Los métodos alternativos a la experimentación animal ante las nuevas normativas internacionales

IV Jornada de REMA, X Aniversario
Colegio Oficial de Veterinarios de Madrid
1 de diciembre de 2009

Documentos disponibles en http://www.remanet.net/
Development and Validation of Alternative Methods
10th Anniversary REMA, Madrid, 1 December 2009

Sharon Munn

Alternative Methods and ECVAM
The Institute for Health and Consumer Protection (IHCP)
Science for a healthier life
OUTLINE

• History
• Validation principles
• Successes and next challenges
• ECVAM Role
Two British scientists, Bill Russell and Rex Burch introduced the “3Rs” as a framework for considering the humane use of animals.

Russell, W. M. S. and Burch, R. L. 1959. The principles of humane experimental technique

Refine

Reduce

Replace

86/609/EEC
ECVAM

- Founded in 1991 to promote 3R methods primarily by confirming their scientific validity


- Since 2009 ECVAM is a Centre hosted by the Institute for Health and Consumer Protection and is served by mainly two scientific Units

- ECVAM has its own scientific advisory committee (ESAC)
ECVAM

• So far developed/optimised and/or validated 34 methods alternative to animal testing according to generally accepted validation principles

• Most methods have similar toxicological endpoints, i.e. skin and eye irritation; many methods are not replacement methods

• Maintains a database on alternative test methods (DB-ALM) and tracking system (TSAR)

• Promotes method development through own research as well as through participation in RTD projects that will yield new methods

• Contributes to the regulatory acceptance of alternative methods
ECVAM’s MISSION STATEMENT

To support the EU policies in the field of Consumer protection, Environmental protection and Animal protection

by validating alternative methods for safety testing that implement the 3Rs and provide the same or a better basis for risk assessment and risk management as current methods

and by promoting their development, their application in industry and their acceptance by regulators.
OUTLINE

• History
• Validation principles
• Successes and next challenges
• ECVAM Role
Research
Development
Prevalidation
Validation
Independent Review
Regulatory Acceptance
Implementation

Research e.g.
DG RTD & MS

Industry
Academia
Regulators
### Information requirements that validation studies endeavour to satisfy

<table>
<thead>
<tr>
<th>Test method definition</th>
<th>Module 1 – Test definition: test system, SOP, prediction model, development, possible use, limitations, etc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reliability</td>
<td>Module 2 – Within laboratory reproducibility: sufficiently standardized to give reproducible results in one lab?</td>
</tr>
<tr>
<td>Concordance/ Discordance</td>
<td>Module 3 – Transferability: transferable, and yes, how readily?</td>
</tr>
<tr>
<td></td>
<td>Module 4 – Between laboratory reproducibility: how reproducible between labs?</td>
</tr>
<tr>
<td>(Predictive) Relevance</td>
<td>Module 5 – Predictive capacity: Specificity, Sensitivity, Overall Accuracy</td>
</tr>
<tr>
<td>Accuracy</td>
<td>Module 6 – Applicability domain/Limitations: Which xenobiotics can NOT be tested?</td>
</tr>
<tr>
<td>Performance criteria</td>
<td>Module 7 – Performance Standards: Performance Acceptance Criteria for new tests that are sufficiently similar to the validated one</td>
</tr>
</tbody>
</table>
OUTLINE

• History
• Validation principles
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• ECVAM Role
### ECVAM activities & involvement, Per endpoint, 2009

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Development</th>
<th>Prevalidation</th>
<th>Validation</th>
<th>Reg. acceptance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin Corrosion</td>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Acute Phototoxicity</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Skin Absorption / Penetration</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Skin Irritation</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Eye Irritation</td>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Acute Toxicity</td>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Genotoxicity / Mutagenicity</td>
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<td>✓</td>
<td>✓</td>
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<tr>
<td>Skin Sensitisation</td>
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<td>✓</td>
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<tr>
<td>Reproductive &amp; Developmental</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Toxicokinetics / Metabolism</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Carcinogenicity</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Subacute &amp; Subchronic Toxicity</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biologicals, vaccines</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

* Reduction / refinement alternatives
Human health effects

10th Anniversary REMA, Madrid 1st December 2009 – Sharon Munn, AM&ECVAM, IHCP, JRC - European Commission

Skin Corrosion
Acute Phototoxicity
Skin Absorption / Penetration
Skin Irritation
Photogenotoxicity
Eye Irritation

Acute Toxicity
Genotoxicity / Mutagenicity
Subacute & Subchronic Toxicity
Skin Sensitisation
Photo-allergy (-sensitisation)
Carcinogenicity-Toxicokinetics & Metabolism
Reproductive & Developmental Toxicity

Biggest challenge
Systemic Toxicity - Systems Biology

- Metabolism
- Multiple modes of action
- Dose/response
- One to one replacement not possible
- Battery of test methods
- Tiered testing strategies
- Combination of disciplines (*in vitro*/*in silico*/*in vivo*/PBPK models)
- Integrated testing strategies
Integrated Testing Strategies

Endpoint-specific strategy

- in vivo data
- (Q)SARs
- read-across & chemical groups
- in vitro tests
- Exposure information
- Other existing information

Classification and Labelling,
Risk Assessment of Chemicals and Persistent Bioaccumulative Toxic Chemicals

Risk Management Measures
New/emerging technologies

- Human cell-based metabolically competent liver cells
- Human stem cell-derived neurons/micro electrode arrays
- 3D in vitro tissue models
- Automation of in vitro methods (HTS/HCS)
- Development of computational methodology (in silico, QSAR)
- ‘Omics’, genomics, proteomics, metabonomics
EC Call for proposals on Alternative Testing Strategies in field of repeated dose systemic toxicity—7th Framework programme

- COLIPA matched funding to EC (Eur 25 + 25 million)
- Open call – 30 July 2009 to 3 Feb 2010
Validation of Integrated Testing Strategies?

- Validate ITS or Building blocks of ITS or both?
- Building blocks (reliability (modules 1-4) sufficient?)
- Predictive capacity – validate against what?
- Validation should be ‘fit for purpose’
- Need case studies

ATLA, 37,437-444,2009
OUTLINE

• History
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• Successes and next challenges
• ECVAM Role
JRC-IHCP Units Supporting ECVAM

Alternative Methods and ECVAM

- Chemicals Assessment and Testing (CAT)
- Systems Toxicology (ST)
- In-Vitro Methods (IVM)
- Nanobiosciences (NBS)
- Molecular Biology and Genomics (MBG)
ECVAM has now full access to the broad range of competencies available across the Institute.

The relevant competencies include:

• at cellular level: in vitro methods, working with many different (human) cell systems and their automation towards high throughput;

• at sub-cellular level: "omics" (metabonomics, genomics);

• at molecular or chemical level: computational chemistry including QSAR.

PBPK and modeling expertise to help connect the cellular and sub-cellular levels with the organ or organism level.

Wealth of experience on validation of alternative methods
Validating alternative methods that provide the same or better basis for risk assessment as current methods

INNOVATION: contribute to methods/testing strategies that reduce reliance on in vivo animal studies even for complex endpoints

VALIDATION: continue to manage and coordinate scientific validation of submitted methods, assessing robustness, reliability, predictive capacity of methods and regulatory relevance, promoting regulatory acceptance

COMMUNICATION: engage with regulators/risk assessors, test developers, test users/risk assessors, promote dialogue/cross talk through workshops, promote uptake of methods though dissemination
Concluding remarks

- Integrated testing strategies required to address complex endpoints
- Emerging technologies give opportunity to make a gear change in progress
- A major challenge lies in the integration of the data and its interpretation in relation to specific regulatory questions
- Risk assessors need to engage in dialogue to give a steer to increase chance of relevant outcomes (both development and validation aspects)
Alternative Methods to Animal Testing: Improving the Scientific Basis for the Protection of Human Health and the Environment while Reducing the Need for Animal Testing

for more information visit

http://ecvam.jrc.ec.europa.eu/
Additional Slides
Workshop report

- The Use of Fish Cells in Ecotoxicology. ECVAM Workshop Report 47. Castaño et al. (2003). ATLA 31, 317-351

Acute aquatic toxicity

- Threshold Approach (*reduction*)
  - proposal as OECD Guidance document
- Zebrafish Embryo Toxicity Test (*replacement*)
  - coordination of study at OECD level

Bioconcentration/accumulation

- Validation (Module 1-4) of in vitro trout S9 fraction assay (end in 2010)
  - in vitro information on metabolic stability of substances will be used to refine bioaccumulation/bioconcentration models for derivation of BAF/BCF values

International collaboration

- HESI, CEFIC-LRI on alternative methods
Acute toxicity - Overview main activities

1. Strategy to replace acute oral toxicity testing – FP6 A-Cute-Tox

2. Prediction of non-toxic substances *in vitro* by the Balb 3T3/NRU cytotoxicity test – Validation Study

3. Retrospective data analysis assessing value of testing by multiple exposure routes (Concordance of acute oral, dermal and inhalation toxicity data)
2. ECVAM follow-up 3T3/NRU validation study

In this class are 86% of all new industrial chemicals

predictive capacity of the 3T3/NRU assay to identify substances with acute oral LD50 > 2000 mg/kg b.w

<table>
<thead>
<tr>
<th>Toxicity class</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category 1</td>
<td>0.05%</td>
</tr>
<tr>
<td>LD₅₀ &lt; 5 mg/kg b.w.</td>
<td></td>
</tr>
<tr>
<td>Category 2</td>
<td>0.31%</td>
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<tr>
<td>LD₅₀ 5-50 mg/kg b.w.</td>
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<tr>
<td>Category 3</td>
<td>3.41%</td>
</tr>
<tr>
<td>LD₅₀ 50-300 mg/kg b.w.</td>
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</tr>
<tr>
<td>Category 4</td>
<td>5.83%</td>
</tr>
<tr>
<td>LD₅₀ 300-2000 mg/kg b.w.</td>
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</tr>
<tr>
<td>Category 5</td>
<td>70.16%</td>
</tr>
<tr>
<td>LD₅₀ 2000-5000 mg/kg b.w.</td>
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</tr>
<tr>
<td>Not classified</td>
<td>15.68%</td>
</tr>
<tr>
<td>LD₅₀ &gt;5000 mg/kg b.w.</td>
<td></td>
</tr>
</tbody>
</table>

EU new chemicals database (27/03/2008) – Prevalence acute oral toxicity
(4773 substances with oral toxicity data) Bulgheroni et al., 2009

From: The Registry of Cytotoxicity
(W. Halle, 2003, ATLA, 31:89-198)
3. Concordance of acute oral, dermal and inhalation toxicity data

Comparison of LD$_{50}$ values/classifications: oral vs. dermal and inhalation
- >1,500 substances from EU New Chemicals Database

• Key findings:
  - Dermal LD$_{50}$ data do not add value for classification & labelling of pure substances;
  - Oral-inhalation concordances require further examination


In collaboration with the EPAA Acute Toxicity Task Force
1. *In Vitro* Strategy to Replace Acute Toxicity Testing

Simple *in vitro* cytotoxicity test:

*in vitro*/*in vivo* correlation ~ 50-60%
certain number of misclassifications

Further needs:
To improve the *in vitro* - *in vivo* correlation by evaluating existing outliers in order to introduce further parameters (ADE, metabolism, organ specificity).
97 reference chemicals

- Generation of an in vivo database and establishment of a depository of reference compounds
- Generation of an in vitro database
- Technical optimisation of the amended test strategy
- Prevalidation of the testing strategy
- WP 2
- WP 4
- WP 5 Role of ADE
- WP 6 Role of metabolism
- Role of target organ toxicity
  - WP 7.2 – nephrotoxicity
  - WP 7.3 – hepatotoxicity
- Analysis and integration of in vitro/in vivo data
- Alerts and correctors in toxicity screening
56 chemicals were selected for testing in 3 labs under blind conditions.

**MT- ECVAM**
P. Prieto; A. Kinsner

- **Observer ICCVAM**

- **Chemicals Selection Committee**
P. Prieto  T. Cole  A. Kinsner  M. Liebsch

- **Buying & Coding Chemicals- ECVAM**
A. Kinsner

- **Distribution Chemicals SIGMA**

- **Lab 1**
HSL, UK
R. Gibson
Validated 3T3/NRU Manual

- **Lab 2**
IHCP-JRC, Italy
S. Coecke, M. Whelan
Validated 3T3/NRU Automated version

- **Lab 3**
IIVS, US
R. Curren  H. Raabe
Validated 3T3/NRU Abbreviated version

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Validations

Three partial replacement methods for skin sensitisation testing developed by Colipa-associated industries and optimised within Colipa ring trials have entered a Phase III prevalidation for the assessment of their reproducibility and preliminary evaluation of their predictive capacity.

- **Direct Peptide Reactivity Assay (DPRA, Procter & Gamble).** Protein binding is a key step in the induction of skin sensitisation, this test uses HPLC to monitor a chemical’s potential to deplete a nucleophile-containing synthetic peptide.

- **Human Cell Line Activation Test (h-CLAT, Kao and Shiseido).** This test monitors, using flow cytometry, the induction of two protein markers on the surface of a human monocytic leukemia cell line following exposure to the chemical.

- **Myeloid U939 Skin Sensitisation Test (MUSST, L’Oréal).** This test monitors, using flow cytometry, the induction of a protein marker on the surface of a human dendritic cell like line following exposure to the chemical.
The sixth Framework Programme sponsored Integrated Project Sens-it-iv aims to make available by 2010 a panel of in vitro assays for the identification of skin and respiratory sensitisers for use in the chemical, cosmetic and pharmaceutical industries. ECVAM is leader of WP1 on chemicals selection, involved in WP8 (in vitro assays development) and WP9 (technology transfer and dissemination), in the management and steering of the project.

**WP1 Activities**

**Compound selection**
The Sens-it-iv list of chemicals comprises 6 respiratory sensitisers, 13 skin sensitisers (including 3 pro-haptens and 1 pre-hapten) and 10 negatives. For the detailed chemical list refer to: [http://www.sens-it-iv.eu/index.php?id=701](http://www.sens-it-iv.eu/index.php?id=701)

**2. Chemicals Repository**
Chemicals are purchased and stored in the ECVAM repository. Aliquots are prepared and distributed to project participants.

**3. Guideline for chemical handling**
General guidelines on how to handle and dissolve chemicals were developed and distributed to participants.

**4. Database of in vivo and in vitro data from the literature**
A literature search is performed on a continuous basis to retrieve in vivo and in vitro data on skin sensitisation
Reduced-LLNA (r-LLNA)

- Retrospective analysis of published data for 211 (169 sensitisers 42 non-sensitisers) chemicals generated with the standard test to explore opportunities to further reduce animals number by limiting the doses necessary to classify chemicals as sensitisers or non-sensitisers.

- rLLNA protocol same as standard LLNA protocol but uses only the equivalent of the highest-dose group in the traditional test.

- Does not provide information of potency.

- For risk assessment purposes a standard LLNA should be conducted.

- ESAC statement: April 2007, currently under evaluation its inclusion in the OECD TG 429

ECVAM evaluation

<table>
<thead>
<tr>
<th>N</th>
<th>Accuracy</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>False Positive</th>
<th>False Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>211</td>
<td>98.6 %</td>
<td>98.2 %</td>
<td>100 %</td>
<td>0 %</td>
<td>1.8 %</td>
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<tr>
<td></td>
<td>(208/211)</td>
<td>(166/169)</td>
<td>(42/42)</td>
<td>(0/42)</td>
<td>(3/169)</td>
</tr>
</tbody>
</table>
**Endpoint Genotoxicity**

**Validation**
- Retrospective validation of *in vitro* micronucleus test

**Testing Strategy**
- Workshop on how to reduce false positives in *in vitro* genotoxicity testing (Kirkland et al., Mut Res, 2007)
- Recommended chemical list for assessment of the performance of new and improved genotoxicity tests (Kirkland et al., Mut Res, 2008)
- Workshop on reduction of animal use in regulatory genotoxicity test (Pfuhler et al, Mut Res, 2009)
Validation

- Prevalidation of micronucleus and comet assays in reconstituted human skin models
  (funded by COLIPA and ECVAM and coordinated by COLIPA)

- Validation of *in vitro* and *in vivo* COMET Assays (coordinated by JaCVAM)

Testing Strategy

- Analysis on the top concentration for genotox testing to reduce false positives

Support research

- Involved in EC/COMICS project (high-throughput comet assay)

- Link with OECD, ICH and IWGT on testing strategy activities above
Endpoint Carcinogenicity

Validation of three cell transformation assays (CTA)
Focus on reproducibility of standardised protocols

- OECD recommendation to develop Test Guidelines on CTA (DRP 31)
- Experimental work finalised
- ESAC peer review (beginning 2010)
- Publication of the study in a special issue on CTA of Mut Res, in preparation

CARCINOGENOMICS FP6 IP

Aim: to develop in vitro toxicogenomics tests to assess carcinogenic potential

- Responsible for workpackage on prevalidation
- Workshop on genomics approaches and cancer risk assessment, Venice, 08/2009
Workshop reports

- 11 workshop reports addressing 3Rs issues in the batch quality control of biologicals (batch safety and batch potency testing)

Validation & ESAC statements

- Serological methods (ELISA) for tetanus & erysipelas vaccines (*refinement, reduction*)
  - accepted in European Pharmacopoeia monographs
- Six in vitro methods based on human fever reaction for pyrogenicity test (*replacement*)
  - accepted in Europe & USA
- Deletion of the target animal safety test for routine quality control of veterinary vaccines in Europe (no longer relevant)
  - currently followed up in collaboration with EMEA at VICH level

ECVAM/EPAA Workshop

- The Consistency Approach for Quality Control of Vaccines – a 3Rs opportunity; Brussels, 11-12 January 2010
Dissemination of Knowledge

- **DB-ALM***:
  - **Increase in registrations** continues (+- 34 new users/month, Σ1772/75 countries)
  - **Online information content** updating and revisions continued (Annex 1)
  - **INVITTOX protocols**:
    - remote data entry facility under development
    - content updating priority on validated and accepted methods

Complementary activities

- “**ECVAM Guide on good search practices**”
  - Help finding the needed information faster and more efficiently

- **Development of on-line test submission**: Work started

- **Tracking of validation and acceptance status of alternative methods** – amending TSAR foreseen to start later in 2009

- **Portal development** for easier access to listed information systems

*http://ecvam-dbalm.jrc.ec.europa.eu*
DB-ALM Online Information Content

- **In vitro methods**
  - Method-Summary Descriptions 139
  - **INVITTOX Protocols**
    - Evaluation Studies 45
    - Formal Validation Studies 16
    - Test Results 8431
    - Bibliographic References 5100

- **Who’s who in the field of alternative methods** 202