ADVANCES IN GENOTOXICITY TESTING, PART 2: The reconstructed human skin micronucleus assay

ASCCT webinar series

Stefan Pfuhler
Procter & Gamble
CE TG Genotoxicity
Agenda

1. Background
2. Concept of the use of 3D skin tissue equivalents in genotoxicity testing
3. Reconstructed skin comet assay
4. Reconstructed skin micronucleus assay
5. Strategic fit of assays in testing strategies and examples
6. Summary/outlook
Dermal route

Dermal Route

- **3D Skin Comet**
  - Reconstructed Skin Comet assay

- **RSMN**
  - Reconstructed Skin Micronucleus test

- Test systems combined with classical read-outs.
- Battery of two assays addresses all three endpoints.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Mutation</th>
<th>Structural</th>
<th>Numerical</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS Comet</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>RSMN</td>
<td></td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

- Assays are intended to follow up on initial positive findings.

Phenion® Full-Thickness Skin Model
www.phenion.com

EpiDerm™ (MatTek)
Understanding skin metabolism

Dermal application of test material, multiple application protocol (enables enzyme induction)

Metabolic competency of 3D skin models similar to human skin

Test Principle: RSMN

- Assay is built on reconstructed human skin tissues using micronucleus OECD 487 technology
- Assay development: Collaboration between IIIVS and P&G (Curren et al., 2006)
- Protocol refinement and start of an international validation effort in 2007

1. EpiDerm™ models are treated topically with test compound.
2. Dose at 24h intervals (48h or 72h total)
3. Precipitation at the beginning and the end of the treatment period is noted.
4. Keratinocytes are released by trypsinization
5. Micronuclei in binucleated cells are counted by visual scoring.

Detailed methodology info:
See Dahl et al, 2011
Experimental design

- OECD 487 cytoB method, modified
- 2 or 3 treatments at -72, -48 and -24h
- Minimum of 3 doses
- 3 tissues/dose (2 acceptable)
- 500 binucleated cells evaluated/tissue
- Maximum dose: 1600 ug/cm²
- If cytotoxic, aiming at:
  - 50 ± 10% (high cytotoxicity)
  - 30 ± 10% (intermediate cytotoxicity)
  - 10 ± 10% (low cytotoxicity)
- Toxicity measures:
  - % binucleation (>40% control)
  - Cell count (>40% control)
  - More sensitive defines cutoff

Decision tree for validation exercise

[Diagram showing decision tree for validation exercise]

Conclusion:
- Positive response
- Negative response
- Equivocal response

2nd Confirmatory: 48h, or 72 h (phases 2b-d)

Adjust dose spacing if applicable
Recommended decision tree, using the 72h protocol only. Decision tree is in line with OECD 487 where clear positive or clear negative results do not need to be reproduced.

**Conclusion:**
- **Positive response**
  - Conclusion: Positive
  - Positive response
  - Confirmatory 72 h
    - Adjust dose spacing if applicable
    - Positive response
    - Equivocal response
      - Conclusion: Equivocal

- **Equivocal response**
  - Conclusion: Equivocal

- **Negative response**
  - Conclusion: Negative

Pfuhler et al., 2020
Validation setup

• International validation team, with involvement of EURL ECVAM from the start
• Substance selection via external subject matter experts
• Steering Team of experts, extended team as needed (e.g., decision making for next steps)
• International laboratories (6 total) experienced in genotoxicity testing and with working with 3D skin models
• Constant discussion/calibration with scientific community (over 100 presentations and publications)
RS assay project – validation outline

**Phase 1**
Optimization and transferability with 2 model genotoxins

**Phase 2**
Intra- and inter-lab reproducibility with 5-10 coded compounds

**Phase 3**
Validation with 30+ coded Compounds per assay

**Selection of compounds:**
Initial selection by international subject matter experts (assay experts, skin metabolism and skin cancer experts): final selection of validation subset by Raffaella Corvi (EURL-ECVAM), David Kirkland (Kirkland consulting)

**Coding & shipment of chemicals:**
EURL-ECVAM, Italy; ZEBET, Germany; Covance, UK; VitroScreen, Italy; Integrated Laboratory Systems, Inc. USA, BioTeSys, Italy

**Decoding:**
Raffaella Corvi (EURL-ECVAM)

**Independent analysis of data:**
Sebastian Hoffmann (seh consulting & services); Ralph Pirow, BfR, Germany
We personally care

Validation timeline

Phase 0
Transferability and assay optimization:
(Dahl et al., 2010)

Phase 2a
Predictive capacity: n = 35, 48h

Phase 1
Intra- and inter-laboratory reproducibility:
n = 3, 48h
(Aardema et al., 2010)

Phase 2b
Predictive capacity: n = 9, re-tested, 72h

Phase 2c
Predictive capacity and reproducibility of 72h protocol:
n = 12, re-tested, 48 & 72h
SC meeting: bridging study needed

Phase 2d
Predictive capacity gap-filling:
n = 5, 48 & 72h
SC/expert meeting: gap-filling required

Validation outcome - Mutagenesis Special Topic “3D Skin”

- Edited by Shareen Doak; Guest Editors: Rafaella Corvi & Stefan Pfuhler
- April 2021
- 5 manuscripts, including the RS Comet and RSMN validation papers
- Volume 36 Issue 1 | Mutagenesis | Oxford Academic (oup.com)
Examples from Validation dataset

a) Figure S5: Colchicine
b) Figure S14: 5-fluorouracil

(Data from: Pfuhler et al, Mutagenesis, 2021, 36, 1–17 – Supplemental figures)
Figure S5: colchicine

% micronucleated binucleate cells (MNNB): mean with range (●)

% relative binucleation: mean - SD (◼)

% relative viable cell count: mean - SD (☉)

A: 48 h (2a)
B: 48 h (2a)
C: 48 h (2a)
D: 48 h (2a)

Lab A
Lab C

Figure S5: colchicine

% relative binucleation: mean - SD (●) and % relative viable cell count: mean - SD (◼)

CA: p<0.05

Lab A
Lab C

Dose (µg/cm²)

A
B
C
D

CA: p<0.05

SCMMC
Figure S14: 5-fluorouracil

Figure S14: 5-fluorouracil
We personally care

See Pfuhler et al, 2021

Table 1. Overview of validation outcome of the RSMN experiments conducted within the coded validation effort in all phases

<table>
<thead>
<tr>
<th>Chemical</th>
<th>CAS No.</th>
<th>Cat</th>
<th>Phase</th>
<th>Lab A</th>
<th>Lab B</th>
<th>Lab C</th>
<th>Lab D</th>
<th>BLR</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Acetylamino-1,4-benzenediol (2-AAF)</td>
<td>53-96-3</td>
<td>TP</td>
<td>2</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2-Amino-3-methylimidazo[4,5-f]quinoline (IQ)</td>
<td>76180-96-6</td>
<td>TP</td>
<td>2d</td>
<td>Pos</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Azidothymidine (AZT)</td>
<td>30516-87-1</td>
<td>TP</td>
<td>2d</td>
<td>Pos</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Cadmium chloride (CdCl₂)</td>
<td>10108-64-2</td>
<td>TP</td>
<td>2a,b,c</td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Colchicine</td>
<td>64-86-8</td>
<td>TP</td>
<td>2a</td>
<td>Pos</td>
<td>Pos</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclopenta[c,d]pyrene (CPPE)</td>
<td>27208-37-3</td>
<td>TP</td>
<td>2a,b</td>
<td>Pos</td>
<td>Neg*</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Cytosine arabinoside</td>
<td>147-94-4</td>
<td>TP</td>
<td>2a,b</td>
<td>Neg</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>2,4-Diaminotoluene (2,4-DAT)</td>
<td>95-80-7</td>
<td>TP</td>
<td>2a,b</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2,3-Dibromo-1-propanol</td>
<td>96-13-9</td>
<td>TP</td>
<td>2a</td>
<td>Pos</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>56-53-1</td>
<td>TP</td>
<td>2a,b</td>
<td>Pos</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>7,12-Dimethylbenz[a]anthracene (DMBA)</td>
<td>57-97-6</td>
<td>TP</td>
<td>2d</td>
<td>Neg</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Ethyl methanesulfonate (EMS)</td>
<td>62-50-0</td>
<td>TP</td>
<td>2a,c</td>
<td>Pos</td>
<td>Pos</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N,N-Dimethyl-N-nitrosourea (ENU)</td>
<td>759-73-9</td>
<td>TP</td>
<td>1</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Etoposide</td>
<td>33419-42-0</td>
<td>TP</td>
<td>2a</td>
<td>Pos</td>
<td>Pos</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>51-21-8</td>
<td>TP</td>
<td>2a,b,c</td>
<td>Pos</td>
<td>Neg</td>
<td>–</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Methyl methanesulfonate (MMS)</td>
<td>66-27-3</td>
<td>TP</td>
<td>2a</td>
<td>Pos</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>N-Nitro-N-nitrosoguanidine (MNNG)</td>
<td>70-25-7</td>
<td>TP</td>
<td>2d</td>
<td>Pos</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>50-07-7</td>
<td>TP</td>
<td>1</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Potassium bromate</td>
<td>7738-01-2</td>
<td>TP</td>
<td>2a,b,c</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Taxol</td>
<td>33069-62-4</td>
<td>TP</td>
<td>2a</td>
<td>Pos</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>4-Vinyl-1-cyclohexene dioxide</td>
<td>106-87-6</td>
<td>TP</td>
<td>2a,b,c</td>
<td>Pos*</td>
<td>Pos</td>
<td>Pos</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ampicillin sodium salt</td>
<td>69-52-3</td>
<td>TN</td>
<td>2a</td>
<td>Neg</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Beclometasone dipropionate</td>
<td>5534-09-8</td>
<td>TN</td>
<td>2a</td>
<td>Neg</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>N-Butyl chloride</td>
<td>109-69-3</td>
<td>TN</td>
<td>2a,c</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Curcumin</td>
<td>458-37-7</td>
<td>MP</td>
<td>2a</td>
<td>Pos</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Cyclohexanone</td>
<td>108-94-1</td>
<td>TN</td>
<td>1</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2,6-Diaminotoluene (2,6-DAT)</td>
<td>823-40-5</td>
<td>MP</td>
<td>2a</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
<td>120-83-2</td>
<td>MP</td>
<td>2a</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Dichlofenac</td>
<td>15307-79-6</td>
<td>TN</td>
<td>2a,c</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ethionamide</td>
<td>536-33-4</td>
<td>MP</td>
<td>2a,c</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Eugenol</td>
<td>97-53-0</td>
<td>MP</td>
<td>2d</td>
<td>Pos</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>148-24-3</td>
<td>MP</td>
<td>2a</td>
<td>Neg</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>d-Limonene</td>
<td>5989-27-5</td>
<td>TN</td>
<td>2a,c</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>d-Mannitol</td>
<td>69-65-8</td>
<td>TN</td>
<td>2a</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Nifedipine</td>
<td>21829-25-4</td>
<td>TN</td>
<td>2a</td>
<td>Neg</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>67-20-9</td>
<td>MP</td>
<td>2a</td>
<td>Neg</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>1-Nitronaphthalene</td>
<td>86-57-7</td>
<td>MP</td>
<td>2a</td>
<td>Neg</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>4-Nitrophenol</td>
<td>100-02-7</td>
<td>MP</td>
<td>2a,c</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>85-01-8</td>
<td>TN</td>
<td>2a,b</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Phenol</td>
<td>108-95-2</td>
<td>MP</td>
<td>2a</td>
<td>Neg</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Propyl gallate</td>
<td>121-79-9</td>
<td>MP</td>
<td>2a</td>
<td>Neg</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Resorcinol</td>
<td>108-46-3</td>
<td>MP</td>
<td>2a,c</td>
<td>Equiv</td>
<td>Neg</td>
<td>–</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Tobutamide</td>
<td>64-77-7</td>
<td>TN</td>
<td>2a</td>
<td>Equiv</td>
<td>Neg</td>
<td>Neg</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>
Follow-up options for *dermally exposed substances*, as a function of the outcome of the 2-test *in vitro* battery

*low priority for follow-up*
Validation outcome - RSMN

Overall Sensitivity of the skin assay battery increases to 89% when endpoint-specific strategy is applied!

(many true pos are double-positive)
Practical use of the RSMN – Case examples

• RSMN (and Comet) assays are offered by CRO’s, under GLP
• Several examples exist of how these assays have been used for (regulatory) decision making:
  o RS Comet examples, as already presented by K. Reisinger
  o Example 1: Use of the RSMN as ‘2\textsuperscript{nd} Tier’ tool in an \textit{in-vitro-only} testing strategy for fragrance materials (concordance with in vivo)
  o Example 2: Use in the context of a hair dye precursor (skin-specific metabolism)
  o Example 3: Use for a nanomaterial (barrier)
  o Example 4: Use for an aneugenic dermal drug (hazard/risk, limitations)

Not discussed today:

Cosmetics Europe project with IIVS to establish a \textbf{photo-RSMN} that enables detection of genotoxins that are activated by UV irradiation
Example 1: Research Institute for Fragrance Materials (RIFM) genotoxicity program

- Part of RIFM screening for genotoxicity potential of >2500 fragrance components
- Bluescreen® used to prioritize for further testing, then a 2-test *in vitro* strategy (Ames plus *in vitro* MN)
- Many fragrance materials are also used as flavor -> EFSA* requires *in vivo*-follow-up testing
- Aspiration to avoid *in vivo* testing in the future also in the context of oral exposure! (HET-MN)
- Manuscript in press

https://doi.org/10.1093/mutage/geab040

*EFSA: European Food Safety Authority
RIFM dataset

- 19 RSMN/in vivo MNT pairs
- 100% concordance
- RSMN = GLP compliant
- 18/19 in vivo MNT are state-of-art, GLP and OECD compliant studies

Table 1. Summary table describing all genotoxicity data for materials

<table>
<thead>
<tr>
<th>Material</th>
<th>CAS #</th>
<th>In vitro MNT</th>
<th>3D Skin MNT</th>
<th>In vivo MNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>sec-Butyl ethyl ether</td>
<td>2679-87-0</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cadinene</td>
<td>29350-73-0</td>
<td>+(^a)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2,3-Dihydro-1,1-dimethyl-1H-indene-ac-propanal</td>
<td>300371-33-9</td>
<td>Equivocal</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1,5-Dimethyl[bicyclo[3.2.1]octan-8-one-oxime]</td>
<td>75147-23-8</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2,2’-(Dithiodimethylene)difuran</td>
<td>4437-20-1</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl formate</td>
<td>109-94-4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-Ethyl-1,3,3-trimethyl-2-norbornanol</td>
<td>18368-91-7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Furfuryl thioacetate</td>
<td>13678-68-7</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Isobornyl methyl ether</td>
<td>3331-32-8</td>
<td>Equivocal</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lauric Aldehyde</td>
<td>112-54-9</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>p-Methoxy cinnamaldehyde</td>
<td>1963-36-6</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6-Methoxy-2,6-dimethylheptan-1-ol</td>
<td>62439-41-2</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-Methyl-2-pentenal</td>
<td>623-36-9</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl beta-phenylglycidate</td>
<td>37161-74-3</td>
<td>+(^a)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nona-2 trans-6-cis-dienal</td>
<td>557-48-2</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-Octenoic acid, 4-ethyl, (2Z)</td>
<td>60308-75-0</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-Octen-4-one</td>
<td>4643-27-0</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4-Phenyl-3-buten-2-ol</td>
<td>17488-65-2</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5-Phenylhex-3-en-2-one</td>
<td>60405-50-7</td>
<td>Equivocal</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4-Thujanol</td>
<td>546-79-2</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3,3,3’-Trimethylcyclohexaneacetic acid</td>
<td>3213-73-8</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Veratraldehyde</td>
<td>120-14-9</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

aResults did not meet all criteria for a positive.
bRead-across analogue is 1-ethyl-3-methoxytricyclo[2.2.1.02,6]heptane (CAS # 31996-78-8).
cRead-across analogue is 4-Phenyl-3-buten-2-one (CAS#122-57-6).
Example 2: Hair dye precursor paraphenylenediamine (PPD)

- Data situation: (from dossier, SCCS/1443/11)
  - pos in *in vitro* standard battery: Ames, CA, MLA tk (new criteria: negative)
    - neg in HPRT assay
- Was assessed non-genotoxic by SCCS since it was:
  - neg *in vivo*: MN (bone marrow), UDS (liver), Comet (8 organs; Sasaki 2000))
- Shown to be N-acetylated when applied to human volunteers in hair dye formulation (Nohynek et al, *Food Chem Toxicol*, 42, 1885-1891)
Case study: PPD

Evaluation of PPD in the 3D Human Reconstructed Skin Micronucleus Assay, 2 independent studies

Legend:
closed squares: % micronucleated cells
open triangles: relative cell counts
closed triangle: % relative binucleation

PPD tested negative in the 3D skin MN test – skin “first pass” effect?

skin = N-Acetyltransferase (NAT) proficient
Case study: PPD

Comet assay with PPD in three different cell lines:
- NAT1 deficient (V79) and NAT1 proficient (V79NAT1*4, HaCaT)

• Genotoxic effect abolished in NAT competent cell lines

From Zeller and Pfuhler, Mutagenesis 29(1):37-48, 2014

Metabolism data support negative result in Skin assay - “first pass” effect!
Formation of Diacetyl-PPD: Comparison between liver S9 and skin S9

- Speed of NAT conversion in skin similar to liver S9!

- PPD disappears at the same rate Diacetyl-PPD (DAPPD) is formed

Data from Cosmetic Europe Metabolism Project; Eilstein et al., 2019
Example 3: Skin models as a penetration barrier

Slides courtesy of Shareen Doak, Swansea University
85nm amorphous silica nanoparticles on skin surface
Topically applied in acetone, 50μg/mL
2D vs 3D micronucleus assay

LINES / POINTS = Cell Viability
BARS = Micronucleus Frequency

85nm-SiO₂

2D  3D  3DM

Binucleated Cell Micronucleus Frequency (%)

Relative Cell Viability (%)

2D (μg/mL) / 3D (μg)
Equivalent Doses

0  / 0  100 / 150  200 / 300  300 / 450  ~ / 1000  +ve
TK6 Cell Uptake (16nm Amorphous Silica)

Clear particle uptake into cells
Uptake into RS (16nm Amorphous Silica)

No particle uptake into the cells – more realistic exposure conditions for dermal route
Experiments in the EpiDerm 3D Skin In Vitro Model and Minipigs In Vivo Indicate Comparatively Lower In Vivo Skin Sensitivity of Topically Applied Aneugenic Compounds

Maik Schuler, Lindsay Tomlinson, Michael Homiski, Jennifer Cheung, Yutian Zhan, Stephanie Coffing, Maria Engel, Elizabeth Rubitski, Gary Seitis, Katherine Hales, Andrew Robertson, Saurabh Vispute, Jon Cook, Zaher Radi, and Brett Hollingshead

Highlights assay limitations:

- Limited selection of qualified solvents available to date
- Aqueous solvents are problematic – solvents like acetone and ethanol force penetration
- Evaluation is time consuming, automation desired!

- Attempt to use RSMN assay for risk assessment
- Authors could rank-order results according to potency of aneugens
  “...demonstrate that the EpiDerm RSMN is sensitive for the hazard identification of aneugens”
- BUT: substance in question was negative in minipig assay \textit{in vivo}
- Also promotes use of flow-based alternative biomarkers
Summary

- Use of RS models considers main route of exposure of cosmetics as well as skin-specific metabolic fate
- The 3D skin comet and micronucleus assays have been successfully validated
- If used as intended: Overall sensitivity = 89%, overall specificity = 79%
- Assays are offered commercially under GLP at several CROs
- 19 fragrance ingredients with positive results in standard *in vitro* genotoxicity assays tested negative in RS assays and *in vivo* (100% concordance)
- Case studies show the relevance as an exposure-route specific tool
- OECD approved the development of 2 separate guidelines
- Currently undergoing formal validation peer-review by ECVAM
- If successful, OECD guideline development will start
Acknowledgements

Cosmetics Europe TF Genotoxicity

Rolf Fautz, Arianna Giusti, Nicola Hewitt, Sebastian Hoffmann, Julia Kenny, Gladys Ouedraogo, Kerstin Reisinger, Stefan Pfuhler, Brian Wall

External Experts
David Kirkland
Raffaella Corvi
Rodger Curren
Jan van Benthem
Sebastian Hoffman
Ralph Pirow
Manfred Liebsch
Tom Slaga
Johannes Döhmer
Peter Kasper
Günter Speit

Collaborators
P&G, USA: Tom Downs, Brenda Barnett
TNO, The Netherlands: Cyrille Krul, Astrid Reuss
Bioreliance: Marilyn Aardema, Shambu Roy
IIVS, USA: Greg Mun; Emilia Costin
L’Oreal, France: Gladys Ouedraogo
Henkel, Germany: Kerstin Reisinger
BfR/FU Berlin, Germany: Frank Henkler, Joop Brinkmann; Andre Said, Monika Schaefer-Korting
BASF, Germany: Markus Schulz, Veronica Blaz
Imperial College London, UK: Rob Edwards
University Duesseldorf: Ellen Fritsche
### References (selected)

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Validation report</td>
<td>Pfuhler S, Downs TR, Hewitt NJ, Hoffmann S, Mun GC, Ouedraogo G, Roy S, Curren RD, Aardema MJ: Validation of the 3D reconstructed human skin micronucleus (RSMN) assay, an animal-free alternative for following-up positive results from standard in vitro genotoxicity assays; Mutagenesis, 2021, 36, 1–1; doi:10.1093/mutage/geaa035</td>
</tr>
<tr>
<td>Supporting literature</td>
<td>SCCS. (2018) Scientific Committee on Consumer Safety Notes of Guidance (NoG) for the Testing of Cosmetic Ingredients and Their Safety Evaluation, 10th Revision, SCCS/1602/18</td>
</tr>
</tbody>
</table>