Scope and ethical principles of toxicology and 3R

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DISCLAIMER

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Presentation overview

1. Scope of Toxicology
2. Ethics: Principal, Practice and Animals
3. 3R: Reduction
4. 3R: Replacement
5. 3R: Refinement
6. 3R in Legislation and Research
7. Mode of Action & Adverse Outcome Pathways

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Scope

• Toxicology is the study of the adverse effects of xenobiotics on living systems.

• Toxicology assimilates knowledge and techniques from biochemistry, biology, chemistry, genetics, mathematics, medicine, pharmacology, physiology, and physics.

• Toxicology applies safety evaluation and risk assessment to the discipline.
Scope

Modern toxicology goes beyond the study of the adverse effects of exogenous agents

Assimilates knowledge and techniques from most branches of biochemistry, biology, chemistry, genetics, mathematics, medicine, pharmacology, physicology, and physics and applies safety evaluation and risk assessment to the discipline.

In all branches of toxicology, scientists explore the mechanisms by which chemicals produce adverse effects in biological systems. Activities in these broad subjects complement toxicologic research, thereby contributing to the application of this knowledge to the science and art of toxicology.
Ethics

- Enormous benefits from rapid advances in science and technology but ...
  creating undesirable hazardous side-effects that impact human health and the environment.

- The impact of Toxicology on society has grown enormously arising financial, legal, and individual implications.

- Decision making has become more difficult and complex.

- It is thus increasingly important to consider the ethical, legal, and social issues that confront toxicologists, public health professionals, and decision makers.
An ethical toxicologist should consider...

<table>
<thead>
<tr>
<th><strong>Dignity</strong></th>
<th>which includes respect for the autonomy of human and animal subjects</th>
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</thead>
<tbody>
<tr>
<td><strong>Veracity</strong></td>
<td>an adherence to transparency and presentation of all the facts so all parties can discover the truth</td>
</tr>
<tr>
<td><strong>Justice</strong></td>
<td>which includes an equitable distribution of the costs, hazards, and gains</td>
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<tr>
<td><strong>Integrity</strong></td>
<td>an honest and forthright approach</td>
</tr>
<tr>
<td><strong>Responsibility</strong></td>
<td>an acknowledgment of accountability to all parties involved</td>
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<tr>
<td><strong>Sustainability</strong></td>
<td>consideration that actions can be maintained over a long period of time</td>
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</table>

(Gilbert and Eaton, 2009)
Ethics

Conflict of interest?

• Transparency

• Confidentiality

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Ethics and Animals’ Use

Millions of lives have been saved, improved and extended thanks to the results of humane scientific research that has relied upon animals at various stages. Without the use of animals, men, women and children around the world would simply not enjoy the quality and length of life they do today.

BUT...

Animals are used in research when there is simply no alternative that will produce the necessary results.
Russel and Burch in 1959

Reduction

Replacement

Refinement

Reduction - To minimize number of animals used
Replacement - To avoid the use of living animals
Refinement - To minimize suffering and distress
Reduction

A reduction alternative decreases the number of animals required for a test method, while remaining consistent with sound scientific practices necessary to obtain valid results.

- Setting specific goals, strategy and study designs in order to avoid unnecessary animal testing
- Use of appropriate statistics for full data analysis and use of existing data with meta-analysis
- Getting the most from each animal used
Replacement

A replacement alternative uses non-animal systems instead of animals, or uses a phylogenetically lower species of live animal than the current test.

- Subcellular fractions
- Cellular fractions
- Tissue slices
- Tissue culture
- Perfused organs
Refinement

- Non-invasive techniques
- Appropriate anaesthetic and analgesic regimes for pain relief
- Training animals to voluntarily co-operate with procedures (e.g. blood sampling) so that they have greater control over the procedure reduce distress
- Provision of species-appropriate housing and environmental enrichment which meet the animals' physical and behavioural needs (e.g. providing opportunities for nesting for rodents)
3R and Risk Assessment

EU has been promoting for many years the 3R

Traditional risk assessment approaches are insufficient to adequately predict the potential risk associated with any given substance, especially when considering normal life low-dose exposure.

Therefore new prediction models are needed in a new intelligent and more efficient safety assessment, based on in vitro testing in combination with computational modelling.
Examples of EU legislation that require, or strongly encourage, the replacement of animal testing

- Directive on the protection of animals used for scientific purposes (2010/63)
- Regulation on cosmetic products (1223/2009)
- Classification, Labelling and Packaging (CLP) (1272/2008)

The EU policies on endocrine disruptors, combination effects of chemicals and nanomaterials are all examples of areas of concern where traditional risk assessment is coming to an end.
3R and the JRC Activity

- New integrated methods based on in-depth biological knowledge are needed. Therefore the JRC is developing and testing new animal-free methods, alternatives to animal-based tests, to be applied in an integrated safety assessment of chemicals. JRC is also providing informatics tools and databases to support this approach.

- EURL ECVAM, the European Union Reference Laboratory for Alternatives to Animal Testing
Human health endpoints that are covered:

- Skin irritation and corrosion
- Serious eye damage and eye irritation
- Skin sensitisation
- Acute systemic toxicity
- Repeat dose toxicity
- Genotoxicity and mutagenicity
- Carcinogenicity
- Reproductive toxicity
- Endocrine disruption relevant to human health
- Toxicokinetics

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Acute systemic toxicity

- Information on the acute systemic toxicity of chemicals is required for human health risk assessments under REACH and the Biocides Products Regulation, and can be used for classification and labelling under CLP.

- Non-standard methods and ITS for acute systemic toxicity should predict LD50 values or official acute toxicity categories, depending on the regulatory application. Ideally, they should eventually be predictive of acute toxicity to humans.

- Despite considerable research efforts over the past 20 years in the area of acute systemic toxicity, a complete mechanistic understanding of the key pathways is lacking, which is hampering the development of non-standard methods and AOP-based ITS.

- Non yet appropriate in vitro for full substitution of in vivo
Acute systemic toxicity - Reduction!

- After many years of controversy and debate, the LD50 test was finally suspended by the end of 2002.

- Three alternative animal tests
  - Fixed Dose Procedure (FDP)
  - Acute Toxic Class Method (ATC)
  - Up and Down Procedure (UDP)
Repeat dose toxicity

- The *in vivo* consequences of the repeat/chronic exposure to chemicals involve integrated processes at the molecular, cellular, organ and system levels.

- There is limited knowledge of the underlying mechanistic pathways and their interactions.

- Many compounds are thought to induce their toxic effects by interfering with basic biochemical functions (e.g. interference with energy production, DNA function, receptor-mediated signalling pathways) or homeostatic mechanisms (e.g. perturbation of calcium homeostasis), resulting in functional impairments at the cell, tissue and organ levels.

- However, a quantitative description of these effects (dose response relationships) is generally lacking.
**3R research and reality: new tools!!**

<table>
<thead>
<tr>
<th>In vitro and High-throughput screening (HTS)</th>
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<tbody>
<tr>
<td>• Cell models and robotic systems</td>
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<table>
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<tr>
<th>Microarray Technologies</th>
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<tbody>
<tr>
<td>• DNA or protein fragments placed onto a slide, which are then used as “miniaturized reaction areas”</td>
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<table>
<thead>
<tr>
<th>-omics</th>
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<tbody>
<tr>
<td>• Chemical analysis of molecular level</td>
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<table>
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<tr>
<th>Non Mammalian Models</th>
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<tbody>
<tr>
<td>• Use of invertebrates such as drosophila, freshwater snails and Caenorhabditis elegans</td>
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<th>ITS</th>
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<td>• Integrated testing strategies</td>
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## 3R research and reality: new tools!!!

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
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<tbody>
<tr>
<td>Kinetics modelling</td>
<td>• physiology-based pharmacokinetic (PBPK) or biokinetic (PBBK) modeling software</td>
</tr>
<tr>
<td>In silico methods</td>
<td>• Computerized modelling, QSAR</td>
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<tr>
<td>Virtual Organs</td>
<td>• Virtual liver</td>
</tr>
<tr>
<td>TTC</td>
<td>• Threshold of Toxicological Concern</td>
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<tr>
<td>Read Across</td>
<td>• Use of knowledge between similar chemicals</td>
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-Omics! Prediction and monitoring

- **Genomics**: The study of genes and their function.
- **Proteomics**: The study of proteins.
- **Metabonomics**: The study of molecules involved in cellular metabolism.
- **Transcriptomics**: The study of the mRNAs.
- **Glycomics**: The study of cellular carbohydrates.
- **Lipomics**: The study of cellular lipids.
Mode of Action & Adverse Outcome Pathways
UNDERSTANDING WHAT A MOA IS ABOUT!

“A common set of physiological and behavioural signs that characterise a type of adverse biological response, where the major (but not all) biochemical steps are understood” (ECETOC Technical Report 102, 2007).

“Mode of action differs from mechanism, in that the mode of action requires a less detailed understanding of the molecular basis of the toxic effect” (Seed, J., et al, Overview: Using mode of action and life stage information to evaluate the human relevance of animal toxicity data. Crit. Rev. Toxicol. 35: 664-672, 2005).
Understanding what a MoA is about!

Representation of the relationships between Toxicity Pathways, Mode of Action Pathways, Adverse Outcome Pathways, and Source to Outcome Pathways.


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What is the value of a MoA/AOP and how can be used?

- AOP Knowledge Base: a web-based platform which aims to bring together all knowledge on how chemicals can induce adverse effects (https://aopwiki.org/wiki/index.php/Main_Page)
- Identification of new biomarker endpoints
- Integrated Assessment and Testing Approaches
- Identification of new methods/profilers for grouping chemicals
- Prioritization in Risk Assessment
- Integrated Testing Strategies, for defined hazard endpoints
- Prediction of adversity based on early stage effects
- establish relationships
- Mixtures Toxicology

http://www.who.int/ipcs/methods/harmonization/en/

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Methodology & Study

MIE | IEs | AO

Molecular | Organelle | Cellular | Tissue | Organ

Diagram & Reporting

Evaluation

Testing & Confirmation

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Establishing a methodology for MoA building

<table>
<thead>
<tr>
<th>Step</th>
<th>Activity</th>
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<tbody>
<tr>
<td>Step 1</td>
<td>Selection of the Adverse Outcome (AO)</td>
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<td>Step 2</td>
<td>Selection of the Molecular Initiating Event (MIE)</td>
</tr>
<tr>
<td>Step 3</td>
<td>Study of the relevant physiology</td>
</tr>
<tr>
<td>Step 4</td>
<td>Determination of the Intermediate Effects (IEs) through literature search</td>
</tr>
<tr>
<td>Step 5</td>
<td>Graphic representation of the MoA</td>
</tr>
<tr>
<td>Step 6</td>
<td>Evaluation</td>
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Step 1: Selection of the Adverse Outcome (AO)

- SEURAT-1 ⇒ hepatic, cardiac, renal, neuronal, muscle and skin tissues
- Hepatotoxicity is of particular interest (Hengstler et al. 2012, Vinken et al. 2011)
- JRC has a liver cell model in-house and is already exploring the development of suitable high content assays for evaluating responses to toxicants.

LIVER FIBROSIS & LIVER STEATOSIS
Step 2 • Selection of the Molecular Initiating Event (MIE)

- SEURAT-1 Gold Compound Working Group selection of reference chemicals for fibrosis and steatosis
- Fibrosis: Two GC WG fibrotic chemicals are protein alkylators
- Steatosis: connection with endocrine disruption due to the globally increased concern to endocrine disrupting chemicals that act mainly through nuclear receptors binding (MIE related to the MoA of EDCs). The one until now proposed EDC is a steatotic LXR activator

PROTEIN ALKYLATION FOR FIBROSIS & LXR ACTIVATION FOR STEATOSIS
Selection of MIE and AO:
From where to where???
Step 3

• Study of the relevant physiology

- Study of physiology as well as biology
- As the study goes in depth, scanning from individual to molecules, the amount of knowledge is becoming extremely big
- Focus on the needed detail

In full detail a very time consuming step demanding an adequate level of expertise
Step 4

- Determination of the Intermediate Effects (IEs) through literature search

- Systematic literature searching from all possible sources (books, peer review papers, etc)
- Searching on bibliographic databases like PubMed and Scopus according to basic terms and their combinations
- Focus on review papers and key studies is helpful
- Searching bottom-up, top-down or both directions
Step 4 (continued)

- Determination of the Intermediate Effects (IEs) through literature search

- Information analysis according to different levels of biological organization and MoAs construction based on the OECD draft guidance document and template (OECD 2012)

- Attention on differences between studies (different species, cell models, concentrations)

- Lack of quantitative data

- Establishment of the needed detail?

In full detail a very time consuming step demanding an adequate level of expertise
Step 5

- **Graphic representation of the MoA**

  - Difficulty on a clear graphic representation of the MoA non-linearity, intersections and loops
  - ...and the difficulty becomes bigger as more IEs become involved in the MoA, especially in the molecular level
  - Focus on the needed detail

**MoA diagrams**

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MoA flow diagram from LXR activation to steatosis

**Biological Organization Level**

- **Molecular**
  - **MIE**
  - **Intermediate Effects**
  - **Key Event**

**Organelle**
- **TGs accumulation**
  - Cytoplasm displacement
  - Nucleus distortion
  - Mitochondrial disruption

**Cellular**
- Fatty liver cells

**Tissue**
- Steatosis > 5–10% by liver weight

**AOP from LXR**
- LXR activation
- Activation of:
  - 1. CHREBP
  - 2. SREBP-1c
  - 3. FAS
  - 4. SCD1
- De novo FA synthesis
- Inhibition of respiration = NAD+ depletion
- Induction of the mitochondrial b-oxidation
- Peroxisomal AOX inhibition

**AOP 1**
- ER binding
- PPAR-α antagonism binding

**AOP 2**
- PXR activation
- Induction of CYP3A4

**AOP 3**
- PPAR-γ activation
- Differentiation
- CD36 up-regulation
- Increase of the FA influx from peripheral tissues

**AOP 4**
- AhR agonism

**AOP 5**

**AOP 6**

**Testing**

- Mechanism elucidation
  - 1. TA and RBA assays
  - 2. Genomics (using positive controls)
  - 3. Imaging techniques

- EDs identification
  - 1. Transcriptional activation assays
  - 2. Binding / Affinity assays

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Corresponding diagram for mechanism of action for steatosis
OECD 2012 guidance for AOP evaluation: elements from the Bradford Hill criteria for a WoE approach and confidence of an AOP

- Concordance of DR relationships: lack of data
- Temporal concordance: lack of data
- Strength, consistency and specificity
- Biological plausibility, coherence, and consistency of the experimental evidence
• Evaluation

- Alternative destructive mechanisms
- Uncertainties, inconsistencies and data gaps
- Assessment of the quantitative understanding of the AOP
- Specificity in relation to certain tissues, life stages, age classes, across taxa

Confidence on MoAs
Reporting?

OECD template specifically developed for formally describing a MoA

- OECD template and related guidance was followed to differing extents
- Overall the draft template proved useful but it needs improvements in order to host all the essential information and to wide applicability
- An appropriate tool for communicating knowledge on MoA in a harmonized way
A new reality!

A suite of AOPs relevant to repeat dose toxicity, including some of those from the above-mentioned research initiative, will be evaluated and published by the OECD.
The vision and hope…

…is that by employing the MoA/AOP framework, it should be possible to identify a limited number of key events that act as common “nodes” in multiple toxicity pathways, with the practical benefit that a limited set of (in vitro and in silico) models based on these key events will provide a sufficient toolkit for quantitative hazard assessment based entirely on non-animal approaches.
Thank you for your attention