Application of bioassays for packaging safety evaluation

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What is the problem?

- Identification
- Quantification
- Migration conditions
- Product applications
- Toxicology

- Not practical
- Not desirable

Is there a role/value for bioassays in safety assessment of such mixtures?
Why not?

Safety

- Novel foods
- Plant extracts
- Medical devices
- Water quality

In vitro tox

- > 1000 (Tox21)
- Human stem cells
- 3-D

mixtures

bioassays

Packaging

- Genotoxicity
- Endocrine act.

ISO

OECD

Research
The topic is increasingly discussed in context of packaging safety

- Quality/validation
- Limitations
- Data interpretation
What are bioassays?
In vitro hazard identification/characterization:

Hazard identification:
- MIEs/KEs (AOP/MOA)
- Stem cells/3D

Hazard characterization:
- Dose responses
- QIVIVE/Reverse dosimetry

Oral Point of departure

Exposure

Safety/MoE

Biodetection (occurrence):

In vitro tools designed:
- Molecular events
- Properties of tox relevance

In vitro tools designed:
- Molecular events
- Properties of tox relevance

In silico:
- Link to chemical structure, SAR
- Similarity

Mitigation

Manage

Exposure

Standard hazard id/RA
Example of nuclear receptor activation
transcriptional activation assay

**CALUX** = Chemical-Activated Luciferase Reporter Gene-Expression Assay

- Several hormone receptors available
- Agonist and antagonist modes

- ERα
- AR
- AhR
- GR
- TR
- PPARγ
- ...

-/+ S9 cytotox
Application of the Calux assay

Migration study:
• Analytical chemistry
• Bioassay

156 µg/L NP (LC-MS)
Example of genotoxicity assay

**Gadd45α induction (Bluescreen)**

- Cell's genotoxic stress response
- Identifies diverse genotoxic agents:
  - Direct acting
  - Others (with threshold)
- Possibility to apply metabolic system (S9)
- Cytotoxicity test included
- High sensitivity: little false negatives
- High specificity: little false positives
- Good within/between lab reproducibility
- Commercially available
- Getting increasing acceptance for screening
- Potential for improvement/optimization
Gadd45α induction in FCM-migrates of experimental material

Gaps and limitations need to be addressed:

- Relevance of migration studies (stability of the materials?)
- Identify causative agent(s)
  - Current analytical data did not reveal chemicals with alert for genotox (DNA-reactivity)
  - Test of identified NIAS ongoing
  - Fractionation planned
- Address mechanisms of genotoxicity
  - Mutagenic
  - No positive samples in Ames (no DNA reactive? Threshold?)
- …..
Why and When?

1. Safety by design
2. Application of the TTC
Safety by design: bioassay data on R&D materials.

<table>
<thead>
<tr>
<th>Biological activity</th>
<th>Coating 1</th>
<th>Coating 2</th>
<th>Coating 3</th>
<th>Coating 4</th>
<th>Coating 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-estrogenic (ERα)</td>
<td>+</td>
<td>-</td>
<td></td>
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<tr>
<td>PPARγ</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Anti-androgenic</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>AhR</td>
<td></td>
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<tr>
<td>Gadd45α</td>
<td></td>
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<tr>
<td>Cytotoxicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMES</td>
<td>*</td>
<td>*</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

EthOH, 95% concentration migration 10 days at 60°C

Bioassays DMSO
Good correlation analytical vs biological profile

GC/MS

N=3

Packaging material

-  -  -  +  ++  ++  +++

50%-Cytotoxicity
50%-Genotoxicity
A way forward (effect directed analysis)

Fractionation:
LC-HRMS platform coupled with a Waters fraction collector

- Monomers
- Additives
- Synthetic oligomers

<table>
<thead>
<tr>
<th>Fraction</th>
<th>genotox</th>
<th>endocrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 6</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Fraction 7</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Fraction 8</td>
<td>--</td>
<td>--</td>
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<tr>
<td>Fraction 9</td>
<td>--</td>
<td>--</td>
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<tr>
<td>Fraction 10</td>
<td>--</td>
<td>✓</td>
</tr>
<tr>
<td>Fraction 11</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Fraction 12</td>
<td>✓</td>
<td>--</td>
</tr>
<tr>
<td>Fraction 13</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
Safety by design:
evaluate raw materials early (e.g. monomers)

**Compound A:**
- PPAR\(\gamma\) antagonist effect
- No antagonistic effect on ER\(\alpha\)
- No agonistic effect on AR
Why and When?

1. Safety by design
2. Application of the TTC
Application of the Cramer class III-TTC to unknown NIAS (safety assessment)?

Exclude member of cohort of concern.
Exclude chemicals not covered by TTC.
TTC = 0.15 μg, if alert of genotox.
TTC = 18 μg, inh. AChE.

AhR; DR-Calux.
ER, AR assays
Ach-esterase inhibition
Genotoxicity assays
### Proposed steps in assessment of unknowns

<table>
<thead>
<tr>
<th>Steps</th>
<th>What?</th>
<th>Why?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Material characterization</td>
<td>Composition, manufacturing, processing, degradation, …</td>
<td>Exclude chemicals of high concern (cohort of concern)</td>
</tr>
</tbody>
</table>
| 2. Analytical methods  | - Sample preparation  
- Chromatographic techniques  
- Detection methods  
- Partial identification |                                                        |
| 3. Targeted analysis   | - Methods for specific chemicals                                     |                                                        |
| 4. Food intake         | Material application, population, dietary habits                      | Estimate exposure                                      |
| 5. Quantification      | Quantification of unknowns                                           |                                                        |

Adapted from Koster et al., *Fd Chem Tox* 49 (2011) 1643-60; Rennen et al., *Fd Chem. Tox* 49 (2011) 933-940
Genotox tests are sensitive:

\[
\text{Sensitivity} = \frac{\text{TP}}{\text{TP} + \text{FN}}
\]

TP: true positive
FN: false negative

Genotox tests are not sensitive:

Limits of detection (LoD) in mixture are currently poor:
- 0.15 μg/person
- risk \((10^{-6})\)
- 10 ppb
- 90 μg/person
### Genotoxic:

- LoD can be improved, observed for key mechanisms
- Important for mixture testing
- Important to discriminate genotoxicity from cytotoxicity
- Depends upon:
  - *Type of assay*
  - *Culture conditions*
  - *Substances*
  - ...  
  - Clearly insufficient (compared with LoD requirements)
  - **Need a breakthrough**

### Other endpoints:

- Similar conclusion for Ach-esterase inhibition
- Less an issue for receptor mediated activation

<table>
<thead>
<tr>
<th>Type MoA Substances</th>
<th>LEC Standard protocol</th>
<th>LEC optimized protocol</th>
<th>Improvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct DNA-damaging compounds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA alkylation or forming DNA adducts</td>
<td>0.1 mg/ml</td>
<td>0.01 mg/ml</td>
<td>10</td>
</tr>
<tr>
<td>AFB1</td>
<td>1.25 mg/ml</td>
<td>0.42 mg/ml</td>
<td>3</td>
</tr>
<tr>
<td>B(a)P</td>
<td>6.25 mg/ml</td>
<td>5.31 mg/ml</td>
<td>1</td>
</tr>
<tr>
<td>CPA</td>
<td>Not detected</td>
<td>0.5 mg/ml</td>
<td></td>
</tr>
<tr>
<td>Pyrene</td>
<td>34.0 mg/ml</td>
<td>1.16 mg/ml</td>
<td>29</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>46.31 mg/ml</td>
<td>25.0 mg/ml</td>
<td>1.8</td>
</tr>
<tr>
<td>N-nitrosodimethylamine (NDMA)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Other endpoints:

- Similar conclusion for Ach-esterase inhibition
- Less an issue for receptor mediated activation
The way forward?
High performance thin layer chromatography (HPTLC)-bioassay

Discovery in surface/waste water

Bioassays: roles in packaging safety

To prioritize structurally uncharacterized chemicals with TTC-class III:
- To contribute to exclusion of chemicals of the cohort of concern
- To exclude ACHE-inhibitors and chemicals with genotoxic alert
- In combination with other parameters
- More sensitive methods required

To test for the presence of chemicals:
- Endocrine activity
- High toxic potency
- To be assessed/managed (early)

HPTLC-bioassay is likely to significantly improve the situation:
- May increase LoDs by orders of magnitude (e.g. genotox, AChE-inh, receptor med, …).
Chemical screening vs biodetection

**final thoughts**

Together with analytical chemistry, bioassays have a role to play in safety assessment of FCMs.
Acknowledgements:

Packaging Safety Group

Early warning Group

Chemical Food Safety Group

Thanks for your attention