Alternative methods for acute systemic toxicity testing

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Use of *in vitro* methods – pharmaceutical industry

- 7 UK companies
- Number of *in vitro* tests carried out
- Genotoxicity, ADME & safety pharmacology

_Goh et al., Toxicol. Res. 2015_
**Introduction – In vitro methods for acute systemic toxicity testing**

- **DB-ALM** (http://ecvam-dbalm.jrc.ec.europa.eu/) – 25 protocols

- **Toxicokinetics**
  - 3 protocols

- **Specific target organs**
  - 18 protocols

- **Basal cytotoxicity**
  - 4 protocols
Research efforts into alternative methods for acute systemic toxicity testing

1989

MEIC programme

Halle's Registry of cytotoxicity

EURL ECVAM 3T3 NRU validation study

2013

NICEATM & ECVAM NRU validation study

ACuteTox
EURL ECVAM strategy paper - 2014

- **3Rs** in acute systemic toxicity testing

- **Aim 1**: reduction & replacement of animal testing

- **Aim 2**: refinement of animal studies
3T3 Neutral Red Uptake (NRU) test method

- BALB/c 3T3 cells + Neutral Red Uptake (fixation of red dye)
- Validated on 56 industrial chemicals
- High sensitivity (92-96%) for identification of non-classified (oral \( \text{LD}_{50} > 2000 \text{mg/kg} \))
- **Take home** – 3T3 NRU as part of WoE/ ITS to identify non-classified chemicals
EURL ECVAM strategy – Aim 1 - Reduction

3T3/NRU basal cytotoxicity assay

- Predicted oral LD_{50} ≤ 2000 mg/kg b.w.
  - Positives
    - TP: Suspected specific MoA
    - FP: Low bioavailability in vivo (e.g. poor gastrointestinal absorption)
  - How to improve the prediction of the true CLP toxicity category?
  - Increased probability for TP

- Predicted oral LD_{50} > 2000 mg/kg b.w.
  - Negatives
    - FP: Suspected detoxification
    - FN: Suspected bioactivation in vivo
    - TN: Evidence of lack of bioactivation
    - Suspected specific MoA and/or detection of acute organ-specific toxicity
    - Nominal concentration > > > free intracellular concentration (e.g. using Virtual Cell Based Assay)
    - Nominal and free intracellular concentration is similar (e.g. using Virtual Cell Based Assay)
    - Increased probability for TN

European Commission
Increasing confidence - 3T3 NRU + QSAR methods

- 3T3 NRU & LD$_{50}$ data for 181 chemicals
- Threshold POS/NEG: LD$_{50}$ = 2000mg/kg
- 5 false negatives results
- "correction" for metabolism

Thanks to Julien Burton
### Increasing confidence - 3T3 NRU + QSAR methods

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Nb unique metabolites</th>
<th>Min predicted LD50 (mg/kg)</th>
<th>Max predicted LD50 (mg/kg)</th>
<th>Avg predicted LD50 (mg/kg)</th>
<th>Oral cat1 &lt;5</th>
<th>Oral cat2 &lt;50</th>
<th>Oral cat3 &lt;300</th>
<th>Oral cat4 &lt;2000</th>
<th>Oral cat5 &lt;5000</th>
<th>In vivo LD₅₀ (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digoxin</td>
<td>777</td>
<td><strong>30.11</strong></td>
<td>1429.32</td>
<td>294.47</td>
<td>0</td>
<td>114</td>
<td>362</td>
<td>301</td>
<td>0</td>
<td>28</td>
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<tr>
<td>Aconitine</td>
<td>677</td>
<td><strong>23.55</strong></td>
<td>9218.3</td>
<td>325.24</td>
<td>0</td>
<td>119</td>
<td>297</td>
<td>243</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>Benzylbenzoate</td>
<td>69</td>
<td><strong>815.43</strong></td>
<td>6511.15</td>
<td>2257.27</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>35</td>
<td>31</td>
<td>1990</td>
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<tr>
<td>5,5-Diphenylhydantoin</td>
<td>25</td>
<td><strong>1088.63</strong></td>
<td>3620.21</td>
<td>1783.76</td>
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<td>0</td>
<td>0</td>
<td>18</td>
<td>7</td>
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<tr>
<td>Disopyramide</td>
<td>188</td>
<td><strong>266.83</strong></td>
<td>6960.22</td>
<td>1393.25</td>
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<td>0</td>
<td><strong>1</strong></td>
<td>159</td>
<td>25</td>
<td>333</td>
</tr>
</tbody>
</table>

- Extended to all negatives (automated process)
- Limitations  - QSAR on oral for rats  
  - Metabolites generated “in situ” (oral model accounts for ingestion of the chemical)

Thanks to Julien Burton
Reduction – use of existing repeated-dose toxicity data

- Analysis of New Chemical Database – relation 28 day oral NOAEL & oral LD$_{50}$ (Bulgheroni et al., 2009)
  - **NOAEL $\geq 200$mg/kg bw - LD$_{50}$ $> 2000$mg/kg bw** (63% correct, n=1436)
- European Chemicals Agency (ECHA) – REACH registration dossiers
  - 28 day oral LOAEL & oral LD$_{50}$
  - Klimisch scores 1 & 2
  - Rat & oral gavage
- 96 chemicals
Reduction – use of existing repeated-dose toxicity data

<table>
<thead>
<tr>
<th>LOAEL (mg/kg b.w./day)</th>
<th>EU CLP categories (LD50 mg/kg b.w.)</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>&lt; 5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 – 50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>50 – 300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>300 – 2000</td>
<td>0</td>
<td>0</td>
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<tr>
<td>&gt;2000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>0</td>
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</table>

<table>
<thead>
<tr>
<th>LOAEL (mg/kg b.w.)</th>
<th>LD50 (mg/kg b.w.)</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>≤ 2000</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>&lt;200</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>≥200</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>47</td>
</tr>
</tbody>
</table>

- poor **direct** correlation between the two data sets
- correctly predict **85% non-classified** substances
3T3 NRU dataset - mechanism mapping

- Lack of mechanistic knowledge for acute systemic toxicity
- **Data rich** set of 181 chemicals
  - $IC_{50}$ values; oral $LD_{50}$ values & functional information
  - 99 industrial chemicals & 82 "others" ie biocides, pharmaceutical
  - 66 non-classified & 115 "toxic"

- 8 target organs:
  - cardiovascular system
  - nervous system
  - lungs
  - immune system
  - blood
  - liver
  - kidneys
  - gastrointestinal tract

- **Aim:** Complement 3T3 NRU results with mechanistically relevant information
How often are the 8 organs the SINGLE targets of toxicity?

- **in vitro** methods for target organ toxicity
- brain aggregates for neurotoxicity
Conclusions & summary

- *In vitro* methods are relevant and useful in safety assessment.

- **3T3 NRU** method could form valuable part of an **integrated testing strategy** to identify non-classified compounds.

- QSAR modelling of metabolism.

- Existing *in vivo* **LOAEL** data from repeated-dose studies.

- Mechanistic data on specific target organs.
Thanks
3T3 NRU dataset - mechanism mapping

Acute toxic categories

- cat 1: 8
- cat 2: 19
- cat 3: 26
- cat 4: 62
- n.c.: 66

Number of chemicals
26 of these chemicals were identified to have a mechanism of general cytotoxicity.
3T3 NRU dataset - mechanism mapping

Blood

- **Organ**: Blood
  - **Effect**: Hypoxia
    - **Mechanism**: 
      1. Interference with haemoglobin, oxidation of the oxygen carrying iron molecule to Fe²⁺
         (methaemoglobin) 1-Naphthylamine, Malononitrile, Resorcinol N-isopropyl-N-phenyl-p-benzenediamine
      2. Interference with haemoglobin, e.g., CO binds to haemoglobin with higher affinity than O₂
      3. Chemicals reacting directly with ion of cytochrome oxidase in mitochondria; Potassium Cyanide
    - **Cell type(s)**: Erythrocytes
  - **Effect**: Haemorrhage
    - **Mechanism**: 
      1. Binding to cell (antigen) triggers immune-mediated destruction Rifampicin
      2. Direct destruction of cells by oxidative damage to cell wall (non-immune mediated destruction) 
         Rifampicin, tert-Butyl hydroperoxide
    - **Cell type(s)**: Erythrocytes, immune cells
  - **Effect**: Infection
    - **Mechanism**: 
      1. Interference with clotting factor production Warfarin, Acetylsalicylic acid
      2. Clotting factor exhaustion from circulation by triggering massive coagulation
    - **Increased capillary fragility Warfarin**: Thrombocytes, liver
    - **Cell type(s)**: Thrombocytes
  - **Effect**: Lysis of cells
    - **Mechanism**: 
      1. Binding to cell (antigen) triggers immune-mediated destruction Cupric sulfate pentahydrate
      2. Direct destruction of cells by oxidative damage to cell wall (non-immune mediated destruction)
      3. Inhibition of normal function such as phagocytosis
    - **Cell type(s)**: Leukocytes, immune cells
Increasing confidence - 3T3 NRU + QSAR methods

Simulations for all 19 true negatives

<table>
<thead>
<tr>
<th>Name</th>
<th>CAS No.</th>
<th>N° of unique metabolites</th>
<th>Min predicted LD&lt;sub&gt;50&lt;/sub&gt; (mg/kg)</th>
<th>Max predicted LD&lt;sub&gt;50&lt;/sub&gt; (mg/kg)</th>
<th>Avg predicted LD&lt;sub&gt;50&lt;/sub&gt; (mg/kg)</th>
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<tbody>
<tr>
<td>Dichloromethane</td>
<td>75-09-2</td>
<td>4</td>
<td>106</td>
<td>567</td>
<td>289</td>
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<tr>
<td>*1,2-Dichlorobenzene</td>
<td>95-50-1</td>
<td>4</td>
<td>248</td>
<td>578</td>
<td>413</td>
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<tr>
<td>Gibberellic acid</td>
<td>77-06-5</td>
<td>5</td>
<td>264</td>
<td>553</td>
<td>418</td>
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<tr>
<td>1,1,1-Trichloroethane</td>
<td>71-55-6</td>
<td>5</td>
<td>263</td>
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<td>Benzene</td>
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<td>536</td>
<td>1219</td>
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<td>*Ethylene glycol</td>
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<td>585</td>
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<td>*2,6-Diethylaniline</td>
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<td>2-Ethylhexyl acrylate</td>
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<td>Tris(nonylphenyl)phosphate</td>
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<td>1,2-Benzenedicarboxylic Acid</td>
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<td>Di-&quot;Isodecyl&quot; phthalate</td>
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<td>2-(2-Butoxyethoxy)ethanol</td>
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