Testing of the predictive power and robustness of an adverse outcome pathway construct for bile salt export pump inhibition to cholestatic injury

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1. Context of AOP development and use

☐ Safety Evaluation Ultimately Replacing Animal Testing (SEURAT)

- Raised in response to European Regulation (EC) No. 1223/2009
  - Cosmetic products and their ingredients
  - Testing and marketing ban

- Public - private research initiative
  - European Commission/FP7 (25 million €)
  - Cosmetics Europe (25 million €)

- Organization
  - 1 January 2011 - 31 December 2015
  - More than 70 research institutions
  - 6 projects and 1 coordinating action

www.seurat-1.eu
SEURAT-1 projects

- **SCR&Tox**: stem cell differentiation for providing human organ-specific target cells
- **HeMiBio**: development of a hepatic microfluidic bioreactor
- **DETECTIVE**: identification and investigation of human biomarkers
- **COSMOS**: delivery of computational tools to predict adverse effects of chemicals
- **NOTOX**: development of systems biology tools for organotypic human cell cultures
- **ToxBank**: supporting integrated data analysis and servicing
- **COACH**: coordinating action
2. AOP selection

☑️ Resource and strategy

Scientific Committee of Consumer Safety (SCCS)
Performs safety evaluations of candidate cosmetic compounds to be included in the annexes of European Regulation (EC) No. 1223/2009
Publication of safety evaluation reports on open website

Screening of SCCS safety evaluation reports published between 2000 and 2009
253 safety evaluation reports covering 220 cosmetic substances
Focus on repeated dose toxicity testing

☑️ Outcome

The liver is the most frequently targeted organ by cosmetics
Cholestasis is a prominent form of liver toxicity induced by cosmetics

3. AOP development

✓ Step 1: identification of AOP anchors

- Adverse outcome (AO): change in morphology or physiology of an organism or system that results in impairment of the functional capacity or the capacity to compensate for stress

- Molecular initiating event (MIE): initial point of chemical-biological interaction within the organism

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OECD (2012) Proposal for a template and guidance on developing and assessing the completeness of adverse outcome pathways.
AOP from bile salt export pump inhibition (MIE) to cholestasis (AO)

Bile salt export pump (BSEP/ABCB11) inhibition

ATP-binding cassette transporter

Located at canalicular membrane surface of hepatocytes

Active transport of bile and drugs from hepatocytes to the bile duct
Cholestasis

Definition

Impairment of bile flow from liver to duodenum

Accumulation of bile plugs in canalicular areas or bile ducts

Types

Intrahepatic: blockage inside the liver

Extrahepatic: blockage outside the liver
Step 2: identification of intermediate steps and key events

- Response matrix between the MIE and the AO

- Key events are intermediate steps that are
  - Toxicologically relevant
  - Experimentally measurable/quantifiable

- Located at different levels of biological organization

- Need for understanding normal physiological pathways

Resources

- Literature data
- *In vivo* data
- *In vitro* data
AOP from BSEP inhibition to cholestasis

Intermediate steps

Cellular level
- Altered expression of drug metabolizing enzymes
- Altered expression of drug transporter proteins
- Mitochondrial disruption
- Apoptotic cell death
- Necrotic cell death

Organ level
- Extracellular leakage from cytosolic enzymes
- Pruritus
- Bilirubinuria
- Bilirubinemia
- Jaundice

Key events (cellular level)
- Bile acid accumulation
- Inflammation
- Oxidative stress
- Activation of nuclear receptors

Step 3: data linkage and representation

- Linear graphical representation

- Linkage of events
  - Established mechanistic links (quantitative data)
  - Plausible links (limited data)
  - Empirical links (quantitative exposure - response data)
  - Predictive model links (quantitative exposure - response data/chemical structure data)
  - Hypothetical links

- Subjective evaluation of the strength of linkage of the AOP components
  - (Very) strong
  - Moderate
  - Weak

AOP from BSEP inhibition to cholestasis

4. AOP evaluation

- **Weight of evidence assessment: Bradford-Hill criteria**
  - Concordance of dose-response relationships?
  - Temporal concordance among the key events and the AO?
  - Strength, consistency and specificity of the MIE-AO association?
  - Biological plausibility, coherence and consistency of the experimental evidence?
  - Alternative mechanisms?
  - Uncertainties, inconsistencies and data gaps?

- **Confidence assessment: OECD key questions**
  - How well characterised is the AOP?
  - How well are the MIE and key events causally linked to the AO?
  - What are the limitations in the supporting evidence?
  - Is the AOP specific to certain tissues, life stages or age classes?
  - Are the MIE and key events expected to be conserved across species?

OECD (2012) Proposal for a template and guidance on developing and assessing the completeness of adverse outcome pathways.
5. AOP project/SEURAT-1 case study

✓ Set - up

3 Liver-based in vitro models
- Human primary hepatocytes
- Human hepatoma HepaRG cells
- Human skin-derived hepatic progenitor cells (hSKP-HPCs)

AOP verification using a compound with a clear-cut toxicological profile
- Bosentan: drug for treatment of pulmonary hypertension

24 hours exposure to 3 concentrations
Detection of established biomarkers
- BSEP inhibition functionality assays
- Reporter gene assay for nuclear receptor activation
- Expression of key events: microarray analysis/qRT-PCR analysis

Characterization of new biomarkers
- Transcriptomics
- Epigenomics
- Metabonomics
- Proteomics

AOP application using a compound with a poorly documented toxicological profile
- Triclosan: antimicrobial agent in consumer products

24 hours exposure to 3 concentrations
Preliminary results

- HepaRG cells: 135 µM bosentan
- Cholestasis is the third most significantly enriched toxicity function in HepaRG cells
5 genes correctly modulated

<table>
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<tr>
<th>Gene</th>
<th>HepaRG vs. control</th>
<th>Prediction</th>
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<tr>
<td>SLCO1B1 (OATP1B1)</td>
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<td>ABCC3 (MRP3)</td>
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molecular interaction
- tight junction disruption
- altered membrane fluidity
- BSEP inhibition
- compromised cytoskeleton
- disturbed vesicle transport

cellular effects
- bile acid accumulation
- MPP
- oxidative stress
- inflammation
- SHP activation
- FXR activation
- PXR activation
- CAR activation

organ effects
- decreased ALP
- increased ALT
- increased AST
- pruritus

organism effects
- bilirubinuria
- bilirubinemia
- jaundice
- increased ALP
- increased 5-NT
- increased GGT
- increased ALT
- increased AST
- pruritus

upregulated CYP2B10 expression
upregulated UGT2B4 expression
upregulated CYP3A4 expression
upregulated SULT2A1 expression
upregulated MRP5 expression

upregulated NTCP expression
upregulated OATP1B1 expression
downregulated CYP7A1 expression
downregulated ATP181 expression
downregulated bilirubinuria
Identification of new (transcriptomics) biomarkers of cholestasis

ABCB4: multidrug resistance protein 3

ADH1C: alcohol dehydrogenase 1C

AKR1D1: aldo-keto reductase 1D1

GPX2: glutathione peroxidase 2

LBP: lipopolysaccharide-binding protein

SLC22A1: organic cation transporter 1
6. AOP use

Chemical categorization/grouping

- Focus on MIE

- Establishment of (quantitative) structure - activity relationships

- Basis for read-across approaches and *in silico* modeling

Example: BSEP inhibition

- Contributing structural determinants: esters bound to a carbon atom of heterocyclic groups

- Counteracting structural determinants: hydroxyl groups bound to aliphatic carbon atoms

Identification of biomarkers and test development

- Focus on MIE and key events

- Establishment of mechanistic \textit{in vitro} methods

- Characterization of \textit{in vivo} - relevant biomarkers

- Examples
  - BSEP inhibition
    - Cholyl - lysyl - fluorescein assay
    - Vesicular transport assay
    - BSEP ATPase assay
  - Nuclear receptor activation: gene reporter constructs

7. Take-home message

✓ Generation of hepatic AOPs in SEURAT-1
  - Cholestasis
  - Fibrosis
  - Steatosis

✓ Fine-tuning of hepatic AOPs
  - More time points (reversibility of effects)
  - More chemicals (structure-activity effects)
  - More analyses (functional and -omics read-outs)

✓ Follow-up
  - Testing in SEURAT-1 case study program
  - Testing in OECD AOP program
  - Included in AOP wiki
8. Acknowledgements

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