Next generation risk assessment (NGRA) case study: use of 0.1% coumarin in face cream

Maria Baltazar & Gavin Maxwell
Outline

9h00 – 9h25 – Introduction to Next generation risk assessment (NGRA): concepts and tools (30 min)

9h25 – 9h35 – Exposure information and Collation of existing information (10 min)

9h35 – 10h – Breakout Discussion (25 min)

10h00 – 10h15 – Break (15 min)

10h15 – 10h55 – In vitro biological activity characterisation (35 min)

10h55 – 11h20 – Breakout Discussion (25 min)

11h20 – 11h30 – Metabolism refinement & Margin of Safety determination & Risk assessment conclusion (10 min)

11h30 – 11h55 – Poll questions & Discussion (25 min) (plenary)

11h55 – 12h00 – Concluding remarks (5 min)
Introduction to Next generation risk assessment (NGRA): concepts and tools (30 min)
The objective of a consumer product risk assessment is to determine if we can safely use x% of ingredient y in product z.
NGRA is defined as \textit{an exposure-led, hypothesis-driven risk assessment approach that integrates New Approach Methodologies (NAMs) to assure safety without the use of animal testing}.

Main overriding principles:
The overall goal is a human safety risk assessment
The assessment is exposure led
The assessment is hypothesis driven
The assessment is designed to prevent harm

Principles describe how a NGRA should be conducted:
Following an appropriate appraisal of existing information
Using a tiered and iterative approach
Using robust and relevant methods and strategies

Principles for documenting NGRA:
Sources of uncertainty should be characterized and documented
The logic of the approach should be transparently and documented
NGRA: The overall goal is a human safety risk assessment

“Advances in toxicogenomics, bioinformatics, systems biology, and computational toxicology could transform toxicity testing from a system based on whole-animal testing to one founded primarily on in vitro methods that evaluate changes in biologic processes using cells, cell lines, or cellular components, preferably of human origin.” 2007
NGRA: The assessment is exposure-led

- Route of exposure
- Consumer use (Habits & Practices)
- Applied dose (external concentration)

ADME parameters

- Skin penetration
- Phys-chem properties
- Hepatic clearance
- Fraction unbound
- blood:plasma ratio

Physiologically-based kinetic (PBK) modelling
- Internal concentration (plasma, urine, organ-level)

Uncertainty analysis
- Population simulation
NGRA: The assessment is designed to prevent harm

The philosophy behind this type of risk assessment aimed at preventing harm is based on the premise of “Protection not Prediction”.

The hypothesis underpinning this type of NGRA is that if there is no bioactivity observed at consumer-relevant concentrations, there can be no adverse health effects.

Slide from Dr Rusty Thomas, EPA, with thanks

NGRA: The assessment is hypothesis driven & should be conducted Using a tiered and iterative approach

NGRA: Using robust and relevant methods and strategies to characterise bioactivity

**In silico tools**

- **ToxTree**
- **Derek Nexus**
- **OECD**
- **QSAR Toolbox**

**In silico models to predict Molecular initiating events (MIEs)**

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**Using 2D Structural Alerts to Define Chemical Categories for Molecular Initiating Events**

Timothy E. H. Allen,† Jonathan M. Goodman,‡,† Steve Gutsell,¶ and Paul J. Russell§

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**Metabolic fate predictions**
NGRA: Using robust and relevant methods and strategies to characterise bioactivity

**OECD test methods**

- OECD TG437
- OECD TG430/431
- OECD TG439
- OECD TG442C
- OECD TG442D

**Skin and eye irritation**

- OECD TG432

**Phototoxicity**

- OECD TG473
- OECD TG471
- OECD TG476

**Skin sensitisation**

**Genotoxicity**

**Receptor-binding assays**

e.g. AR-CALUX® assay to measure androgen receptor activity

- Ibuprofen – Cox-1.

NGRA: Using robust and relevant methods and strategies to characterise bioactivity

Tox21/ToxCast
~700 HTS Biological Pathways Assays

- Nuclear receptors
- Transcription factors
- Cell stress/mitochondrial tox
- Enzymatic assays
- Receptor binding
- DNA damage/cell cycle
NGRA: Using robust and relevant methods and strategies to characterise bioactivity

High-throughput transcriptomics and High-throughput phenotypic profiling developed to increase biological coverage

Harrill J et al. 2019. Considerations for strategic use of high-throughput transcriptomics chemical screening data in regulatory decisions. Current Opinion in Toxicology 15, 64-75


NGRA: Using robust and relevant methods and strategies to characterise bioactivity

36 biomarkers identified that were representative of key stress pathways, mitochondrial toxicity and cell health.
For some chemicals pathway-based risk assessment might be needed

Examples of Adverse Outcome Pathway (AOP) risk assessment

Induction of skin sensitisation that leads to allergic contact dermatitis

Anti-androgenic and estrogenic effects

Employing Dietary Comparators to Perform Risk Assessments for Anti-Androgens Without Using Animal Data

Matthew P. Dent,⁎⁎, Hequn Li,⁎ Paul L. Carmichael,⁎ and Francis L. Martin⁎

Regulatory Toxicology and Pharmacology

An exposure:activity profiling method for interpreting high-throughput screening data for estrogenic activity—Proof of concept

Richard A. Becker⁎⁎, Katie Paul Friedman⁎, Ted W. Simon⁎, M. Sue Marty⁎, Grace Patlev J. Craig Rowlands⁎
NGRA: the margin of safety (MoS) approach and decision making

Is it safe?

- **Amount/Conc. of ingredient due to exposure** → **Hazard Characterisation** → **Adverse Organism Reporres** → **Species Extrapolation** → **NOAEL**
- **Targeted Testing**
  - e.g. 90 Day Repeat Dose Study

**PoD**

**NOAEL** + 10 - 1000

**Uncertainty Factors**

**Safe Dose in Humans**

Range of *in vitro* AC50 values converted to human *in vivo* daily dose

Safety margin

Actual Exposure (est. max.)
NGRA: Sources of uncertainty should be characterized and documented

Exposure models (PBK, free/total concentration)

Point of departure derived from concentration-response data

Uncertainty in the PBK inputs
Population variability

Plasma Cmax as a distribution

Point of Departure as a distribution

Variability in the data
Plate effects
Etc.
NGRA: the margin of safety (MoS) approach and decision making

- Point of departure derived from concentration-response data
- Exposure models (PBK, free/total concentration)
- Exposure estimation: Plasma $C_{\text{max}}$
- Calculation of Margin of Safety (MoS) distribution

The MoS is defined as the ratio the PoD and the relevant plasma $C_{\text{max}}$ estimate.
The margin of safety covers off various sources of uncertainty in translating NAMs and a safety decision. These include:

- Exposure
  - Applied dose
  - Clearance
  - Metabolism
  - Cmax/AUC

- POD
  - Biological coverage
  - Time-dependence
  - Cell/tissue sensitivity

NGRA: Sources of uncertainty should be characterized and documented
NGRA: Making sense of margins of safety by benchmarking


Exposure + Bioactivity data (substance and comparators)

Exposure: activity ratios = 
Exposure (plasma exposure in µM) / Activity (IC\textsubscript{50} µM) 
Dietary comparator = ratio
EAR (test substance) / EAR (dietary comparator)

A case study approach – human health safety assessment required for...

0.1% COUMARIN IN FACE CREAM FOR EU MARKET (NEW FRAGRANCE)

Assumed that:
- Coumarin was 100% pure
- no in vivo data was available such as animal data, History of Safe Use (HoSU) info. or Clinical data
- no use of animal data in Read Across
- In silico alerts known to be based on animal or in vivo data or on the structure of Coumarin itself were excluded
Next-Generation Risk Assessment case study workflow for 0.1% coumarin in face cream

https://doi.org/10.1093/toxsci/kfaa048
Exposure information and Collation of existing information (10 min)
Next-Generation Risk Assessment case study workflow for 0.1% coumarin in face cream
NGRA for 0.1% coumarin in face cream: exposure estimation

Assessment is exposure-led and uses available habits and practices data.
NGRA for 0.1% coumarin in face cream: exposure estimation - Internal concentration using PBK modelling - Model Inputs

NGRA for 0.1% coumarin in face cream: exposure estimation - Internal concentration using PBK modelling - Model Inputs

**Level 2.**

- **In vitro data generation** for parameters with **high sensitivity &/or low confidence** in the predicted values require further refinement through

- **Update the model** with new parameters

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NGRA for 0.1% coumarin in face cream: exposure estimation - Internal concentration using PBK modelling - Model Outputs

Level 2 - Simulated plasma concentration of coumarin after dermal exposure.

Level 2. Uncertainty and population variability
Distribution of Cmax values after performing Monte Carlo simulation.

<table>
<thead>
<tr>
<th>Total Plasma C&lt;sub&gt;max&lt;/sub&gt; (µM)</th>
<th>Mean</th>
<th>Median</th>
<th>90th percentile</th>
<th>95th percentile</th>
<th>97.5th percentile</th>
<th>99th percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Face Cream</td>
<td>0.0022</td>
<td>0.0021</td>
<td>0.004</td>
<td>0.0043</td>
<td>0.0046</td>
<td>0.005</td>
</tr>
</tbody>
</table>

NGRA for 0.1% coumarin in face cream: exposure estimation

- Total plasma Cmax values obtained from PBK model: 0.002 µM (mean), 0.005 µM (99th percentile)
- Stability assays indicated coumarin is rapidly metabolized mainly via CYP2A6
Next-Generation Risk Assessment case study workflow for 0.1% coumarin in face cream
NGRA for 0.1% coumarin in face cream: in silico predictions

- Coumarin might bind to proteins - MIE for induction of skin sensitisation
- DNA binding alert + epoxide formation MIE for genotoxicity
- Reactive metabolites might be formed with alerts for both genotoxicity and skin sensitisation
- No binding alerts for the 39 targets in MIE atlas

*Allen THE et al., 2018. Using 2D Structural Alerts to Define Chemical Categories for Molecular Initiating Events. Toxicol Sci. 2018 Sep 1;165(1):213-223*
NGRA for 0.1% coumarin in face cream: in silico predictions - Metabolism

- Hydroxylation predicted as main route of biotransformation
- Reactive metabolites (e.g. epoxides) predicted.
 NGRA for 0.1% coumarin in face cream: in vitro existing information

Identification of potential biological targets – PubChem and ToxCast

Only few active assays among multiple assays (≈ 5000)

Coumarin inhibited both Monoamine oxidases and Carbonic anhydrases at concentrations between 3 µM- 40 µM

The AC50 from dose-response curves was used a PoD for MoS calculation
NGRA for 0.1% coumarin in face cream: exposure estimation

Total plasma Cmax values obtained from PBK model: 0.002 µM (mean), 0.005 µM (99th percentile)
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Genotoxicity and skin sensitisation alerts for parent compound
- Hydroxylation predicted as main route of biotransformation
- Reactive metabolites (e.g. epoxides) predicted.
- Low bioactivity in ToxCast and Pubchem: binding to Carbonic Anhydrases and MAO-A/B reported
- Lowest PoD was 3 µM for carbonic anhydrase I (Figure 7)
Breakout Discussion (25 min)
Breakout group questions

1. Do you agree with the interpretation of the data/information? (Poll in menti, yes/no/not sure)

2. What other data/information would you like to generate/see? (please add your comment in Menti)

3. Any other questions? (please add your question in Menti)

10 min breakout discussion

15 min plenary discussion
Break (15 min)
In vitro biological activity characterisation (35 min)
Next-Generation Risk Assessment case study workflow for 0.1% coumarin in face cream
NGRA for 0.1% coumarin in face cream: In vitro biological activity characterisation: Genotoxicity assessment: ToxTracker

Initial hypothesis:

- DNA binding alerts for coumarin and metabolites

Results:

- ToxTracker negative
- Reactive coumarin metabolite(s) could induce DNA lesions secondary to oxidative stress
NGRA for 0.1% coumarin in face cream: In vitro biological activity characterisation: Skin sensitisation assessment

Initial hypothesis:

- Protein binding alerts for coumarin and metabolites

NGRA for 0.1% coumarin in face cream: In vitro biological activity characterisation: Skin sensitisation assessment

**Step 1: Generation of in vitro results for Coumarin**

<table>
<thead>
<tr>
<th>Call</th>
<th>DPRA (TG442C)</th>
<th>KeratinoSen (TG 442D)</th>
<th>h-CLAT (TG 442E)</th>
<th>U-SENS (TG 442E)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Model Input</td>
<td>%cys depletion</td>
<td>%lys depletion</td>
<td>EC1.5 (µM)</td>
<td>CD54 (EC200 µg/mL)</td>
</tr>
<tr>
<td>RUNs</td>
<td>1.0 0.7 2.2</td>
<td>0 0 0</td>
<td>200 175 NA</td>
<td>&gt;637 &lt;178 &gt;637</td>
</tr>
</tbody>
</table>

**Initial results:**

- Coumarin is a skin sensitiser
- Likely to be due to metabolites (-ve DPRA)
NGRA for 0.1% coumarin in face cream: In vitro biological activity characterisation: Skin sensitisation assessment

**Step 2. Generation of PoD for risk assessment- Skin allergy risk assessment (SARA) Defined approach (DA)**

- The **SARA DA** is a Bayesian probabilistic model, which estimates the human sensitiser potency via a prediction of a HRIPT 1% sensitising dose (ED$_{01}$) (i.e PoD) for a selected chemical.

**SARA Model Inputs**

- Historical Local lymph node assay (LLNA)
- Historical Human repeated insult patch test (HRIPT)
- *In vitro* data: DPRA (TG442C), KeratinoSens (TG 442D), h-CLAT (TG 442E), U-SENS (TG 442E)
- First publication dataset of 30 chemicals – expanded to 53 core + 49 *in vitro* only

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* Reynolds, J, MacKay C, Gilmour N, Miguel-Vilumbrales D and Maxwell G (Submitted for publication: Computational Toxicology) Probabilistic prediction of human skin sensitiser potency for use in next generation risk assessment
NGRA for 0.1% coumarin in face cream: In vitro biological activity characterisation: Skin sensitisation assessment

**Step 2: PoD for risk assessment**

The PoD for coumarin has a central 95% credible interval ranging from 546 - 217,603 µg/cm²

**Results:**
- Exposure is much lower than the predicted PoD
- MoS = 400 - 160 000
- Low risk conclusion
NGRA for 0.1% coumarin in face cream: Key results

**Exposure Estimation**
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**Collate Existing Information**
- Genotoxicity and protein binding alerts for parent compound
- Hydroxylation predicted as main route of biotransformation
- Reactive metabolites (e.g. epoxides) predicted.
- Low bioactivity in ToxCast and Pubchem: binding to Carbonic Anhydrases and MAO-A/B reported
- Lowest PoD was 3 µM for carbonic anhydrase I (Figure 7)

**In Vitro Biological Activity Characterisation**
- ToxTracker negative; weak activation of DNA damage reporters (only +S9).
- Predicted MoS (400-160 000) suggests that the risk of inducing skin allergy is low at the consumer exposure.
NGRA for 0.1% coumarin in face cream: In vitro biological activity characterisation: In vitro binding and enzymatic assays: Eurofins SafetyScreen44

To investigate possible interactions between coumarin and the 44 key targets involved in drug attrition

Results:

All binding and enzymatic assay results were negative at 10 µM
NGRA for 0.1% coumarin in face cream: In vitro biological activity characterisation: Immunomodulatory screening assay: BioMap Diversity 8 Panel

To investigate possible effects on vascular inflammation, immune activation and tissue remodelling

Data suggested that coumarin has no immunomodulatory effects at relevant concentrations and is not an anti-inflammatory compound.
NGRA for 0.1% coumarin in face cream: In vitro biological activity characterisation:
In vitro cell stress panel

To characterize non-specific biological activity which is not mediated via a specific protein/receptor interaction - covering ~10 cell stress pathways using high content imaging analysis

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Cell type</th>
<th>Stress pathway</th>
<th>PoD (µM)</th>
<th>Effect</th>
<th>Concentration dependency score (CDS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (6h)</td>
<td>HepG2</td>
<td>cell health</td>
<td>794 (363-977)</td>
<td>down</td>
<td>0.98</td>
</tr>
<tr>
<td>ATP (24h)</td>
<td>HepG2</td>
<td>cell health</td>
<td>617 (282-891)</td>
<td>down</td>
<td>1</td>
</tr>
<tr>
<td>Phospholipidosis (24h)</td>
<td>HepG2</td>
<td>cell health</td>
<td>759 (437-977)</td>
<td>down</td>
<td>0.93</td>
</tr>
<tr>
<td>GSH (24h)</td>
<td>HepG2</td>
<td>oxidative stress</td>
<td>851 (301-1000)</td>
<td>up</td>
<td>0.92</td>
</tr>
<tr>
<td>IL-8 (24h)</td>
<td>HepG2</td>
<td>inflammation</td>
<td>912 (575-1000)</td>
<td>down</td>
<td>0.61</td>
</tr>
<tr>
<td>OCR (1h)</td>
<td>NHEK</td>
<td>mitochondrial toxicity</td>
<td>62 (2.6-776)</td>
<td>down</td>
<td>0.6</td>
</tr>
<tr>
<td>OCR (6h)</td>
<td>NHEK</td>
<td>mitochondrial toxicity</td>
<td>468 (214-794)</td>
<td>down</td>
<td>1</td>
</tr>
<tr>
<td>OCR (24h)</td>
<td>NHEK</td>
<td>mitochondrial toxicity</td>
<td>309 (138-1000)</td>
<td>down</td>
<td>0.52</td>
</tr>
<tr>
<td>Reserve capacity (1h)</td>
<td>NHEK</td>
<td>mitochondrial toxicity</td>
<td>44 (23-96)</td>
<td>down</td>
<td>1</td>
</tr>
<tr>
<td>Reserve capacity (6h)</td>
<td>NHEK</td>
<td>mitochondrial toxicity</td>
<td>759 (302-1000)</td>
<td>down</td>
<td>0.9</td>
</tr>
<tr>
<td>Reserve capacity (24h)</td>
<td>NHEK</td>
<td>mitochondrial toxicity</td>
<td>794 (295-1000)</td>
<td>down</td>
<td>0.55</td>
</tr>
</tbody>
</table>

NGRA for 0.1% coumarin in face cream: In vitro biological activity characterisation: In vitro cell stress panel

Results:
Coumarin not very active in comparison to known “high risk compounds” like doxorubicin
• PoDs shown for HepG2 only

NGRA for 0.1% coumarin in face cream: In vitro biological activity characterisation: High-Throughput Transcriptomics (HTTr) using TempO-SEQ technology

Transcriptomics was applied as a broad nontargeted biological screen

Differential expression analysis using DESeq2 analysis

Results:
Across the cell lines, treatment with coumarin resulted in limited gene-expression changes at concentrations below 100 µM, suggesting limited cellular effects at lower concentrations
NGRA for 0.1% coumarin in face cream: In vitro biological activity characterisation: High-Throughput Transcriptomics (HTTr), TempO-SEQ technology

Transcriptomics was applied as a broad nontargeted biological screen

<table>
<thead>
<tr>
<th>Pathway level tests PoD₇ (µM)</th>
<th>HepG2</th>
<th>MCF7</th>
<th>HepaRG 2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 pathways with the lowest p value</td>
<td>(308 pathways)</td>
<td>(0 pathways)</td>
<td>(17 pathways)</td>
</tr>
<tr>
<td>Reactome</td>
<td>70</td>
<td>NA</td>
<td>58*</td>
</tr>
<tr>
<td>20 pathways with the lowest BMD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reactome</td>
<td>44</td>
<td>NA</td>
<td>58*</td>
</tr>
<tr>
<td>BMD of Reactome pathway with lowest BMD that meets significance threshold criteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene level tests PoD₇ (µM)</td>
<td>(1570 genes)</td>
<td>(47 genes)</td>
<td>(87 genes)</td>
</tr>
<tr>
<td>Mean BMD of 20 genes with largest fold change</td>
<td>6</td>
<td>3</td>
<td>54</td>
</tr>
<tr>
<td>Mean BMD of genes between 25th and 75th percentile</td>
<td>17</td>
<td>1</td>
<td>59</td>
</tr>
</tbody>
</table>

Results:

- The MCF7 PoD₇ were not considered to be sufficiently robust to derive a MoS
- The lowest PoDT for each cell model was selected for the MoS calculation

NGRA for 0.1% coumarin in face cream: Key results

**Exposure Estimation**
- Total plasma Cmax values obtained from PBK model: 0.002 µM (mean), 0.005 µM (99th percentile)
- Stability assays indicated coumarin rapidly metabolized mainly via CYP2A6

**Collate Existing Information**
- Genotoxicity and protein binding alerts for parent compound
- Hydroxylation predicted as main route of biotransformation
- Reactive metabolites (e.g. epoxides) predicted.
- Low bioactivity in ToxCast and Pubchem: binding to Carbonic Anhydrases and MAO-A/B reported
- Lowest PoD was 3 µM for carbonic anhydrase I (Figure 7)

**In Vitro Biological Activity Characterisation**
- ToxTracker negative; weak activation of DNA damage reporters (only +S9)
- The probability of coumarin inducing skin sensitisation at the consumer exposure is low
- No immunomodulation potential
- Low bioactivity confirmed by binding/enzymatic assays, HTTr and cell stress panel.
- PoD range: 6-912 µM
NGRA for 0.1% coumarin in face cream: Preliminary Margin of Safety – How MoS is calculated
NGRA for 0.1% coumarin in face cream: Preliminary Margin of Safety

<table>
<thead>
<tr>
<th>Technology</th>
<th>Cell line/ Enzyme/Biomarker</th>
<th>Face cream Min. 5th percentile MoS</th>
<th>PoD provided as distribution?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell stress panel</td>
<td>HepG2 (ATP, 24h)</td>
<td>96738</td>
<td>Yes</td>
</tr>
<tr>
<td>Cell stress panel</td>
<td>NHEK (OCR 1h)</td>
<td>1330</td>
<td>Yes</td>
</tr>
<tr>
<td>HTTr</td>
<td>HepG2 (24h)</td>
<td>7223</td>
<td>No</td>
</tr>
<tr>
<td>HTTr</td>
<td>HepaRG (24h)</td>
<td>8864</td>
<td>No</td>
</tr>
<tr>
<td>Toxcast</td>
<td>MAO B (rat brain)</td>
<td>3711</td>
<td>No</td>
</tr>
<tr>
<td>PubChem</td>
<td>Carbonic Anhydrase Type I</td>
<td>706</td>
<td>No</td>
</tr>
<tr>
<td>PubChem</td>
<td>Carbonic Anhydrase Type II</td>
<td>2140</td>
<td>No</td>
</tr>
<tr>
<td>PubChem</td>
<td>Carbonic Anhydrase Type VI</td>
<td>14652</td>
<td>No</td>
</tr>
</tbody>
</table>

*Based on total concentrations for both \( C_{\text{max}} \) and PoDs*

- The lowest MoS across all assays was derived using the PoD (represented by Ki) for the inhibition of carbonic anhydrase I
- All PoD are higher than predicted exposure
NGRA for 0.1% coumarin in face cream: Key results

**Exposure Estimation**
- Total plasma Cmax values obtained from PBK model: 0.002 µM (mean), 0.005 µM (99th percentile)
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**Collate Existing Information**
- Genotoxicity and protein binding alerts for parent compound
- Hydroxylation predicted as main route of biotransformation
- Reactive metabolites (e.g. epoxides) predicted.
- 90-100% coumarin predicted to be freely available in vitro
- Low bioactivity in ToxCast and Pubchem: binding to Carbonic Anhydrases and MAO-A/B reported
- Lowest PoD was 3 µM for carbonic anhydrase I (Figure 7)

**In Vitro Biological Activity Characterisation**
- ToxTracker negative; weak activation of DNA damage reporters (only +S9)
- The probability of coumarin inducing skin sensitisation at the consumer exposure is low
- No immunomodulation potential
- Low bioactivity confirmed by binding/enzymatic assays, HTTr and cell stress panel.
- PoD range: 6-912 µM
- Potential metabolite-driven bioactivity not addressed

**Determine Margin of Safety**
- Preliminary MoS
- 706 - 96738
Breakout Discussion (25 min)
Breakout group questions

1. Do you agree with the interpretation of the data/information? (Poll in menti, yes/no/not sure)

2. What other data/information would you like to generate/see to increase your confidence in the conclusions? (please add your comment in Menti)

3. Any other questions? (please add to the chat)

10 min breakout discussion

15 min plenary discussion
Metabolism refinement & Margin of Safety determination & Risk assessment conclusion (10 min)
Next-Generation Risk Assessment case study workflow for 0.1% coumarin in face cream
NGRA for 0.1% coumarin in face cream: Next steps for refinement


2. Short and long-term exposure in 3D tissues - longer exposure durations in a 3D HepaRG model with potentially higher metabolic capacity and in vivo-like physiology than HepG2 cells
NGRA for 0.1% coumarin in face cream: Coumarin metabolism in primary human hepatocytes

In silico biotransformation

Two approaches:
1. A high (1 mM) concentration of coumarin was used to saturate the CYP2A6 pathway.
2. A lower concentration of coumarin (10 µM) was used, both with and without inhibition of CYP2A6 (using either 0.5 or 2 µM tranylcypromine)

In vitro stability assays: CYP2A6 driven metabolism
NGRA for 0.1% coumarin in face cream: Coumarin metabolism in primary human hepatocytes

Metabolism study to investigate if reactive metabolites are likely to be formed at consumer relevant concentrations

Results:

• Coumarin is preferentially detoxified to hydroxycoumarins and respective glucuronides

• Reactive metabolites such as the epoxide, o-HPAA and o-HPA were only detected at the highest concentration (1mM)

• Not expected to be formed in vivo for our consumer exposure scenario
NGRA for 0.1% coumarin in face cream: Short and long-term exposure in 3D tissues

To increase our confidence in the initial PoDs from the 2D cell models

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<tr>
<th>Technology</th>
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<tr>
<td>Cell stress panel</td>
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NGRA for 0.1% coumarin in face cream: Key results

**Exposure Estimation**
- Plasma Cmax obtained (range 0.002-0.02 µM) from PBK models (Table 2)
- Stability assays indicated coumarin rapidly metabolized mainly via CYP2A6

**Collate Existing Information**
- Genotoxicity and protein binding alerts for parent compound
- Hydroxylation predicted as main route of biotransformation
- Reactive metabolites (e.g. epoxides) predicted.
- 90-100% coumarin predicted to be freely available in vitro
- Low bioactivity in ToxCast and Pubchem: binding to Carbonic Anhydrases and MAO-A/B reported
- Lowest PoD was 3 µM for carbonic anhydrase I (Figure 7)

**In Vitro Biological Activity Characterisation**
- ToxTracker negative; weak activation of DNA damage reporters (only +S9)
- The probability of coumarin inducing skin sensitisation at the consumer exposure is low
- No immunomodulation potential
- Low bioactivity confirmed by binding/enzymatic assays, HTTr and cell stress panel.
- PoD range: 6-912 µM

**Metabolism refinement**
- Hydroxylation confirmed as main route of biotransformation at 10 µM
- Reactive metabolites not formed at consumer relevant exposures
- Low bioactivity also found in a metabolic competent cell model (HepaRG 3D)
- PoDs range: 41-871 µM (Table 4 and 5).

**Determine Margin of Safety**
- Updated MoS 9538-9601
- Preliminary MoS 706-96738
The predicted $C_{\text{max}}$ values for face cream were lower than all PoDs with a MoS (the 5th percentile) higher than 100.

Coumarin is not genotoxic, does not cause skin sensitisation, does not bind to any of the 44 targets and does not show any immunomodulatory effects at consumer relevant exposures.

Weight of evidence suggests that the inclusion of 0.1% coumarin in these products is safe for the consumer.
Poll questions & Discussion (25 min)
Discussion questions

1. Do you agree with the low risk decision? (Menti Poll: yes/no/not sure)

2. What additional data/information would you like to generate/see to increase your confidence in the decision? (Menti: post it note)

3. Has this case study increased your confidence in non animal approaches? (Menti Poll: yes/no/not sure)

10 min breakout

15 min discussion
Concluding remarks

1. Available tools can be integrated to make a safety decision; multidisciplinary team needed!

2. NGRA is a framework of non-standard, bespoke data-generation, driven by the risk assessment questions

3. Need to ensure quality/robustness of the non-standard (non-TG) work and to characterise uncertainty to allow informed decision-making

4. Rethinking MoS/MoE – future evaluation of the approach to infer a low risk space

5. Shortcomings will be addressed by current and future research

6. More research, creativity and examples needed to land this successfully across the community

7. Progress is only possible with a change in mindset (protection not prediction)
Acknowledgements

Core Team:

• Maria Baltazar, Alistair Middleton, Tom Cull, Joe Reynolds, Beate Nicol, Mi-Young Lee, Predrag Kukic, Alexis Nathanail, Sophie Cable, Georgia Reynolds, Mona Delagrange, Tom Moxon, Hequn Li, Mabel Cotter, Jade Houghton, Andy White, Matthew Dent, Paul Carmichael, Sarah Hatherell, Sophie Malcomber, Richard Cubberley, Ruth Pendlington

Extended Team:

• Carl Westmoreland, Paul Russell, Gavin Maxwell, Ian Sorrell, Sam Piechota, Juliette Pickles, Karen Bonner, Sandrine Spriggs, Iris Muller, Katarzyna Przybylak, Paul Walker, Caroline Bauch, Rebecca Beaumont, Steve Clifton, Katie Paul-Friedman, Julia Fentem
BACKUP SLIDES
Recent research has shown that for 417 out of 448 chemicals tested the point of departure derived (PoD) from NAMS was more conservative than the in vivo PoD.

Backup slides- Toxtracker

![Graph showing GFP Fold Induction vs Concentration (µM) for different reporters with and without S9 extraction.](image-url)
NGRA: dose-response analysis and PoD derivation

Example dose response data

1. Fit different parametric models to the data
2. Identify the one with the ‘best’ fit
3. Use this to calculate the PoD...

Candidate dose-response models

- Hill function
- Exponential
- Gain-loss model
NGRA: dose-response analysis and PoD derivation

Example dose response data

Candidate dose-response models

1. Fit different parametric models to the data
2. Identify the one with the ‘best’ fit
3. Use this to calculate the PoD...
4. Different PoDs exist, e.g:
   - AC50
   - BMD10
NGRA: dose-response analysis and PoD derivation

1. Challenges with this can arise when e.g. none of the candidate models provide a good fit, or noise (e.g. outliers) in the data leads to spurious PoD estimates.

2. In NGRA it is important to quantify the uncertainty in a) whether there is a concentration-dependent response and b) the PoD estimate, if there is one.

3. Instead we used a non-parametric model (Gaussian processes) within a Bayesian statistical framework to model to data.

NGRA: dose-response analysis and PoD derivation

NGRA for 0.1% coumarin in face cream: In vitro biological activity characterisation: High-Throughput Transcriptomics (HTTr)

- Transcriptomics was applied as a broad nontargeted biological screen of in vitro cellular perturbation following coumarin treatment

**Generation of HTTr using the TempO-SEQ technology**

- TempO-SEQ technology advantages include simple sample preparation, high throughput, high accuracy and sensitivity, simplified bioinformatics analysis
- HepG2, MCF, and HepaRG 2D cell lines
- 24h exposure
- 7 concentrations

**Data analysis: Differential expression analysis, pathway analysis and PoD determination**

- Differential expression analysis was performed using DESeq2 analysis
- Concentration response analysis using BMDexpress2
- PoD was determined based on a subset of methods (1,3,4,5,9) outlined in (Farmahin et al. 2017)
NGRA for 0.1% coumarin in face cream: In vitro biological activity characterisation: In vitro cell stress panel

- Cellular stress response assays are useful to characterize non-specific biological activity which is not mediated via a specific protein/receptor interaction
- Measures a range of biomarkers covering ~10 cell stress pathways
- Single exposure; 8 concentrations; 1h, 6h & 24hr timepoints; HepG2 & NHEK cells

- Mitochondrial Toxicity: MitoSOX, PGC1α, MMP, ATP, Glu/Gal
- Oxidative Stress: GSH, ROS, SRXN1, NRF2
- DNA damage: pH2AX, p53
- Inflammation: TNFAIP3, ICAM1, NFKB p65, IL-1β, IL-8, HMGB1
- ER Stress: PERK, ATF4, CHOP, XBP1, BiP, ER Tracker
- Metal Stress: MTF-1, Metallothionein
- Osmotic Stress (NFAT5); Heat Shock (HSP70); Hypoxia (HIF1α)
- Cell Health: LDH, Phospholipidosis, Steatosis, pHrodo indicator, apoptosis (caspase-3/7) & necrosis (ToPro-3)

NGRA for 0.1% coumarin in face cream: Short and long-term exposure in 3D tissues

PoD for cell stress biomarkers single dose up to 7 days in HepaRG 3D:

- Early signs of cell damage were observed at low concentrations (PoD= 56 µM) after 168h incubation.
- ATP decrease at 72 and 168h (PoD= 190 and 144 µM)
- At concentrations >700 µM) a mixture of biomarkers related to mitochondrial toxicity, oxidative stress and cell health were affected

HTTr in a HepaRG 3D model where cells were exposed to coumarin for 24h

- The response observed was very limited for DeSeq2 with only 4 genes meeting the padj value of 0.05, all seen at the top dose (200 µM)
- Lowest PoD across all methods was 41 µM