EFSA Symposium

“NOVEL CHEMICAL HAZARD CHARACTERISATION APPROACHES “

Section 2: Systems biology approach and predictive toxicology

16 October 2015 – Milano at EXPO2015

Alternative and Integrated Testing Strategies

Horst Spielmann
Professor for Regulatory Toxicology
Freie Universität Berlin
& State Animal Welfare Officer, Berlin
2002 ECVAM - proposal for integrated testing scheme for chemicals
2002 OECD - sequential testing strategy eye & skin irritation/corrosion
2004 BfR - “Concept” for *in vitro* eye & skin irritation testing
2005 ECVAM - “Top-Down” & “Bottom-Up” approaches for eye irritation testing
2009 ECVAM - WS validation of Integrated Testing Strategies (ITS)
2014 OECD - GD 203 “Integrated approach on testing and assessment (IATA) for skin corrosion & irritation
2012 OECD - AOP for skin sensitization *an ITS approach*
2012 ROCHE - Embryonic Stem cell Test (EST) *an ITS approach*
2013 EU - ban on animal testing for cosmetics
Principles of the Future Chemicals Policy of the EU (White Paper) 2002 ➤REACH !!

Action proposed in the EU White Paper

1. Identical amount of testing for new & existing chemicals
   ➤more information on existing chemicals and less on new ones

2. Steps proposed for the testing of 30,000 existing chemicals until 2012
   ➤high production volume chemicals will be tested first

3. Only in vitro/non-animal methods will be used in the basic test set
   ➤they are faster and cheaper to perform, will the information be sufficient for risk assessment for humans and the environment?

➤ CONSEQUENCES in 2002:
The 6th Framework Program of the EU Commission includes funding of research for development and validation of new non-animal methods.
In 2007 the 7th FP included major funding for alternative methods.
ECVAM WG proposal of an integrated testing scheme for Existing and New Chemicals in the EU 2002

**Background Data**
- Name
- Chemical Structure
- CAS number
- Proposed use
- Scale of production
- Producers
- Exposure
- Transport

**Physiochemical Properties**
- Volatility, Solubility (aqueous)
- Melting point, Boiling point
- Stability, Log P
- $p_k a$
- Henry’s partition coefficient
- UV - visible absorption
- Surface tension

**Existing Toxicological Data** (Review)
- Testing required?
  - YES
  - DATA SUFFICIENT FOR CLASSIFICATION CRITERIA to be defined
  - In vitro Testing (Tier 1)
    - Cytotoxicity, Genotoxicity ± S9
    - (Q)SAR, Predict. metabolism
    - Percutaneous. absorption, Corrosivity / Irritancy, Sensitisation
  - Is further information required?
    - YES
    - Priorities for further testing (Tier 2)
    - NO
OECD 2002: Sequential Testing Strategy

- Agreed stepwise decision logic
- Mandatory to follow all steps
- Increasingly more complicated
- From *in vitro* to *in vivo* approaches
- Consideration of the hazard after each step
- Works best for less complicated hazards (e.g. local effects, single endpoints)
- Is a relatively rigid approach
- Is relatively easy to harmonise internationally.
Testing Strategy: Skin and Eye Effects
(TG 404, TG 405)
(adopted 24 April 2002)

Existing human experience
↓
(Q)SAR
(eye irritation/corrosion)
↓
(Q)SAR
(skin corrosion)
↓
pH and buffering capacity
↓
evaluate dermal route toxicity data
↓
*In vitro* Test
(eye irritation/corrosion)
↓
*In vitro* Test
(skin irritation/corrosion)
↓
*In vivo* 1 Rabbit
↓
*In vivo* 2 Rabbits

Reduces by about 90% that eye corrosives damage a rabbit eye
FIGURE

TESTING AND EVALUATION STRATEGY FOR EYE IRRITATION/CORROSION

<table>
<thead>
<tr>
<th>Activity</th>
<th>Finding</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Existing human and/or animal data showing effects on eyes</td>
<td>Severe damage to eyes</td>
<td>Apical endpoint; consider corrosive to eyes. No testing is needed.</td>
</tr>
<tr>
<td></td>
<td>Eye irritant</td>
<td>Apical endpoint; consider irritating to eyes. No testing is needed.</td>
</tr>
<tr>
<td></td>
<td>Not corrosive/not irritating to eyes</td>
<td>Apical endpoint; considered non-corrosive and non-irritating to eyes. No testing required.</td>
</tr>
<tr>
<td>Existing human and/or animal data showing corrosive effects on skin</td>
<td>Skin corrosive</td>
<td>Assume corrosivity to eyes. No testing is needed.</td>
</tr>
<tr>
<td>Existing human and/or animal data showing severe irritant effects on skin</td>
<td>Severe skin irritant</td>
<td>Assume irritating to eyes. No testing is needed.</td>
</tr>
<tr>
<td>no information available, or available information is not conclusive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Perform SAR for eye corrosion/irritation</td>
<td>Predict severe damage to eyes</td>
<td>Assume corrosivity to eyes. No testing is needed.</td>
</tr>
<tr>
<td></td>
<td>Predict irritation to eyes</td>
<td>Assume irritating to eyes. No testing is needed.</td>
</tr>
<tr>
<td>Perform SAR for skin corrosion</td>
<td>Predict skin corrosivity</td>
<td>Assume corrosivity to eyes. No testing is needed.</td>
</tr>
<tr>
<td>No predictions can be made, or predictions are not conclusive or negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Measure pH (buffering capacity, if relevant)</td>
<td>pH ≤ 2 or ≥ 11.5 (with high buffering capacity, if relevant)</td>
<td>Assume corrosivity to eyes. No testing is needed.</td>
</tr>
<tr>
<td>2&lt; pH &lt; 11.5, or pH ≥ 2.0 or ≥ 11.5 with low/no buffering capacity, if relevant</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4. Evaluate systemic toxicity via the dermal route

Highly toxic at concentrations that would be tested in the eye.

Such information is not available, or substance is not highly toxic

5. Perform validated and accepted in vitro or ex vivo test for eye corrosion

Corrosive response

Substance is not corrosive, or internationally validated in vitro or ex vivo testing methods for eye corrosion are not yet available

6. Perform validated and accepted in vitro or ex vivo test for eye irritation

Irritant response

Substance is not an irritant, or internationally validated in vitro or ex vivo testing methods for eye irritation are not yet available

7. Experimentally assess in vivo skin irritation/corrosion potential (see OECD Guideline 404)

Corrosive or severe irritant response

Substance is not corrosive or severely irritating to skin

8. Perform initial in vivo rabbit eye test using one animal

Severe damage to eyes

No severe damage, or no response

9. Perform confirmatory test using one or two additional animals

Corrosive or irritating

Not corrosive or irritating

Substance would be too toxic for testing. No further testing is needed.

Assume corrosivity to eyes. No further testing is needed.

Assume irritancy to eyes. No further testing is needed.

Assume corrosivity to eyes. No further testing is needed.

Consider corrosive to eyes. No further testing is needed.

Consider corrosive or irritating to eyes. No further testing is needed.

Consider non-irritating and non-corrosive to eyes. No further testing is needed.
PERTPECTIVE:

BfR View of a Strategy for Skin and Eye Irritation/Corrosion Assessment without Use of Animals

Conclusions
Assessment of the potential of a chemical to induce local lesions
We have shown that
► structural alerts for the prediction of a potential to cause local lesions and
► physicochemical limit values (DSS) for the prediction of the absence of such a potential
provide testing and assessment strategies which use only (Q)SARs and results of in vitro testing instead of the current employment of test animals for the purpose of classification and labelling of acute local health hazards.
Rules appropriate for all groups of chemicals:

Basis: Evaluation of data of 1627 chemicals with purity > 95%

Attention: Rules are valid exclusively for the Risk phrases mentioned within this specific "exclusion rule". This is due to the fact that acute local tissue lesions called "irritation" or "corrosion" and specified by the respective R-phrase of the EU are in reality based on a great variety of totally different biochemical reactions (depending on the chemical reactivity of the molecule which contacts the biological medium or structure first).

IF melting point > 200°C   THEN NOT (skin corrosion R34 or R35)
(is true for 245/252 chemicals tested = 97%)

(7 skin corrosive substances are organic salts which release strong inorganic acids or bases when getting in contact with aqueous substrates/organic media)

IF log P<sub>ow</sub> > 9   THEN NOT (lesions R34,R35,R36 or R41)
(is true for 32/32 chemicals tested = 100%)

IF log P<sub>ow</sub> < -3.1   THEN NOT (skin corrosion R34 or R35)
(is true for 53/53 chemicals tested = 100%)

IF lipid solubility < 0.01 g/kg   THEN NOT (skin corrosion R34 or R35)
(is true for 58/58 chemicals tested = 100%)

IF aqueous solubility < 0.00002 g/l   THEN NOT (eye irritation R41)
(is true for 109/109 chemicals tested = 100%)

IF aqueous solubility < 0.000005 g/l   THEN NOT (eye irritation R36)
(is true for 38/38 chemicals tested= 100%)

IF molecular weight > 650 g/Mol   THEN NOT (eye irritation R36)
(is true for 139/139 chemicals tested= 100%)

Attention:
chemicals with molecular weight > 650 g/Mol may elicit severe tissue damage resulting in local corrosion!
Assessment strategy for local irritation/corrosion
EU (& OECD) 2004
Proposed new BOTTOM-UP Approach BfR 2004
A proposed eye irritation testing strategy to reduce and replace *in vivo* studies using Bottom-Up and Top-Down approaches

Laurie Scott\(^a\), Chantra Eskes\(^b\), Sebastian Hoffmann\(^b\), Els Adriaens\(^c\), Nathalie Alepée\(^d\), Monica Bufo\(^e\), Richard Clothier\(^f\), Davide Facchini\(^g\), Claudine Faller\(^h\), Robert Guest\(^i\), John Harbell\(^j\), Thomas Hartung\(^b\), Hennicke Kamp\(^k\), Béatrice Le Varlet\(^l\), Marisa Meloni\(^m\), Pauline McNamee\(^n\), Rosemarie Osborne\(^o\), Wolfgang Pape\(^p\), Uwe Pfannenbecker\(^p\), Menk Prinsen\(^q\), Christopher Seaman\(^r\), Horst Spielmann\(^s\), William Stokes\(^t\), Kevin Trouba\(^o\), Christine Van den Berghe\(^d\), Freddy Van Goethem\(^u\), Marco Vassallo\(^e\), Pilar Vinardell\(^v\), Valérie Zuang\(^b,\(^*\)

Agnieszka Kinsner-Ovaskainen,1 Zerrin Akkan,2 Silvia Casati,1 Sandra Coecke,1 Raffaella Corvi,1 Gianni Dal Negro,3 Jack De Bruijn,4 Odile De Silva,5 Laura Gribaldo,1 Claudius Griesinger,1 Joanna Jaworska,6 Joachim Kreysa,1 Gavin Maxwell,7 Pauline McNamee,6 Anna Price,7 Pilar Prieto,1 Roland Schubert,8 Luca Tosti,1 Andrew Worth1 and Valerie Zuang1

Report of the EPAA–ECVAM Workshop on the Validation of Integrated Testing Strategies (ITS)

Agnieszka Kinsner-Ovaskainen,1 Gavin Maxwell,2 Joachim Kreysa,1 João Barroso,1 Els Adriaens,3 Nathalie Alépée,4 Ninna Berg,5 Susanne Bremer,1 Sandra Coecke,1 José Z. Comenges,1 Raffaella Corvi,1 Silvia Casati,1 Gianni Dal Negro,6 Monique Marrec-Fairley,7 Claudius Griesinger,1 Marlies Halder,1 Eckhard Heisler,8 Doris Hirmann,9 André Kleensang,1a Annette Kopp-Schneider,10 Silvia Lapenna,1 Sharon Munn,1 Pilar Prieto,1 Len Schechtman,11 Terry Schultz,12 Jean-Marc Vidal,13 Andrew Worth1 and Valérie Zuang1
2009
In the context of safety assessment, an ITS is a methodology which integrates information for toxicological evaluation from more than one source, thus facilitating decision-making. This should be achieved whilst taking into consideration the principles of the Three Rs (reduction, refinement and replacement)

It was also agreed that, in line with the proposal put forward during the OECD Workshop on Integrated Approaches to Testing and Assessment, held in December 2007, a good ITS should be structured, transparent and hypothesis driven.

2012
During this workshop it was recognized that there is a fundamental difference between:

a) **Type 1 ITS**, i.e. strategies to gather and analyze a broad range of data coming from different sources (epidemiological studies, animal data, in vitro data, read-across methodologies, etc) and used to draw conclusions based on weight-of-evidence (WoE) approaches; and

b) **Type 2 ITS**: testing strategies composed of e.g. a number of in vitro and in silico methods that, combined and weighted in a fixed way, would serve to replace some or all in vivo experimentation for a given toxicity endpoint.

This distinction is essential, when the validation of ITS is under consideration.
### Table 2: Requirements for formal validation of ITS

<table>
<thead>
<tr>
<th></th>
<th>Formal validation of ITS component</th>
<th>Formal validation of ITS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening</td>
<td>Not required</td>
<td>Not required</td>
</tr>
<tr>
<td>Hazard classification &amp; labelling</td>
<td>Not required</td>
<td>Not required</td>
</tr>
<tr>
<td>Replacement of Test Guideline used for regulatory purposes</td>
<td>Required <em>(data requirements are different than in validation of 1-to-1 replacement methods)</em></td>
<td>Required <em>(the principles of ITS validation need to be established)</em></td>
</tr>
<tr>
<td>Risk assessment</td>
<td>Not required</td>
<td>Not required</td>
</tr>
</tbody>
</table>
### Table 1: Parts and Modules of the IATA

<table>
<thead>
<tr>
<th>Part (a)</th>
<th>Module</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>Existing information</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Existing human data</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>a) Non-standardised human data on local skin effects</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b) Human Patch Test (HPT)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>- In vivo skin irritation and corrosion data (OECD TG 404)</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>- In vitro skin corrosion data</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a) OECD TG 430</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b) OECD TG 431</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c) OECD TG 435</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>- In vitro skin irritation data (OECD TG 439)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Other in vivo and in vitro data</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a) In vitro skin corrosion or irritation data from test methods not adopted by the OECD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b) Other in vivo and in vitro dermal toxicity data</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Physico-chemical properties (existing, measured or estimated)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- e.g., pH, acid/alkaline reserve</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>Non-testing methods</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- for substances: (Q)SAR, read-across, grouping and prediction systems;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- for mixtures: bridging principles and theory of additivity</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>Phases and elements of WoE approaches</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>Other in vivo and/or in vitro dermal toxicity testing (if required by other regulations)</td>
</tr>
<tr>
<td>(5b)</td>
<td></td>
<td>In vitro skin corrosion testing</td>
</tr>
<tr>
<td>(3)</td>
<td></td>
<td>In vitro skin irritation testing</td>
</tr>
<tr>
<td>(4)</td>
<td></td>
<td>In vitro skin irritation testing in test method not adopted by the OECD</td>
</tr>
<tr>
<td>(5a)</td>
<td></td>
<td>In vivo skin irritation and corrosion testing</td>
</tr>
<tr>
<td>(2)</td>
<td></td>
<td>In vivo skin irritation and corrosion testing</td>
</tr>
</tbody>
</table>
COMPOSITION OF THE IATA FOR SKIN CORROSION AND IRRITATION
“..... a not-so-distant future where all routine toxicity testing will be conducted in human cells or cell lines *in vitro* by evaluating perturbations of cellular responses in a suite of toxicity pathway assays.....”

We propose a shift from primarily in vivo animal studies to in vitro assays, in vivo assays with lower organisms, and computational modeling for toxicity assessments.
Perturbation of Toxicity Pathways

- Exposure
- Tissue Dose
- Biologic Interaction
- Perturbation

Biologic Inputs

Early Cellular Changes

Adaptive Stress Responses

Cell Injury

Normal Biologic Function

Morbidity and Mortality

Higher yet
Figure 3. ToxCast™ is using a variety of HTS assays to develop bioactivity signatures that are predictive of effects in traditional toxicity testing approaches.
Toxicity Testing and Risk Assessment
(from Krewski et al., 2010, Annual Review of Public Health, in press)
An adverse outcome pathway (AOP) is the sequence of events from chemical structure through the molecular initiating event to the \textit{in vivo} outcome of interest. AOPs are representations of existing knowledge concerning the linkage(s) between a molecular initiating event and an adverse outcome at the individual or population level. As such, AOPs delineate the documented, plausible, and testable processes by which a chemical induces molecular perturbation and causes effects at the subcellular, cellular, tissue, organ, and whole animal levels of observation.
OECD 2012: AOP for skin sensitization initiated by covalent binding to protein
OECD 2012: AOP for skin sensitization initiated by covalent binding to protein

Figure 1. The Induction Phase of Skin Sensitisation.
OECD 2012: AOP for skin sensitization initiated by covalent binding to protein.
**Adverse Outcome Pathway (AOP) for Skin Sensitization**

1. Skin Penetration
2. Electrophilic substance: directly or via auto-oxidation or metabolism
3-4. Haptenation: covalent modification of epidermal proteins
5-6. Activation of epidermal keratinocytes & Dendritic cells
7. Presentation of haptenated protein by Dendritic cell resulting in activation & proliferation of specific T cells
8-10. Allergic Contact Dermatitis: Epidermal inflammation following re-exposure to substance due to T cell-mediated cell death

Key Event 1

Key Event 2 + 3

Key Event 4

Adverse Outcome
Strategy for Testing Skin Sensitisation Potential Without Animals
Combining Different Methods Addressing the Adverse Outcome Pathway
Susanne Kolle and colleagues 2012  BASF

Adverse outcome pathway

- Protein reactivity
- Keratinocyte activation
- DC activation
- LuSens or KeratinoSens™
- MUSST (or h-CLAT)

If both results are negative:
 NON-SENSITIZER
 (High Sensitivity, 100%)

If positive:
 SENSITIZER
 (High Specificity, 100%)

If: results of protein reactivity and DC activation are contradicting
Or: the h-CLAT is being used instead of the MUSST assay

Use weight of evidence:
Results of 2 out of 3 tests determine the overall result
High Overall Accuracy (94%)
Test Battery and Weight of Evidence Assessment (Bauch et al. 2012)

Sensitivity 93%
Specificity 95%
Accuracy 94%

Test strategy compared to human data

Putting the parts together: Combining *in vitro* methods to test for skin sensitizing potentials

Caroline Bauch, Susanne N. Kolle, Tzutzuy Ramirez, Tobias Elze, Eric Fabian, Annette Mehling, Wera Teubner, Bennard van Ravenzwaay, Robert Landsiedel

*Elsevier*
The validated embryonic stem cell test to predict embryotoxicity in vitro hanging drop culture. Embryoid bodies for the differentiation of embryonic stem cells in the embryonic stem cell test are generated by pipetting a single-cell suspension onto the lid of a cell culture dish. The cells aggregate at the bottom of the drop by gravitational force, thereby forming the embryoid body.
Embryonic stem cells develop spontaneously into contracting myocard, this endpoint of prenatal differentiation is used in the mouse EST.

Mouse Embryonic Stem cell Test (mEST)

Day 0: undifferentiated D3-mouse ES cell line

Day 3-5: in vitro differentiation into "embryoid bodies"

Day 5-10: in vitro differentiation into contracting myocard
Endpoints of the Embryonic Stem Cell Test (mEST)

Endpoints: assessment from concentration response curves

1. inhibition of differentiation in ES cells \( \rightarrow \) ID\(_{50}\)
2. cytotoxic effects on ES cells \( \rightarrow \) IC\(_{50}\) D3
3. cytotoxic effects on 3T3 cells \( \rightarrow \) IC\(_{50}\) 3T3

Mouse ES cell line D3

Endpoint 1: inhibition of differentiation

Endpoint 2: cytotoxic effects

Endpoint 3: cytotoxic effects

Differentiated cells

3T3 cells
In Vitro - In Vivo Concordance of Proprietary and Marketed Compounds

- **36 Roche proprietary compounds with in house in vivo data** (10+/26-)
  - Diverse set of structures from 24 different projects

- **16 Marketed drugs**: known teratogens/non teratogens (11+/5-)
  - HIV drugs, 5HT2 antagonists, kinase inhibitor, anticoagulants, statins...

<table>
<thead>
<tr>
<th>Specificity</th>
<th>100.00 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>76.19 %</td>
</tr>
<tr>
<td>Concordance</td>
<td>87.04 %</td>
</tr>
</tbody>
</table>

Overall, satisfactory performance of manual EST with > 85% concordance
Findings from EST assay used to
- rank-order compounds during lead optimization
- follow up on in silico flags
- frontload in vivo studies
Roche Hanging Droplet Culture Plate [HDCP]

- In-house developed injection-molded proprietary labware for culturing cells in 3D
- Precise engineering allows excellent droplet stability
- 24well currently used for automated EST assay.
- 96well design finalized & manufactured. Implementation ongoing.
Automated EST Platform Set-up at Roche

Implemented since Q1, 2012

**Compound liquid handling**
for preparing serial dilutions and compound plates

**Centrifuge**
spins down EB into well for adherence and further differentiation

**Staeubli Robotic Arm**
turns HDCP lid over for liquid handling/media changes

**Warming rack**
for buffer, media, cells

**8-channel pipettor**
uses sterile, disposable tips

**Position for HDCP plate**
for EB culture/differentiation

**Cytomax Incubator**
for 10 d cytotox/differentiation incubations

**RoMa Arm**
moves plates in/out of incubator

Slide Claudia McGinnis
EUROPEAN COMMISSION
PRESS RELEASE

Brussels, 11 March 2013

Full EU ban on animal testing for cosmetics enters into force

Today the last deadline to phase out animal testing for cosmetic products in Europe enters into force. As of today, cosmetics tested on animals cannot be marketed any more in the EU.

A Communication adopted by the Commission today confirms the Commission’s commitment to respect the deadline set by Council and Parliament in 2003 and outlines how it intends to further support research and innovation in this area while promoting animal welfare world-wide.

European Commissioner in charge of Health & Consumer Policy, Tonio Borg, stated: "Today’s entry into force of the full marketing ban gives an important signal on the value that Europe attaches to animal welfare. The Commission is committed to continue supporting the development of alternative methods and to engage with third countries to follow our European approach. This is a great opportunity for Europe to set an example of responsible innovation in cosmetics without any compromise on consumer safety."

The Commission has thoroughly assessed the impacts of the marketing ban and considers that there are overriding reasons to implement it. This is in line with what many European citizens believe firmly: that the development of cosmetics does not warrant animal testing.