Proposed *In Silico/In Vitro* Approach for Botanical Mixtures

Presenter name: Dr. Catherine Mahony
Affiliation: Procter & Gamble
Contact information: mahony.c@pg.com
Conflict of Interest Statement

Presenter is employed by Procter & Gamble. Procter & Gamble provided financial support to the research presented.
Outline of Presentation

• Goals and overall strategic approach

• Constituent characterisation and *in silico* approach

• High content and high throughput *in vitro* approaches

• Conclusions and outlook
Goals of Botanical Safety Research

⇒ Tools and Strategies for Botanical Safety Assessment = Better predictions of toxicity and risk

Support the increasing business interest in botanicals

Focused on challenges that botanicals represent
- Identity/adulteration and complex chemistry
- Data gaps/limitations
- Herb-Drug Interactions (HDIs)
Botanical Safety Strategic Approach

Raw Material Identity and Quality are cornerstones

Tier 1: Based on low level of exposure & Threshold of Toxicological Concern (TTC) approach *(for botanical mixture)*

Tier 2: Based on significant human use history *(consider delayed effects and concomitant meds)*

Tier 3: Based on the in-depth characterization of botanical constituents & evaluation of toxicity
Focus on Tier 3

Where data is insufficient from Tier 1 and Tier 2 assessment, i.e., higher exposures and toxicological data are lacking; differing extractions etc.
1 Collecting Existing Data

2 Generating New Data

3 Analyzing These New Data

4 Resolution or Support for Next Steps
Focus on Tier 3

Safety Assessment of Botanical Preparation

Pre-Work

Unresolved Endpoint Gaps or Different Preparation/Dose*

Advanced Analytical Instrumentation UHPLC w/UV, CAD, and HRMS detection

Obtain Comprehensive compositional, structural and quantification data*

Sample Acquisition & Characterization

Safety Dossier to Support Human Use in Market

Botanical Cleared

Complete Pre-Work

1. Build hazard assessment dossier from reliable sources (Evidence based support not absence of evidence)
2. Evaluate botanical for endpoints (Toxicology Review)
3. Determine: Adequate or Gap for each end point

Safety Assessment of Botanical Preparation

Pre-Work Botanical Preparation

Characterization (Analytical Data)

(F) Dose at dietary exposure levels?

YES

NO

(B) Commonly consumed in diet?

YES

(A) Constituent Structure known?

NO
Focus on Tier 3

Safety Assessment of Botanical Preparation → Pre-Work → Safety Dossier to Support Human Use in Market → Botanical Cleared

Unresolved Endpoint Gaps or Different Preparation Formulations

Advanced Analytical Instrumentation UHPLC w/UV, CAD, and HRMS detection → Sample Acquisition & Characterization

Obtain Comprehensive compositional, structural and quantification data

(F) Dose at dietary exposure levels? YES (B) Commonly consumed in diet? YES (A) Constituent Structure known? NO

Unresolved Gaps

Examples:
- Different Solvent Systems
- Natural Variation
- Target Audience
- Change dose/exposure > dietary/traditional
- Efficacy vs. Safety
Focus on Tier 3

Safety Assessment of Botanical Preparation

Pre-Work

Unresolved Endpoint Gaps or Different Preparation/Dose*

Advanced Analytical Instrumentation
UHPLC w/UV, CAD, and HRMS detection

Sample Acquisition & Characterization

Obtain Comprehensive compositional, structural and quantification data*

Safety Dossier to Support Human Use in Market

Botanical Complete Safety Assessment

Constituent Characterization and Identification (CCID)

- Need sample intended for human exposure
- Reference samples helpful to confirm ID of botanical and any constituents
- Need both qualitative data and quantitative data
- Results of genetox data and level of indented dose will drive lower limit of detection required—"no need to chase ZERO".

(F) Dose at dietary exposure levels? YES
(B) Commonly consumed in diet? YES
(A) Constituent Structure known? NO
Focus on Tier 3

Safety Assessment of Botanical Preparation

Pre-Work

Unresolved Endpoint Gaps or Different Preparation/Dose*

Advanced Analytical Instrumentation
UHPLC w/ UV, CAD, and HRMS detection

Constituent Structure known?

Commonly consumed in diet?

Dose at dietary exposure levels?

Established Complete Safety Endpoint Assessment

YES

NO

Unresolved Endpoint Gaps or Different Preparation/Dose*

Sample Acquisition & Characterization (Analytical Data)

Instrumentation

(U)HPLC pump 0.4 mL/min → PDA-UV → Inverse gradient pump 0.4 mL/min

HR-AM/MS* Component Identification

Charged Aerosol Detector (CAD) Quantitation

Inverse Gradient Pump Mobile Phase Composition

Mobile Phase Composition at CAD and MS Detectors

% Organic

Time

Hazard Analysis: Identify Constituent of the Botanical Cleared

Safety Assessment of Botanical Preparation

Pre-Work Safety Dossier to Support Human Use in Market

Published Data
Focus on Tier 3

Obtain copies of posts to assess structural alerts or unfavorable data. Generate data via high-throughput expert systems (i.e., DEREK) to assess constituents.

For each structure defined by Analytical you must know:
1. If constituent is > TTC, structure and dose
2. If constituent is < TTC follow TTC Rules

For each structure defined by Analytical you must know:

- (YES) Constituent(s) Cleared
- (E) Exposure level below relevant TTC?
- (STOP) Reduce dose to supportable level (based on % constituent(s) driving safety assessment) or obtain additional safety data.

Use Expert Tools: Cramer Classification, DEREK Alerts, DART Decision Tree, Expert Opinion.

Complete Safety Endpoint Assessment (Gentox, DART Systemic, etc).

Verify Identity and exposure levels for any unfavorable alerts.

Established tox profile and suitable MoS/MoE? ; SAR possible & suitable MoS/MoE? ; YES Constituent(s) Cleared.

Constituent Structure known?

Commonly consumed in diet?

Dose at dietary exposure levels?

Exposure level below relevant TTC?

Resolution, Redesign or Testing

Hazard Analysis: Identify Constituent of the Botanical Driving Safety Assessment (In Silico Data)
Focus on Tier 3

Obtain copies of the structural alerts or unfavorable data

Generate data via high throughput expert systems (i.e. DEREK)

Assess constituents with structural alerts or unfavorable data

Constituent Structure known?

YES

(a) Endpoint Assessment
   Known Data
   (Gentox, DART Systemic, etc)

(b) Commonly consumed in diet?

YES

(c) Establish toxin profile and suitable MoS/MoE?

NO

(d) SAR possible & suitable MoS/MoE?

NO

(e) Exposure level below relevant TTC?

YES

(f) Dose at dietary exposure levels?

NO

(g) Constituent(s) Cleared

Complete Safety Endpoint Assessment

(YES)

For each structure defined by Analytical you must know:

1. If constituent is > TTC, then need structure and dose
2. If constituent is < TTC, follow TTC Rules
Determine if exposures is already established in diet:

- What qualifies as dietary intake?
- “Dietary exposures are supported by a genetically diverse and sufficiently large population”
If exposure cannot be supported by dietary intake, then safe human use must be estimated based on toxicology data.

Two Challenges:
1. Adequate data w/o over reliance on history of use
2. Generating more data to fill gaps w/o defaulting to traditional tox studies

With adequate toxicology data for each end point

If exposure cannot be supported by dietary intake, then safe human use must be estimated based on toxicology data.

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With adequate toxicology data for each end point
Focus on Tier 3

When data is not adequate to support safe human exposure than additional assessment approaches may be needed.

1. In silico Approaches
   E.g., DEREK and customized alerts
   E.g., SAR

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1. In silico Approaches
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Focus on Tier 3

Assess Constituents with Structural Alerts or Unfavorable Data (E)

Exposure level below relevant TTC?

(D) SAR possible & suitable MoS/ MoE?

Complete: Analogue Identification & SAR Assessment for Constituents of Concern

Use Expert Tools: Cramer Classification, DEREK Alerts, DART Decision Tree, Expert Opinion

Verify Identity and Exposure Levels for Any Unfavorable Alerts

Options and next steps:

- Reduce dose until safety data can support exposure
- Data generated to this point is useful to design further studies if needed
- Genetox data is often needed

(YES) Constituent(s) Cleared

(STOP) Reduce dose to supportable level (based on % constituent(s) driving safety assessment) or obtain additional safety data

Complete: Analogue Identification & SAR Assessment for Constituents of Concern
Examples Where *In Silico* Decision Tree is Used

1. Benchmarking constituent safety between the diet and botanical supplements

2. Applying constituent analysis to justify bridging safety data between different methods of botanical preparation

3. Establishing exposure thresholds for individual constituents with limited human use data using *in silico* toxicology assessment tools

4. Informing design of follow up safety studies where needed
Summary of CCID-*In Silico* Approach

- Thoroughly vet the scientific literature—define the question(s)!
- Advanced multi-detector analytical characterization technique to establish botanical constituent composition (simultaneous ID and quantitation)
- Each identified constituents is processed through a decision-tree to resolve questions
  - Close safety gaps, inform supportable exposure levels, or need for safety studies
- ⇒ a focused approach for detecting possible bad actors in botanical extracts, the variables involved
Case Study—Artichoke Leaf

- Food-Like
- Insoluble
- Eleutheroside B
- Tannins
- Unknown <180 ug
- Sesquiterpene Lactones

13 mg/dose

Cynaropicrin
(Sesquiterpene lactone)
In Vitro Testing Approaches: Informing DART Potential at a MOA Level

- High-throughput approaches
  - HTS batteries: Customised Cerep Panel
  - Global gene expression analysis: CMAP in 4 cell types

- Decision-making
  - By lack of response on developmentally relevant targets
  - By functional comparisons
How Many Developmentally Relevant MOAs?

• Cataloging via DART ontology project
• 19 major categories, multiple subcategories
• Focus on
  ─ Mechanisms not involving reactivity
  ─ Mechanisms that are unique to development or where development is most sensitive or response most severe
Cerep Assay Selections

- Steroid and retinoic acid receptors (8)
- Neurotransmitter receptors and transporters (32)
- Ca, K, Na ion channels (3)
- DART-relevant enzymes
  - Cox 1 and 2
  - ACE
  - Cholesterol synthesis
  - Tubulin
  - MAO-A
  - Phosphodiesterases
- Run botanical extracts with and without human liver S9 (with NADPH)
Botanicals Tested in Cerep

• Selected based on
  — Presumed lack of DART because of dietary use
  — Anecdotal information of DART
  — Pharmacology that might preclude use in pregnancy

• Concentration based on a multiple of measured Cmax of an active marker compound, or conservative assumptions to estimate a Cmax
Top two. The more red, the higher the inhibition

Bottom. Difference between parent and metabolite where blue means less activity from the metabolites, and red means more activity with the metabolite
## Cerep Results

### Botanical

<table>
<thead>
<tr>
<th>Botanical</th>
<th>Parent hits</th>
<th>Metabolite hits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chaste Tree</td>
<td>$CCK_1$ ($CCK_a$) (h), D2S (h), PR (h)</td>
<td>$CCK_1$ ($CCK_a$) (h), D2S (h)</td>
</tr>
<tr>
<td>St. John’s Wort</td>
<td>N neuronal $\alpha_4\beta_2$ (h), 5-HT2B (h), $ER\beta$</td>
<td>$A_{2A}$ (h), BZD (central), N neuronal $\alpha_4\beta_2$ (h), $\mu$ (MOP) (h), 5-HT1A (h), 5-HT2B (h), $ER\beta$</td>
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<tr>
<td>Cat’s Claw</td>
<td>PR (h), N neuronal $\alpha_4\beta_2$ (h)</td>
<td>N neuronal $\alpha_4\beta_2$ (h)</td>
</tr>
<tr>
<td>Ashwagandhha</td>
<td>$CCK_1$ ($CCK_a$) (h), PR (h), 5-HT1B</td>
<td>$CCK_1$ ($CCK_a$) (h), 5-HT1B</td>
</tr>
<tr>
<td>Green Tea</td>
<td>$CCK_1$ ($CCK_a$) (h), Erbeta, HMG-CoA Reductase</td>
<td>$CCK_1$ ($CCK_a$) (h), Erbeta, HMG-CoA Reductase</td>
</tr>
<tr>
<td>Ginger extract</td>
<td>$CCK_1$ ($CCK_a$) (h), D2S (h), PR (h), PPAR$\gamma$ (h)</td>
<td>$CCK_1$ ($CCK_a$) (h), PPAR$\gamma$ (h)</td>
</tr>
<tr>
<td>Artichoke Leaf</td>
<td>$CCK_1$ ($CCK_a$) (h)</td>
<td></td>
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**Case study interpretation—**artichoke leaf**—**receptor activity consistent with traditional use (relief of digestive disorders) BUT lack of response on developmentally relevant targets.
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IC50 Follow Up – PR Activity

Can inform potency differences; early pregnancy abortifacient → contraceptives → endogenous hormones → botanicals (food vs. non-food)
Connectivity Mapping (CMAP) Approach

MCF7 cells, rich in nuclear hormone receptors

HepG2 cells, hepatic characteristics, including limited xenobiotic metabolism

A549 cells, respiratory epithelium

CDI (iPS-derived) cardiomyocytes, numerous ion channels and neurotransmitter receptors
St. John Wort (SJW)

• Cerep activity and CMAP – where is the commonality?

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</thead>
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<tr>
<td>St. John's Wort USP – 1607506</td>
<td>357 ug/mL</td>
<td>N neuronal α4β2 (h), 5-HT2B (h), ERbeta</td>
<td>A2A (h), BZD (central), N neuronal α4β2 (h), µ (MOP) (h), 5-HT1A (h), 5-HT2B (h), ERbeta</td>
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</tbody>
</table>

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<tr>
<th></th>
<th>250 ug/ml</th>
<th>25 ug/ml</th>
<th>2.5 ug/ml</th>
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<tbody>
<tr>
<td>A549</td>
<td>306</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Ishikawa</td>
<td>321</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>HepG2</td>
<td>326</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>MCF7</td>
<td>291</td>
<td>1</td>
<td>0</td>
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Number of Land Mark genes (FDR <0.05) whose expression was modified by the indicated botanical extracts.

• CMAP top hits were for Phenothiazine antipsychotic drugs
  — serotonin activity (a hit with Cerep)
  — dopamine and adrenergic receptors (not a fit with Cerep but a fit with literature for SJW actives)

• Consistent with mode of action of SJW which is related to the nervous system, psychiatric action
Comparing Active Concentrations

- CMAP effect concentration
  - 25-250 ug/ml, or 1.8-18 uM hyperforin, a marker compound for SJW

- Ki for key receptor for functional analog
  - 2uM Chlorpromazine

- Relative potency is 0.9 - 9

- Developmental NOAEL for functional analog
  - E.g., chlorpromazine: 20 mg/kg/day (rat)
    - Cmax at this dose is approx. 0.5-1uM

- Adjust for potency: 0.45 - 9 uM hyperforin

- Therapeutic dose of SJW gives a Cmax for hyperforin of 280 nM
  - Margin is ~1.6
  - Animal studies have shown equivocal results. The potential risk for humans is unknown. In the absence of sufficient clinical data, the use during pregnancy and lactation is not recommended.
Summary of *In Vitro* MoA Approach

- Biological activity of botanicals can be characterized through *in vitro* approaches

- Best approach will include both methods
  - CMAP to identify functional analogs (but more complex analysis)
  - Cerep to provide better focus (esp. to rule out false positives)

- Further work ongoing to utilize data for risk assessment purposes
  - Comparing concentrations to assess relative potency
    - CMAP effect/no-effect concentration; Ki/IC50 for key receptors
  - Extending both panels for greater coverage of systemic toxicity MoA
Conclusions and Outlook

• Constituent level characterization and *in silico* approach can resolve botanical safety gaps, refine supportable exposure levels, or inform need for safety studies.

• High content and high throughput *in vitro* approaches can inform botanical mode of action or lack of response on toxicologically relevant targets.

• Botanically-derived ingredients are extremely challenging from a Safety POV, but good science can save time and money!
• Vandermolen, K. et, al. Safety Assessment of Mushrooms in Dietary Supplements by Combining Analytical Data with In Silico Toxicology Evaluation. Food and Chemical Toxicology 103 (2017) 133-147

Acknowledgements

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    o Annie Otto-Bruc (AnnieOtto-Bruc@eurofins.com)
    o Kevin Kennedy (KevinKennedy@eurofins.com)
  – Jorge Naciff
  – George Daston
Thank you for your attention!