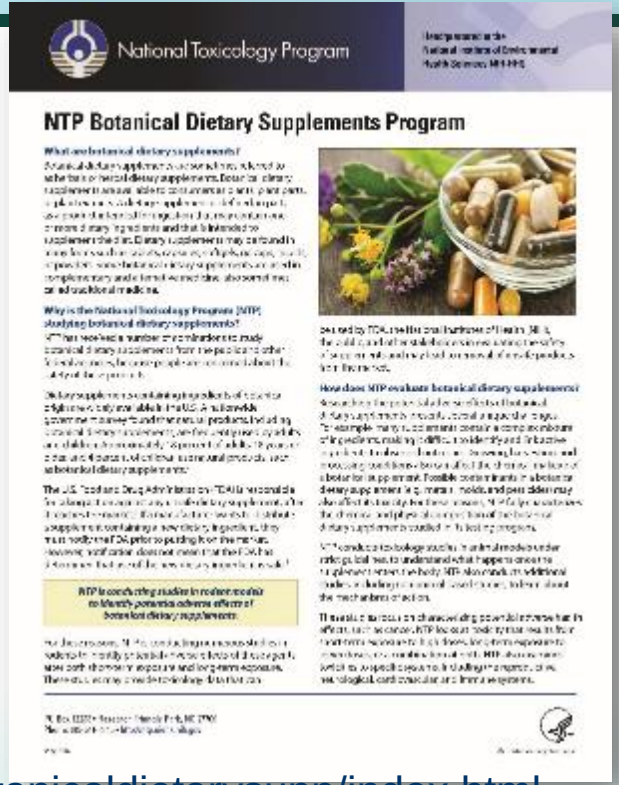
Botanical Dietary Supplements at NTP

Completed

- *Aloe vera* noncolorized whole leaf extract
- Bitter orange extract
- Crude *Ephedra* (Ma Huang) extract
- Ginseng root extract
- *Ginkgo biloba* extract
- Goldenseal root powder
- Kava kava extract
- Milk thistle extract
- *Senna*
- *Usnea* lichen
- Valerian root extract

Ongoing

- Black cohosh extract
- Dong quai (root powder or extract)
- *Echinacea purpurea* extract
- Evening primrose oil
- *Garcinia cambogia* extract
- Green tea extract
- Gum guggul extract
- *Usnea* lichen
- Valerian root extract


National Toxicology Program

NTP Botanical Dietary Supplements Program

http://ntp.niehs.nih.gov/results/areas/botanicaldietarysupp/index.html

Challenges with Botanicals

- Complexity
  - Many constituents
  - Multiple “active” constituents
  - Large unidentified fraction
- Variability across marketplace
  - Differences in raw material due to source, season, plant part
  - Processing/manufacturing
  - Adulteration or combination

Challenges with Studying the Safety of Botanicals

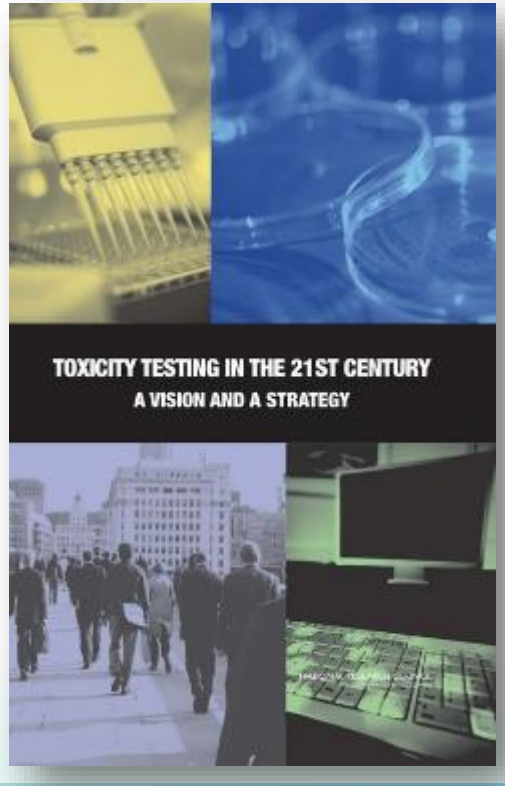
NTP Perspective

- Primary challenges
  - Test article selection
  - Relating animal doses to human intake
- Secondary challenges
  - Extrapolating findings to other botanicals (e.g., combination botanicals)
  - Identifying active constituent(s)
  - Drug-botanical interactions

Botanicals in Tox21

High-Throughput Screening

- Tox21 Community: NCATS, 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*
- Phases
  - Phase 1 (2005-2010) – ~3000 chemicals
  - Phase 2 (2011-2014) – 10K library
  - Phase 3 (present -)



Case Study 2: 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)
- Curcumin (1)
- *Echinacea purpurea* (1)
- Emodin (6)
- Eugenol (1)
- Gallic acid (1)
  - Pyrogallol (3)
- *Ginkgo biloba* extract (4)
  - Kaempferol (2)
  - Quercetin (3)
- Goldenseal root powder (9)
  - Berberine (1)
- Grape seed extract (4)
- Gum guggal extract (5)
  - Gugulipid (3)
- Kava Kava extract (6)
- Methyleugenol (4)
- Milk thistle extract (6)
  - Silybin (3)
- Olive oil (1)
- Pine bark extract (2)
- Pulegone (3)
- Resorcinol (3)
- Resveratrol (3)
- Safflower oil (2)
- Turmeric (4)
  - Curcumin (1)

Assays

- Estrogen receptor alpha
- Androgen receptor
- Aromatase

Endocrine activity assays

- Aryl hydrocarbon receptor
- Peroxisome proliferator-activated receptor gamma

Nuclear receptor assays

- Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (Nrf2/ARE)
- Heat shock factor response element
- Mitochondrial membrane potential (MMP)

Stress response assays

- ATAD5
- P53

Genetox assays

- Cytotoxicity
- Cell viability

Cell death assays

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
- Identify active constituent(s)
- Identify endpoints that could be used in future *in vitro* evaluations of botanicals

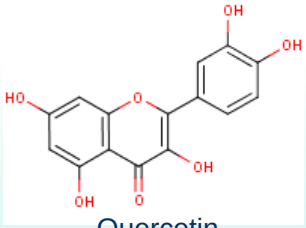
HTS data

Note: Preliminary analysis, subject to revision

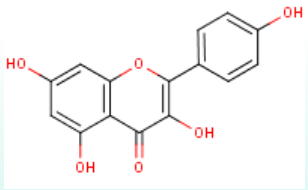
HTS Results - GBE

In vivo: Toxicity/carcinogenicity in nose, thyroid, and liver

	AhR	ARE	AR	Aro	Elg1	ER	HSE	MMP	P53	PPARγ	Hek293	HepG2
GBE lot 1	-	-	-	ER+	-	-	-	-	-	-	-	-
GBE lot 2	+	-	-	-	-	-	-	-	-	-	-	-/+
GBE lot 3	+	-	-	-	-	-	-	-	-	-	-	-/+
Ginkgo leaf powder	-	-	-	-	-	-	-	-	-	-	-	-
Kaempferol lot 1	+	-	AR-	ER+	-	ER+	-	+	-	-	+	-/+
Kaempferol lot 1	+	-	AR-	ER+	-	ER+	-	+	+	+	+	+
Quercetin lot 1	-	-	-	ER+	+	ER+	-	+	+	-	+	+
Quercetin lot 2	+	-	-	ER+	-	ER+	-	+	+	-	+	+
Quercetin lot 3	+	-	-	ER+	+	ER+	-	+	+	-	+	+



Quercetin



Kaempferol

Note: Preliminary analysis, subject to revision

Conclusions

- Botanicals can be tested in an HTS platform
  - Activity observed
- Levels of activity *in vitro* seem to generally correspond to levels of activity *in vivo*
  - Caveat: There is not a direct correlation
- Differences in activity patterns are observed between lots within a botanical class

Case Study 3: Ginkgo biloba Extract and Sufficient Similarity

Good starter project

- What are we comparing?
  - Reference *Ginkgo biloba* extract – assessed in 90-day and 2-year studies
  - 20 *Ginkgo biloba* extract samples
  - 2 NIST *Ginkgo biloba* extract Standard Reference Materials (1 extract, 1 tablet)
  - 4 Formulated *Ginkgo biloba* extract products containing EGb761® (gold standard)

- How are we comparing?
  - Chemical comparison
    - Untargeted chemistry – chromatographic profiles
    - Targeted chemistry – quantification of marker constituents
  - Biological comparison
    - *In vitro* assays – liver models
    - *In vivo* rat study – liver weight and gene expression
  - Combining chemical and biological information

Whole Mixtures

Sufficient similarity

Sufficient similarity=phytoequivalence

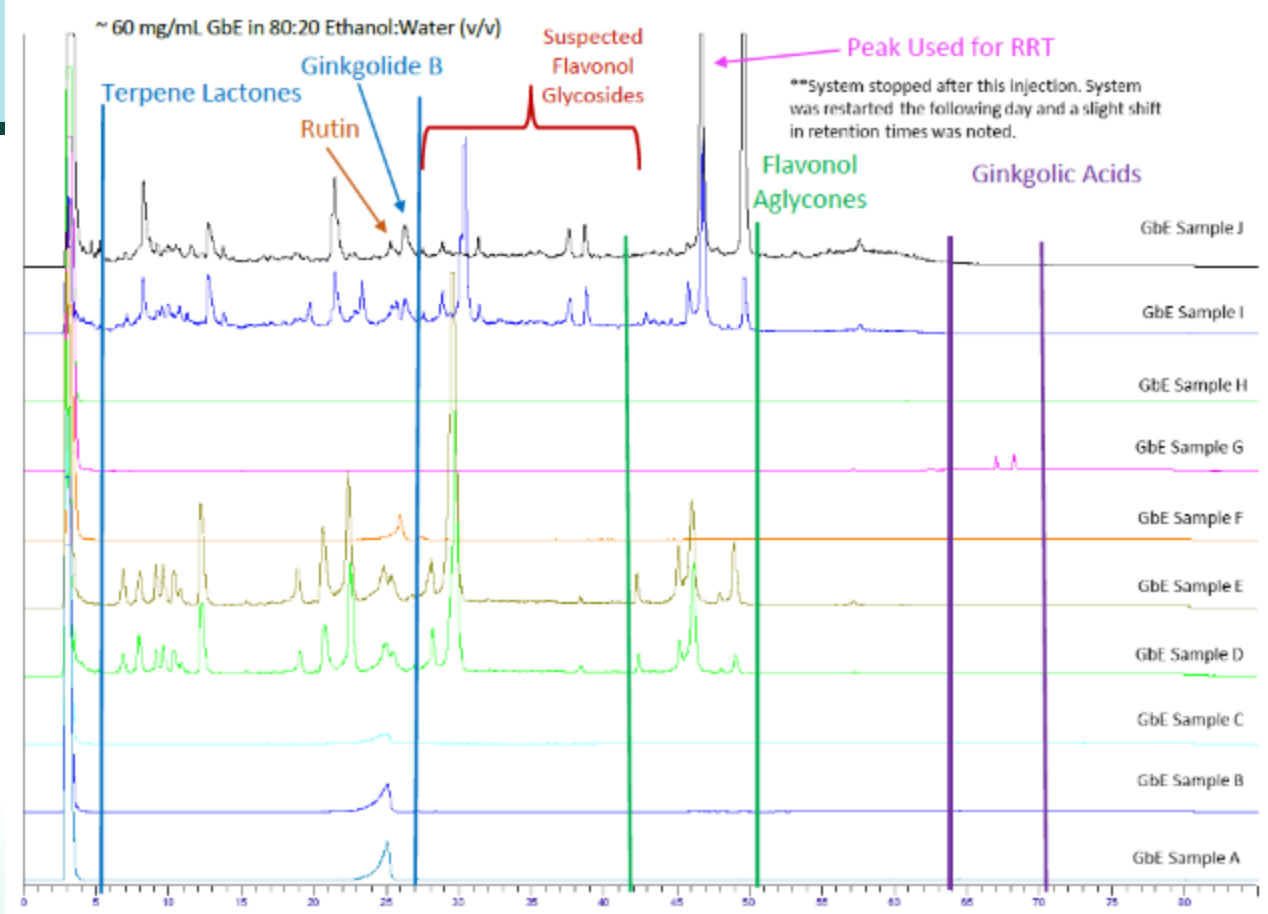
Two mixtures are similar enough that data from one of the mixtures (*reference mixture*) is transferable to the other (*mixture of interest*).

Why is this important?

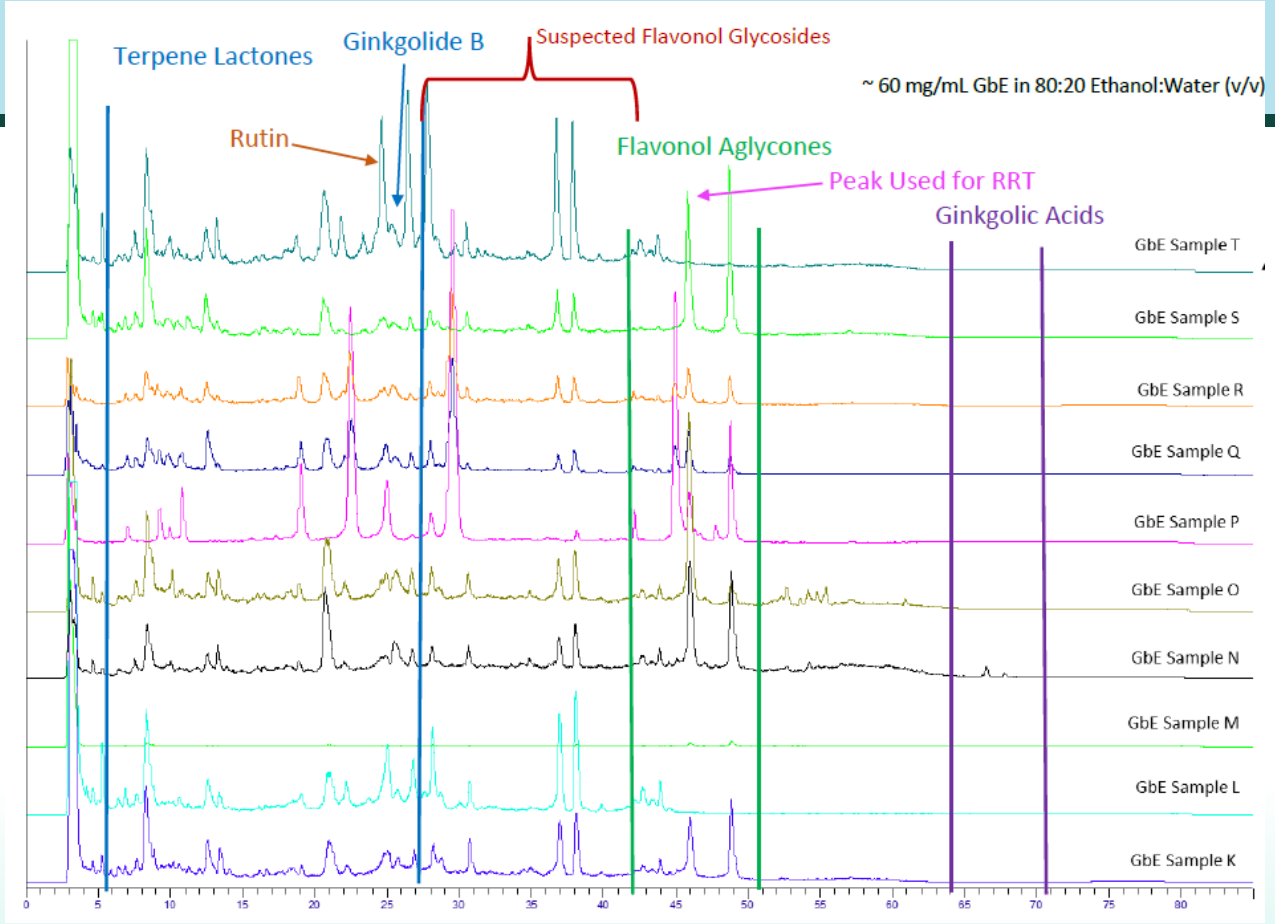
There are thousands of products in the marketplace and we are not going to test all of them

Ginkgo biloba extract

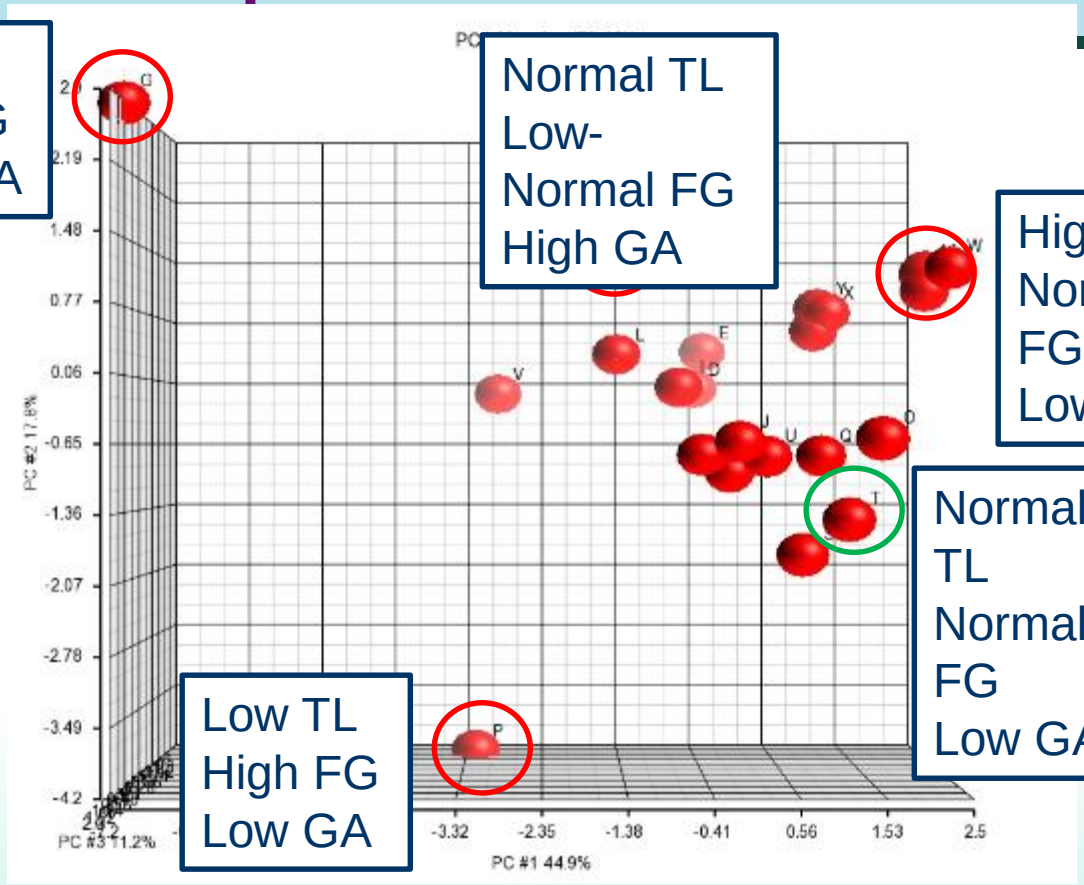
First 10 Lots



Second 10 Lots



Quantitative Comparison



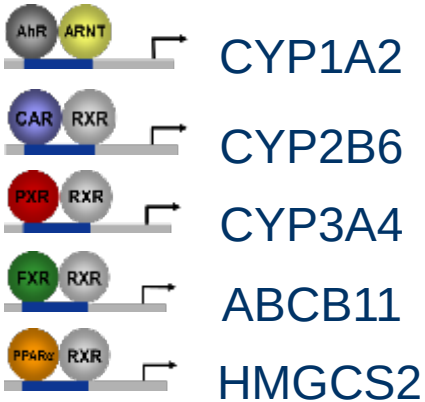
A sufficient similarity case study

Research program

- Chemical analysis of approximately 20 GBE lots
  - Comparison of chemical fingerprints
  - Analysis of chemical markers
- All 20 lots, standard reference materials, NTP test article and 11 marker compounds will be assessed in *in vitro* assays (human hepatocytes) to measure nuclear receptor activation (AhR, CAR, PXR, FXR, PPARα), cytotoxicity, and stress
- 5 chemically-divergent GBE lots and 2 additional botanical test articles (e.g., goldenseal extract and green tea extract) will be tested in 5-day *in vivo* rat studies to assess gene expression changes in liver

Primary Human Hepatocyte Data

- Area under the curve



CYP1A2

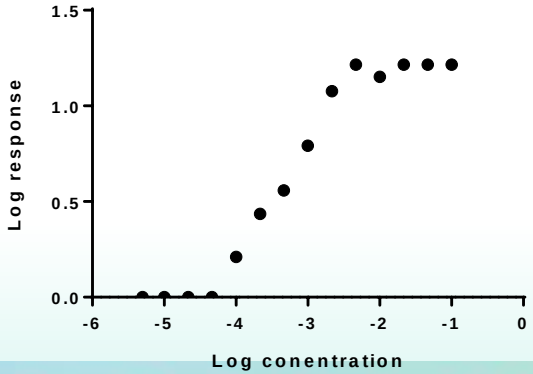
CYP2B6

CYP3A4

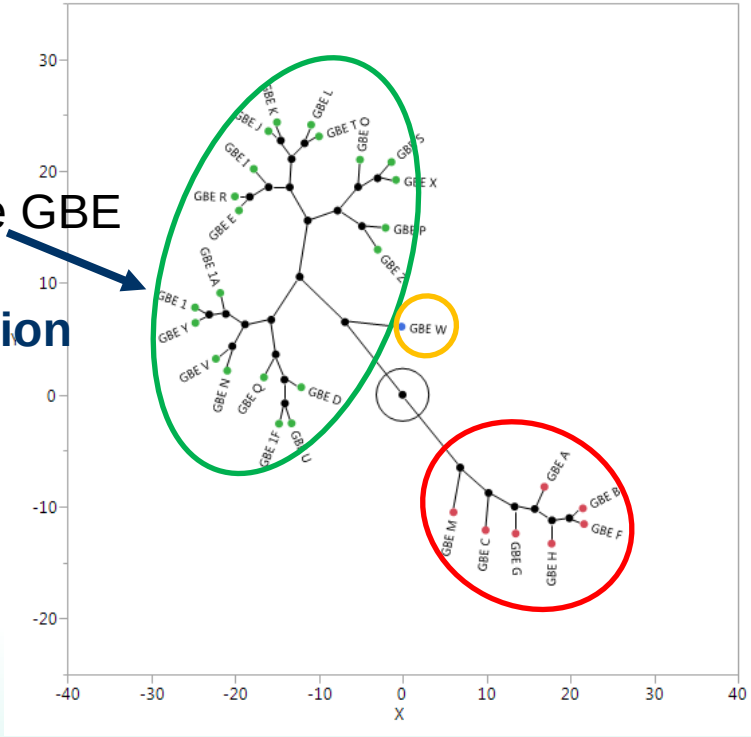
ABCB11

HMGCS2

Sample U - CAR expression



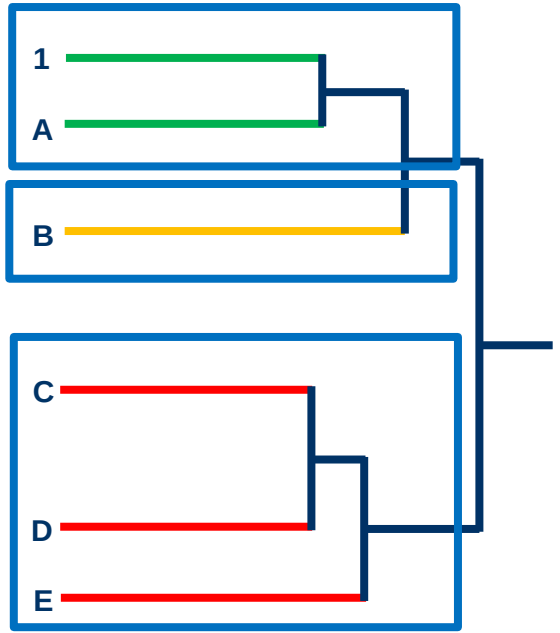
Reference GBE



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”



Strength-of-Evidence

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
Untargeted – unhydrolyzed	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0	0	-1	-1	-1	-1	-1	-1	-1	0	1	1	1	1	1	1
Untargeted - hydrolyzed	-1	-1	-1	-1	-1	-1	-1	-1	0	0	0	0	0	0	0	-1	0	0	0	0	1	1	1	1	1	1
Untargeted score	-1	-1	-1	-1	-1	-1	-1	-1	-0.5	-0.5	0	0	-0.5	-0.5	-0.5	-1	-0.5	-0.5	-0.5	0	1	1	1	1	1	1
Targeted – unhydrolyzed	-1	-1	-1	0	0	-1	-1	-1	0	0	0	0	-1	0	0	0	0	0	0	0	0 1	-1	1	1	1	1
Targeted – hydrolyzed	-1	-1	-1	1	1	-1	-1	-1	0	0	1	0	-1	1	0	1	1	1	0	1	0 1	0	0	0	0	0
Targeted score	-1	-1	-1	0.5	0.5	-1	-1	-1	0	0	0.5	0	-1	0.5	0	0.5	0.5	0.5	0	0.5	0.5	-0.5	0.5	0.5	0.5	0.5
PHH	-1	-1	-1	1	1	-1	-1	-1	1	1	1	1	-1	1	1	1	1	1	1	1	1	1	0	1	1	1
PHH score	-1	-1	-1	1	1	-1	-1	-1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	-1	1	1	1
Attagene – cis-factorial	-1	-1	-1	1	1	-1	-1	-1	1	0	0	0	-1	-1	0	0	1	1	0	0	0	-1	1	1	1	1
Attagene – trans-factorial	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	-1	0	1	1
Attagene – GPCR	0	0	0	-1	-1	0	0	0	-1	1	1	1	1	1	-1	-1	-1	-1	-1	1	1	0	-1	0	0	0
Attagene score	-0.3	-0.3	-0.3	0.0	0.0	-0.3	-0.3	-0.3	0.0	0.3	0.3	0.3	0.3	0.3	-0.3	-0.3	0.0	0.0	-0.3	0.3	0.3	-0.3	-0.3	0.3	0.7	0.7
In vivo							-1							1		-1				1						
In vivo score							-1							1		-1				1						
Average score	-0.8	-0.8	-0.8	0.1	0.1	-0.8	-0.9	-0.8	0.1	0.2	0.5	0.3	-0.5	0.5	0.0	-0.2	0.3	0.3	0.0	0.6	0.7	0.3	0.3	0.7	0.8	0.8

Conclusions

Ginkgo biloba extract

- There is a clear difference between *Ginkgo biloba* extract samples that resemble the reference sample (NTP test article) and other high quality samples (standard reference material and EGb761® containing formulations)
- Findings are relatively consistent across chemistry, *in vitro*, and *in vivo* data with differences only in gray areas (medium quality samples)
- The untargeted chemistry and human hepatocyte data were judged to be the most informative and cost effective combination for determining sufficient similarity
  - Untargeted chemistry is effective regardless of how large the unidentified fraction is and performed comparably to the targeted approach
  - *In vitro* human hepatocyte data reflected the in vivo and chemistry findings for a fraction of the cost (caveat: limited biological coverage)

Case Study 3: Conclusions and Future Directions

- The more you know about chemical composition, active constituents, and biological effects of a mixture, the easier it is to determine sufficient similarity of other, “related” mixtures
- When active constituents are unknown, biological measures of similarity should take precedence over chemical measures
- Biological assays to determine similarity in a hazard identification context should be connected to the observed toxicity

Summary and Conclusions

- HTS can provide screening level information on biological activity.
- Moderate throughput screening (libraries of 100 test articles or less) has advantages in that these efforts are more hypothesis based and can more easily be replicated in an iterative process.
- Combining chemical and biological data enhance our ability to implement sufficient similarity approaches.
- Sufficient similarity approaches all us to use prototype mixtures that have sufficient toxicological data and apply that data to untested mixtures that are deemed “sufficiently similar.”
- Alternative approaches can provide useful information for hazard assessment of complex mixtures in the context of sufficient similarity

Acknowledgements

NTP staff

Scott Auerbach, Chad Blystone, Brad Collins, Mike DeVito, Paul Dunlap, Stephen Ferguson, Paul Foster, Jui-Hua Hsieh, Esra Mutlu, Fred Parham, Sreeni Ramaiahgari, Julie Rice, Nisha Sipes, Ray Tice, Molly Vallant, Suramya Waidyanatha, Nigel Walker

NCATS

Ruili Huang

Menghang Xia

FDA

Paul Howard

Office of Dietary Supplements - NIH

Joe Betz

MRI

Season Kerns

Joe Algaier

Kristin Aillon

USDA

James Harnly