Modernizing the “six-pack” testing strategy: influx of modern in vitro techniques

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The 3Rs

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Perspectives, challenges, common goals and working together
Presentation Outline

Current regulatory climate – global acceptance of *in vitro* methods

The reductionist concept of *in vitro* methods

Drivers of *in vitro* methods advancement

Beyond the “six-pack” battery of acute toxicity tests

- Acute oral toxicity
- Acute dermal toxicity (*oral vs dermal route comparison*)
- Acute inhalation toxicity
- Ocular irritation (*the EPA OPP testing strategy*)
- Skin irritation/corrosion
- Skin sensitization

Modernizing the “six-pack” testing strategy: influx of modern *in vitro* techniques
Current regulatory climate – global acceptance of *in vitro* methods
The reductionist concept of *in vitro* models

<table>
<thead>
<tr>
<th>1940s</th>
<th>1990s</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole animal</strong>&lt;br&gt;(Rabbit)</td>
<td><strong>Cell culture</strong>&lt;br&gt;(Statens Seruminstitut Rabbit cornea cells)</td>
</tr>
<tr>
<td><strong>Organ - Eyeball</strong>&lt;br&gt;(Enucleated chicken or rabbit eye)</td>
<td><strong>Tissue - Cornea</strong>&lt;br&gt;(Resected bovine cornea)</td>
</tr>
</tbody>
</table>

“Less is more”

<table>
<thead>
<tr>
<th>2000s</th>
<th>2010s</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body-on-a-chip</strong>&lt;br&gt;(Human organotypic microtissues)</td>
<td><strong>Organ-on-a-chip</strong>&lt;br&gt;(Human retina)</td>
</tr>
<tr>
<td><strong>Tissue construct</strong>&lt;br&gt;(Human EpiCorneal™ model)</td>
<td><strong>Cell culture</strong>&lt;br&gt;(Normal human corneal epithelial cells)</td>
</tr>
</tbody>
</table>


Drivers of *in vitro* methods advancement

Ongoing evolution on so many levels

- Improve scientific basis for testing using human-derived test models
- Reduce the number of animals for testing
- Increase predictivity
- Reduce time, price
- Harmonize requirements and prediction models

http://alttox.org/mapp/table-of-validated-and-accepted-alternative-methods/
Beyond the “six-pack” battery of acute toxicity tests

PESTICIDES

Acute oral rat

Acute dermal rabbit

Skin irritation rabbit

Ocular irritation rabbit

Skin sensitization guinea pig mouse

Acute inhalation rat

EPA Health Effects Test Guidelines OPPTS 870.1000 Acute Toxicity Testing-Background.

The modern *in vitro* toxicology perspective
Acute oral toxicity – CRO’s perspective

Test system: normal human keratinocytes (NHEKs) or Balbc 3T3 mouse fibroblasts
Assay endpoints: cell viability [by Neutral Red Uptake (NRU) assay]
Data calculation: estimated log LD$_{50}$ mmol/kg = 0.435 x log mean NRU50 mM + 0.625
For initial dose setting
OECD Guidance Document 129

Acute dermal toxicity – Regulatory perspective
(oral vs dermal route comparison)

Efforts to use a single route

- 2013, US EPA OPP, National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM): retrospective analysis of oral and dermal acute lethality studies focused on formulated pesticides products considering the EPA pesticide categorization scheme.
Acute dermal toxicity — Regulatory perspective
(oral vs dermal route comparison)

The dataset of rat acute oral and acute dermal LD$_{50}$ studies included:
- 592 formulated pesticide products, representing 316 active ingredients
- all four Toxicity Categories
- 13 different formulated pesticide product types

Data analysis
- For 57% of the 592 formulations, the results of both oral and dermal acute toxicity studies fall within the same Toxicity Category.
- For 38% of the formulations, the oral study falls within a lower (i.e., more protective) Toxicity Category.
- Thus, for 95% of the formulations in the analysis, if the dermal study had not been available and labelling had been based only on the Toxicity Category for the oral acute toxicity study, the PPE requirements on the labelling would have been equally protective or more protective.
- For the remaining 5%, factors other than the dermal acute toxicity may influence PPE labeling requirements.

The agency has used this analysis to support a policy statement in Section 3.0 to waive all acute lethality dermal studies for formulated pesticide products.

**Acute inhalation toxicity – CRO’s perspective**

**Efforts for *in vitro* methods development**

**Cell lines/tissues/explants**
- Bronchial epithelial cells
- Reconstructed tissues

**Endpoints**
- Cytotoxicity
- mRNA (MUC5AC)
- Protein expression: IL-6, IL-8, MMP-1
- Gene expression and microarrays
- Cellular glutathione levels

**Smoke exposure systems**

**EpiAirway model (MatTek Corporation)**
Normal, human-derived tracheal/bronchial epithelial cells

**MucilAir model (Epithelix)**
Primary epithelial cells co-cultured with human airway fibroblasts

**Precision Cut Lung Slice (PCLS)**
Alveolar spaces; H&E staining, 40x magnification*

http://www.mattek.com
http://www.epithelix.com

*Image courtesy of Dr. Khalid Amin, University of Minnesota, Department of Laboratory Medicine and Pathology*
Voluntary pilot program underway where registrants may send the in vivo acute lethality study for oral and inhalation formulation/product testing as currently required and simultaneously submit the calculations using the GHS dose additive mixtures equation.

The acute toxicity estimate (ATE) of ingredients should be considered as follows:

- Include ingredients present at 1% or greater with a known acute toxicity, which fall into any of the GHS acute toxicity categories.
- Ignore ingredients that are presumed not acutely toxic (e.g., water, sugar).
- Ignore ingredients if the oral limit test does not show acute toxicity at 2,000 mg/kg/body weight.

The ATE of the mixture is determined by calculation from the ATE values for all relevant ingredients according to the following formula below for Oral, Dermal or Inhalation Toxicity:

\[
\frac{100}{ATE_{mix}} = \sum_{i} \frac{C_i}{ATE_i}
\]

where:
- \(C_i\) = concentration of ingredient \(i\)
- \(n\) ingredients and \(i\) is running from 1 to \(n\)
- \(ATE_i\) = Acute Toxicity Estimate of ingredient \(i\)
Currently, one in vitro assay is not sufficient for all eye irritation categories—therefore a bottom-up/top-down strategy was proposed.
Antimicrobial Cleaning Products (AMCP)
Labeling Requirements

**AMCPs quick facts**

- Contain ~275 different active ingredients.
- Are marketed as sprays, liquids, concentrated powders, and gases.
- More than 5000 are currently registered with the U.S. Environmental Protection Agency (EPA) and sold in the marketplace.

The vast majority of the household and commercial cleaning products do not have to go through a registration process before they are marketed.

Companies decide how to assure safety — generally without using animals (*in vitro*).

The product is now EPA regulated (animal testing for safety is required).

Both EPA and industry agreed to work together to build a predictive and *conservative* *in vitro* strategy designed to replace the requirement for Draize rabbit eye irritation data with non-animal methods.

BOVINE CORNEAL OPACITY AND PERMEABILITY (BCOP) ASSAY

Test system: Viable corneas maintained in culture

Assay endpoints: opacity and permeability

Data calculation: *In Vitro Score = Opacity + (15 x Fluor OD\textsubscript{490})*

OECD TG 437


**BCOP Assay Overall Performance**

**PREDICTIVITY**
- Only 2 of 61 materials (8%) were under-predicted.
- All of the EPA toxicity Category IV materials are over-predicted as Category III since the BCOP does not seem to be able to differentiate between materials at this lower end of the toxicity scale.

**LIMITATIONS**
- If the anti-microbial cleaning product is a High Solvent (>5 solvent) formulation, it should be tested in the BCOP assay using a 3 minute exposure instead of the normal 10 minute exposure.
- Testing of ketones and alcohols in the BCOP has been shown to result in high false positive rates for the assay, but not all ketones or alcohols are over-predicted.

**LABELING APPLICABILITY**
- The BCOP assay does differentiate between EPA Category I and II materials, so it is most useful in this higher range.
Ocular irritation - the EPA OPP testing strategy

3D EPIOCULAR™ (EO) ASSAY

**Test system:** Human three dimensional (3D) reconstructed tissue model (keratinocytes)

**Assay endpoints:** tissue viability

**Data calculation:** the exposure time required to reach a 50% reduction in tissue viability (ET$_{50}$ value) dependent on cytotoxic potential and rate of penetration

**US EPA OPP policy (3-2-2015):** Use of an alternate testing framework for classification of eye irritation potential of EPA pesticide products - 40CFR Part 158W for AMCPs

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**EpiOcular™ Assay Overall Performance**

**PREDICTIVITY**
- There was only one **under-prediction** for the 41 total materials.
- The EO method was able to clearly separate a few EPA Category IV materials, although most Category IV materials will be **over-predicted** as Category III.

**LIMITATIONS**
- Oxidizing materials should not be tested in the EO, but both water soluble and water insoluble materials can be tested.

**LABELING APPLICABILITY**
- The EO assay should be useful in clearly identifying materials as EPA Category III or Category IV, but cannot separate EPA toxicity Category I from Category II.
Ocular irritation - the EPA OPP testing strategy

CYTOSENSOR MICROPHYSIO METER (CM) ASSAY
Test system: Mouse fibroblasts (L929)
Assay endpoints: real-time measurement of cellular metabolism
Data calculation: dose calculated to reduce the population metabolic rate to 50% of the initial rate (MRD$_{50}$)

OECD TG: draft (2012)


Cytosensor Assay Overall Performance

PREDICTIVITY
- There were no under-predictions of EPA toxicity categories (of a total of 108 cleaning products tested).
- 89% of the Category IV materials were over-predicted as Category III or higher. However, the CM was able to clearly identify some Category IV materials.

LIMITATIONS
- Oxidizing materials, or materials not completely aqueous soluble at the highest dilution, should not be tested in the CM.

LABELING APPLICABILITY
- The CM should be useful in clearly identifying materials as EPA Category III or Category IV, but cannot separate EPA toxicity Category I from Category II.
BCOP scores vs. EPA Category (Draize) - example
**Ocular irritation**

**Outline of the in vitro testing strategy**


- **Evaluate components**
- **Oxidizing chemistry?**
- **Expected severe or moderate?**
- **Water soluble?**

**BCOP**

- **In vitro score**
  - <25: Default Category III; To distinguish Category IV from III, conduct CM or EO
  - ≥25 but <75: Category II
  - ≥75: Category I

**CM**

- **In vitro score**
  - ≥80 mg/ml: Category IV
  - 2 but <80 mg/ml: Category III

**EO**

- **In vitro score**
  - ≥70 min: Category IV
  - <4 min: Category I
In 2009, the US EPA instituted a 18 month Pilot Program in which manufacturers of AMCP submitted data using the proposed *in vitro* testing strategy for ocular irritation in place of animal testing for product registration.

The program became permanent in 2013.

The policy was updated in March 2015.

Selected reasons for success

- Limited applicability domain (AMCP)
- Cooperation among companies provided a larger test substances set
- Continued interactive discussions with EPA’s Office of Pesticide Programs
- Animal test variability highlighted
- Purpose of test (hazard labeling, not preclinical) clearly understood
- Approach proposed was very conservative (few false negatives, but many over-predictions)
Skin irritation/corrosion

3D RhE ASSAY

Test system: Reconstructed human epidermis (RhE) tissue model (keratinocytes)
Assay endpoints: tissue viability
Data calculation: % viability

OECD TGs: 431 (corrosion; updated 2016); 439 [Skin Irritation Test (SIT); updated 2015]

Receipt of tissues → Treatment of tissues → Rinsing of tissues and MTT reduction → Isopropanol extraction of MTT → Reading of plates

Prediction Model: Skin Corrosion

<table>
<thead>
<tr>
<th>Viability measured after exposure time points (3, 60 and 240-minutes)</th>
<th>Prediction to be considered UN GHS Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>EpiSkin™ (SM)</td>
<td></td>
</tr>
<tr>
<td>&lt; 35% after 3-minutes exposure</td>
<td>Corrosive: Optional Sub-category 1A</td>
</tr>
<tr>
<td>≥ 35% after 3-minutes exposure AND ≤ 35% after 60-minutes exposure OR ≥ 35% after 60-minutes exposure AND &lt; 35% after 240-minutes exposure</td>
<td>Corrosive: A combination of optional Sub-categories 1B-and-1C</td>
</tr>
<tr>
<td>≥ 35% after 240-minutes exposure</td>
<td>Non-corrosive</td>
</tr>
</tbody>
</table>

Prediction Model: Skin Irritation

<table>
<thead>
<tr>
<th>In vitro result Mean tissue viability</th>
<th>In vivo prediction</th>
<th>UN GHS CATEGORY</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 50%</td>
<td>Irritant (I)</td>
<td>Category 2</td>
</tr>
<tr>
<td>&gt; 50%</td>
<td>Non-irritant (NI)</td>
<td>No Category</td>
</tr>
</tbody>
</table>

Skin irritation: US EPA registration of products
Decision process using the rabbit Draize skin irritation test

Chemical hazard classification and labeling

Primary Dermal Irritation Index (PDII)

Sum erythema (1/24/48/72 hr) + Sum oedema (1/24/48/72 hr)
4 intervals (1/24/48/72 hr) x no. of animals

<table>
<thead>
<tr>
<th>US EPA</th>
<th>Hazard Category</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>PDII</td>
<td></td>
</tr>
<tr>
<td>Irritation Potential</td>
<td>Corrosive</td>
</tr>
<tr>
<td>Signal Word</td>
<td>DANGER</td>
</tr>
</tbody>
</table>

Skin irritation: US EPA registration of products

Assessment of an alternative approach using OECD validated in vitro assays

US EPA Hazard Categories

- II: > 5
- III: 2.1-5.0
- IV: 0-2

US EPA PDII

- II: > 5
- III: 2.1-5.0
- IV: 0-2

UN GHS Reaction Scores

- Skin irritation: OECD TG 431
- Draize irritation scale
- OECD TG 439


Optimization of the validated *in vitro* Skin Irritation Test (SIT)

<table>
<thead>
<tr>
<th>Exposure Time (EX)</th>
<th>Post-Treatment (PT)</th>
<th>Assay Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>42 hr</td>
<td>EX15/PT42</td>
</tr>
<tr>
<td>24 hr</td>
<td></td>
<td>EX15/PT24</td>
</tr>
<tr>
<td>60 min</td>
<td>42 hr</td>
<td>EX60/PT42</td>
</tr>
</tbody>
</table>

**Tissue Receipt (EpiDerm™)**
**Tissue Treatment**
**Tissue Rinsing**
**Post-Treatment Incubation**
**MTT Reduction**
**Isopropanol Extraction**
**Spectrophotometric Quantification**

**Assay Modification (Tissue Treatment)**

**Assay Modification (Post-Treatment Incubation)**

**Retrospective Data**

*Companies mentioned in the image:*
- Clorox
- Ecolab
- Procter & Gamble
- Reckitt Benckiser
- S. C. Johnson & Son
- Sealed Air
### Performance of the proposed US EPA Prediction Model

<table>
<thead>
<tr>
<th>US EPA Category determined <em>in vivo</em></th>
<th>US EPA Category determined <em>in vitro</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>II</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100%</td>
</tr>
<tr>
<td>Category under predicted</td>
<td>0</td>
</tr>
<tr>
<td>Category over predicted</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Proposed in vitro testing strategy for assignment of US EPA hazard categories for skin irritation**

- **Category II**
  - EX15/PT42
  - if < 20%

- **Category III**
  - EX15/PT42
  - if ≥ 20%

- **Category IV**
  - EX60/PT42
  - if > 20%

- **Category II**
  - EX15/PT24
  - if > 20%

- **Category III**
  - EX15/PT24
  - if > 20%

- **Category IV**
  - EX15/PT24
  - if > 20%
Mechanisms of skin sensitization

Chemical (hapten) penetrates the skin and reacts with protein(s)

Chemical is recognised by Langerhans cells (LC) which then migrate from the skin to the draining lymph node

Mature LC presents chemical to T cells

Increased number of chemical-specific T-cells released into the systemic circulation

This causes proliferation of specific T cells

Subsequent skin contact with chemical activates the T cells and leads to clinical manifestation

INDUCTION
ELICITATION

Inflammation

Courtesy of Dr. D. Basketter
Adverse Outcome Pathway (AOP) for skin sensitization

**INDUCTION**

- Molecular properties
  - Penetration into the viable epidermis
- Molecular Initiating Event
  - Electrophilic reactivity
  - Covalent interaction with proteins
- Cellular Response
  - Expression of cell surface markers and cytokines

**ELICITATION**

- Organ Response
  - Proliferation of T-cells in lymph nodes
- Organism Response
  - Dermal inflammation (after challenge)

**Test method**

- In silico: QSAR
- In chemico: Peptide reactivity DPRA
- In vitro: Keratinocyte activation KeratinoSens LuSens
  - LC activation h-CLAT
- In vivo: LLNA, GPMT
- Clinical: HIRPT

**Biological event**

- Expression of cell surface markers and cytokines
- Penetration into the viable epidermis
- Electrophilic reactivity
- Covalent interaction with proteins
- Penetration into the viable epidermis

**Molecular Initiating Event**

- Electrophilic reactivity
- Covalent interaction with proteins
- Expression of cell surface markers and cytokines

**Cellular Response**

- Expression of cell surface markers and cytokines
- Proliferation of T-cells in lymph nodes

**Organ Response**

- Dermal inflammation (after challenge)

Skin sensitization – CRO’s perspective

DIRECT PEPTIDE REACTIVITY (DPRA) ASSAY
Test system: in chemico
Assay endpoints: HPLC determination of peptide depletion

OECD TG 442C

Sample preparation → Separation Module → Data analysis

Mean of Cysteine % Depletion

| Reactivity  | Prediction
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0% - 13.89%</td>
<td>Minimal</td>
</tr>
<tr>
<td>13.89% - 23.09%</td>
<td>Low</td>
</tr>
<tr>
<td>23.09% - 98.24%</td>
<td>Moderate</td>
</tr>
<tr>
<td>98.24% - 100%</td>
<td>High</td>
</tr>
</tbody>
</table>

Mean of Cysteine and Lysine % Depletion

| Reactivity  | Prediction
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0% - 6.38%</td>
<td>Minimal</td>
</tr>
<tr>
<td>6.38% - 22.62%</td>
<td>Low</td>
</tr>
<tr>
<td>22.62% - 42.47%</td>
<td>Moderate</td>
</tr>
<tr>
<td>42.47% - 100%</td>
<td>High</td>
</tr>
</tbody>
</table>

Peak Area of un-reacted peptide is compared to peak area of reacted peptide.
Skin sensitization – CRO’s perspective

KERATINOSENS ASSAY

Test system: HaCaT cells (immortalized keratinocytes containing a reporter construct with a copy of the Antioxidant Response Element (ARE) of the human AKRIC2 gene upstream of a luciferase gene.


Data calculation: EC1.5 value (test substance concentration for induction 1.5 fold time above threshold).

\[ I_{\text{max}} \] (the largest average gene fold induction above 1.5 by the test substance).

\[ C_{\text{i, max}} \] (the test substance concentration at which the largest average fold induction value is achieved).

OECD TG 442D

A test substance will be considered to have sensitization potential if:

1) The EC1.5 value falls below 1000 µM (or 200 µg/mL) in at least 2 of 3 repetitions;
2) At the lowest concentration with a gene induction above 1.5, cellular viability should be greater than 70%.
3) An apparent overall dose response should be similar between repetitions.
Human Cell Line Activation Test (hCLAT)  
CRO’s perspective

hCLAT ASSAY
Test system: human monocytic leukemia cell line (THP-1 cells)
Assay endpoints: CD86 and CD54 cell surface marker expression
Data calculation: Relative Fluorescence Intensity (RFI) using flow cytometry
Prediction model: CD86 ≥150% and CD54 ≥200 with cell viability of ≥50% in at least 2 independent repetitions relative to vehicle controls

OECD TG 442F

Chemical allergen

Prediction Model
CD54 > 200%
CD86 > 150%

CD54/APC -
CD86/PE-CY7 -

CD54/APC +
CD86/PE-CY7 +

Flow Cytometry
Human Cell Line Activation Test (hCLAT)  
Regulatory perspective  

International Cooperation on Alternative Test Methods (ICATM)

- First International Cooperation on Alternative Test Methods (ICATM) Workshop was held in 2016 and was focused on the international regulatory applicability and acceptance of alternative non-animal approaches. The countries participating were USA, EU, Japan, Korea, Canada, Brazil and China.

- Multiple non-animal testing strategies incorporating *in vitro*, *in chemico*, and *in silico* inputs demonstrate comparable or superior performance to the LLNA.

- A planned product of the ICATM workshop is the development of an assessment framework for integrated non-animal approaches that could serve as replacements for the current animal test, the LLNA for multiple chemical sectors (pesticides, cosmetics, pharmaceuticals, industrial chemicals, etc.)
Modernizing the “six-pack” testing strategy: influx of modern in vitro techniques

- Acute oral rat
- Acute dermal rabbit
- Acute inhalation rat
- Skin sensitization guinea pig mouse
- Skin irritation rabbit
- Ocular irritation rabbit

PESTICIDES

OECD 431
OECD 439
OECD 435
OECD 129
OECD 437
OECD 438
OECD 460
OECD 442C
OECD 442D
OECD 432

CROs
Industry
Trade Associations
Animal Welfare Groups
Public
Regulatory Agencies
Academia
Perspectives, challenges, common goals and working together

- Industry/Manufacturer
- Safety/Testing Labs
- Trade Associations
- Animal Welfare Groups
- Academia
- Labeling/Regulatory Agency
- Consumer/End-user Safety

Validated
Transferable
Specific
Sensitive
High-throughput
Sensitive
Reproducible
Reliable
Relevant
Easy to perform
Affordable

Dear Stakeholders:

Rapid advancements in science and new technologies give us the opportunity to evaluate more pesticides across a broader range of potential effects in less time, using fewer animals and reducing costs for everyone. The U.S. Environmental Protection Agency’s Office of Pesticide Programs (OPP) is evaluating and adopting alternative approaches to more traditional methods of toxicity testing and using integrated approaches to testing and assessment (IATA) (see http://www.epa.gov/pesticides-risk-assessment/pesticides-testing-testing) and OPP’s 21st Century Science Initiative (http://www.epa.gov/pesticides-risk-assessment/pesticides-testing-testing). OPP will continue to host stakeholder meetings on pesticide alternatives over the course of the year, and I look forward to discussing progress on these initiatives with you.

Sincerely,
[Signature]
[Title]

United States Environmental Protection Agency
Washington, D.C. 20460
Acknowledgments

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Clorox
Colgate Palmolive
Dial
EcoLabs
SealedAir
P&G
Reckitt Benckiser
SC Johnson
The Accord Group

US EPA OPP
Jennifer McLain
Anna Lowit

The Accord Group
Pat Quinn

Institute for In Vitro Sciences
Advancing Science & Animal Welfare Together

National Capital Area Chapter of the Society of Toxicology

Northern California Regional Chapter of the Society of Toxicology

Society of Toxicology
Creating a Safer and Healthier World by Advancing the Science and Increasing the Impact of Toxicology