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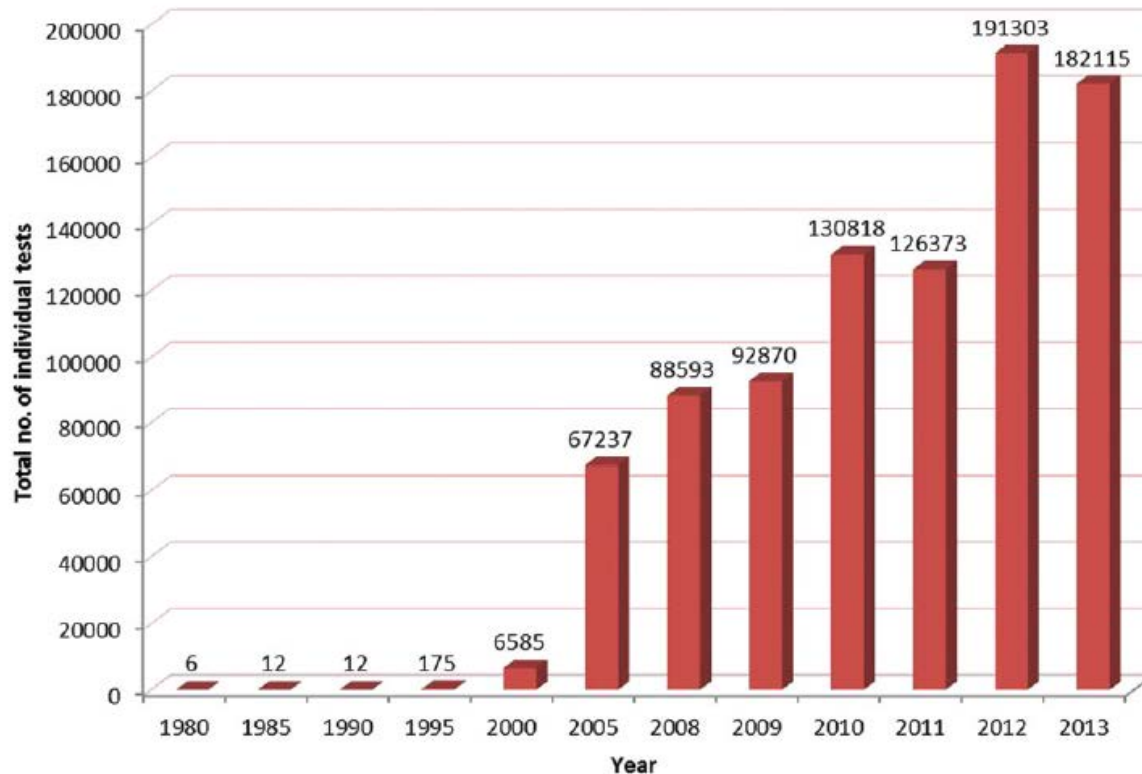
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Stimulating innovation
Supporting legislation*

Alternative methods for acute systemic toxicity testing

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EURL ECVAM

www.ec.europa.eu/jrc

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)

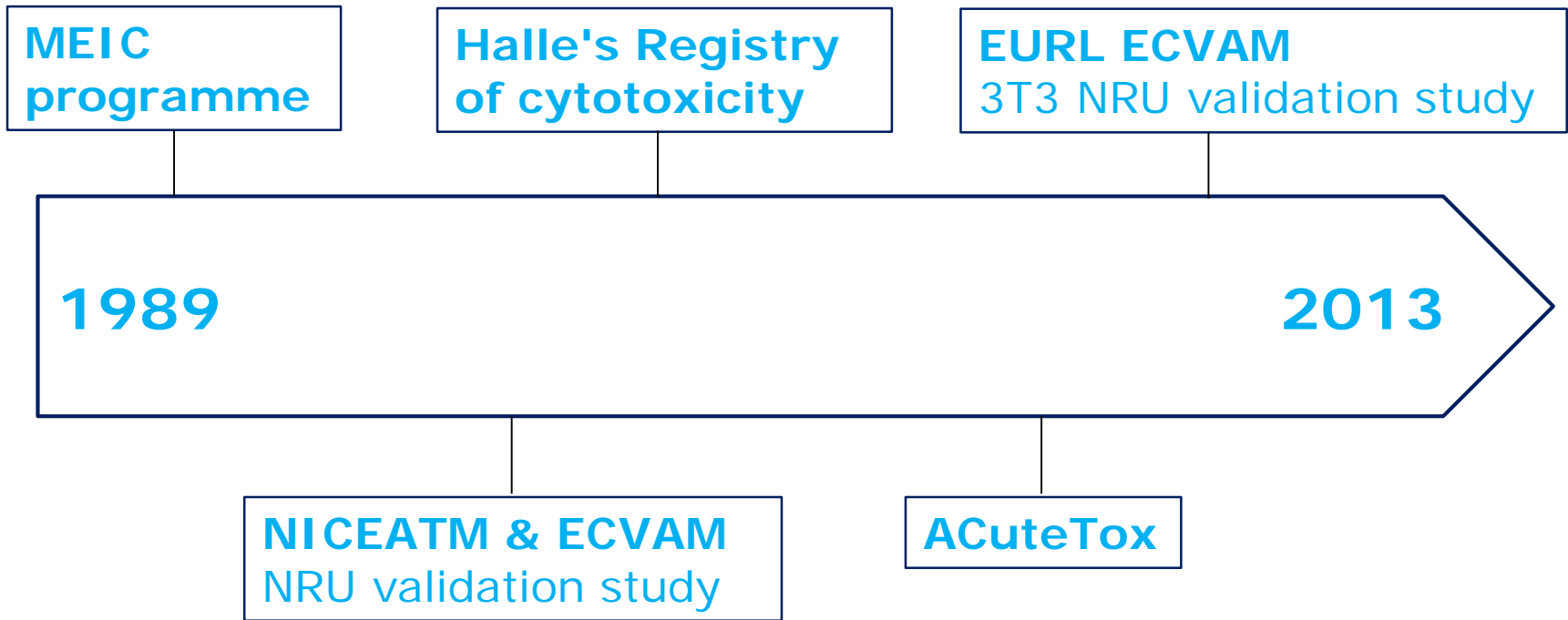
**Specific target
organs**

(18 protocols)

**Basal
cytotoxicity**

(4 protocols)

Research efforts into alternative methods for acute systemic toxicity testing

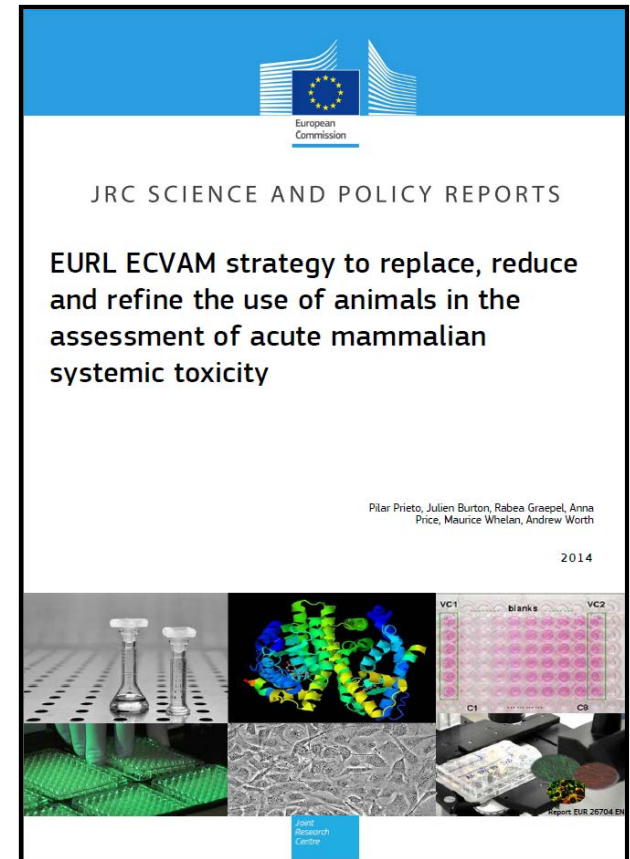


EURL ECVAM strategy paper - 2014

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

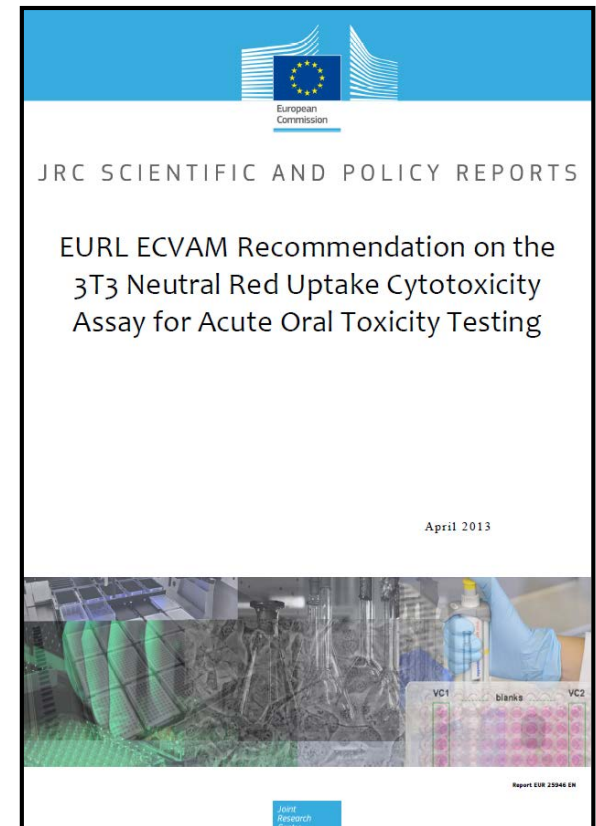
- **Aim 1: reduction & replacement** of animal testing

- **Aim 2: refinement** of animal studies

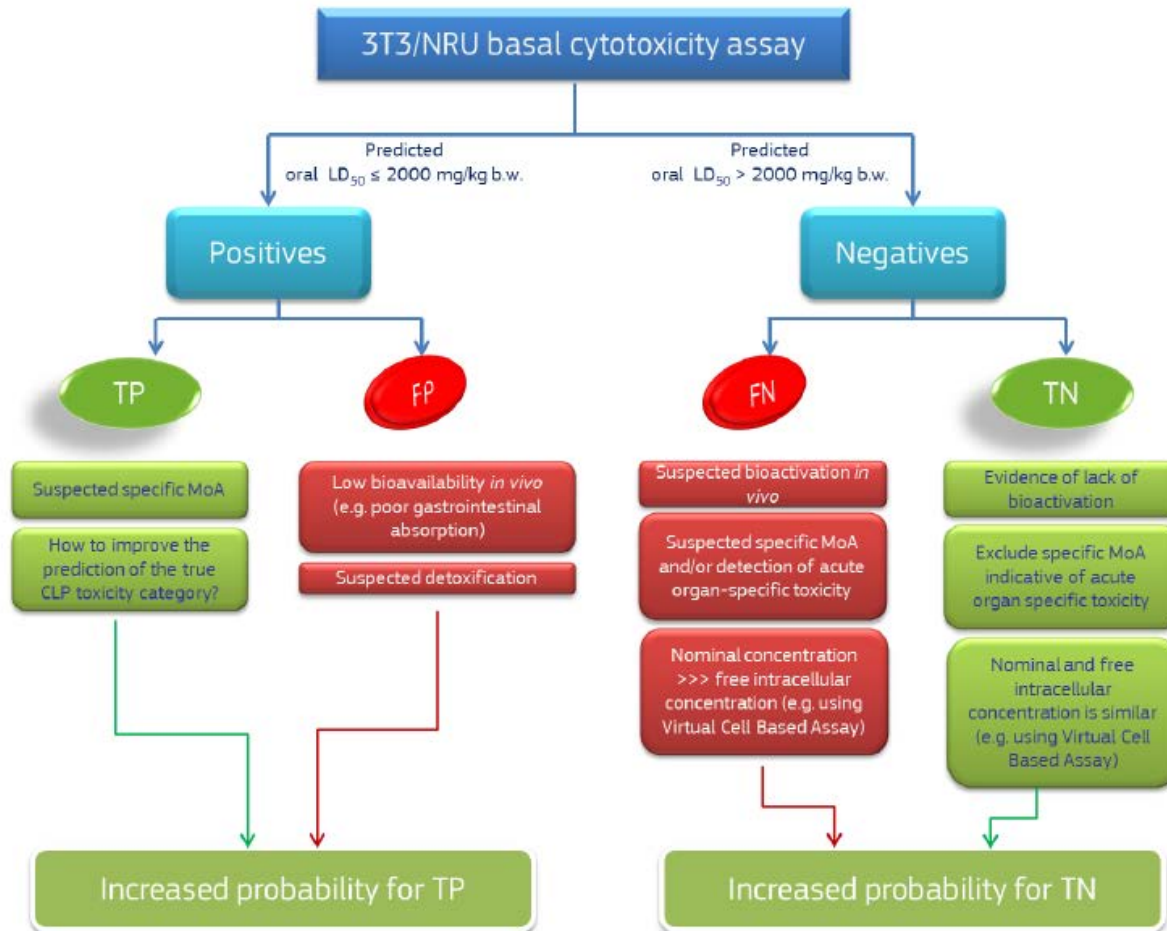


3T3 Neutral Red Uptake (NRU) test method

- 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** $LD_{50} > 2000\text{mg/kg}$)
- **Take home** – 3T3 NRU as part of WoE/ ITS to identify non-classified chemicals



EURL ECVAM strategy – Aim 1 - Reduction



Increasing confidence - 3T3 NRU + QSAR methods

- 3T3 NRU & LD₅₀ data for **181** chemicals
- Threshold POS/NEG: LD₅₀=2000mg/kg
- 5 false negatives results
- "correction" for metabolism



Increasing confidence - 3T3 NRU + QSAR methods

| Compound name | Nb unique metabolites | Min predicted LD50 (mg/kg) | Max predicted LD50 (mg/kg) | Avg predicted LD50 (mg/kg) | Oral cat1 < 5 | Oral cat2 <50 | Oral cat3 <300 | Oral cat4 <2000 | Oral cat5 <5000 | In vivo LD ₅₀ (mg/kg) |
|-----------------------|-----------------------|----------------------------|----------------------------|----------------------------|---------------|---------------|----------------|-----------------|-----------------|----------------------------------|
| Digoxin | 777 | 30.11 | 1429.32 | 294.47 | 0 | 114 | 362 | 301 | 0 | 28 |
| Aconitine | 677 | 23.55 | 9218.3 | 325.24 | 0 | 119 | 297 | 243 | 15 | 6 |
| Benzylbenzoate | 69 | 815.43 | 6511.15 | 2257.27 | 0 | 0 | 0 | 35 | 31 | 1990 |
| 5,5-Diphenylhydantoin | 25 | 1088.63 | 3620.21 | 1783.76 | 0 | 0 | 0 | 18 | 7 | 1360 |
| Disopyramide | 188 | 266.83 | 6960.22 | 1393.25 | 0 | 0 | 1 | 159 | 25 | 333 |

- 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 LD₅₀ (Bulgheroni *et al.*, 2009)
 - **NOAEL ≥ 200mg/kg bw - LD₅₀ > 2000mg/kg bw** (63% correct, n=1436)
- European Chemicals Agency (ECHA) – REACH registration dossiers
 - 28 day oral LOAEL & oral LD₅₀
 - Klimisch scores 1 & 2
 - Rat & oral gavage
- **96 chemicals**

Reduction – use of existing repeated-dose toxicity data

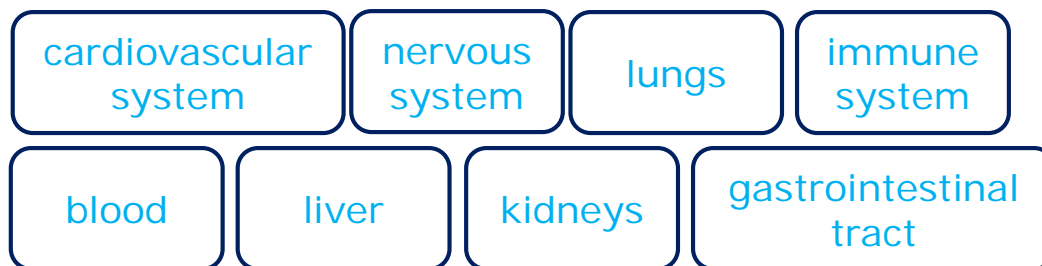
| LOAEL (mg/kg b.w./day) | EU CLP categories (LD50 mg/kg b.w.) | | | | | Total |
|---------------------------|-------------------------------------|----------|--------------|---------------------|---------------|-----------|
| | 1 (<5) | 2 (5-50) | 3 (50 – 300) | 4 (300- 2000) | NC (>2000) | |
| < 5 | 0 | 0 | 0 | 1 | 0 | 1 |
| 5 – 50 | 0 | 0 | 1 | 5 | 0 | 6 |
| 50 – 300 | 0 | 0 | 2 | 10 | 3 | 15 |
| 300 – 2000 | 0 | 0 | 0 | 4 | 17 | 21 |
| >2000 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 0 | 0 | 3 | 20 | 20 | 43 |

| LOAEL (mg/kg b.w.) | LD50 (mg/kg b.w.) | | Total |
|-----------------------|-------------------|-----------|-----------|
| | ≤ 2000 | >2000 | |
| <200 | 20 | 7 | 27 |
| ≥200 | 18 | 40 | 58 |
| Total | 38 | 47 | 85 |

- poor **direct** correlation between the two data sets
- correctly predict **85% non-classified** substances

3T3 NRU dataset - mechanism mapping

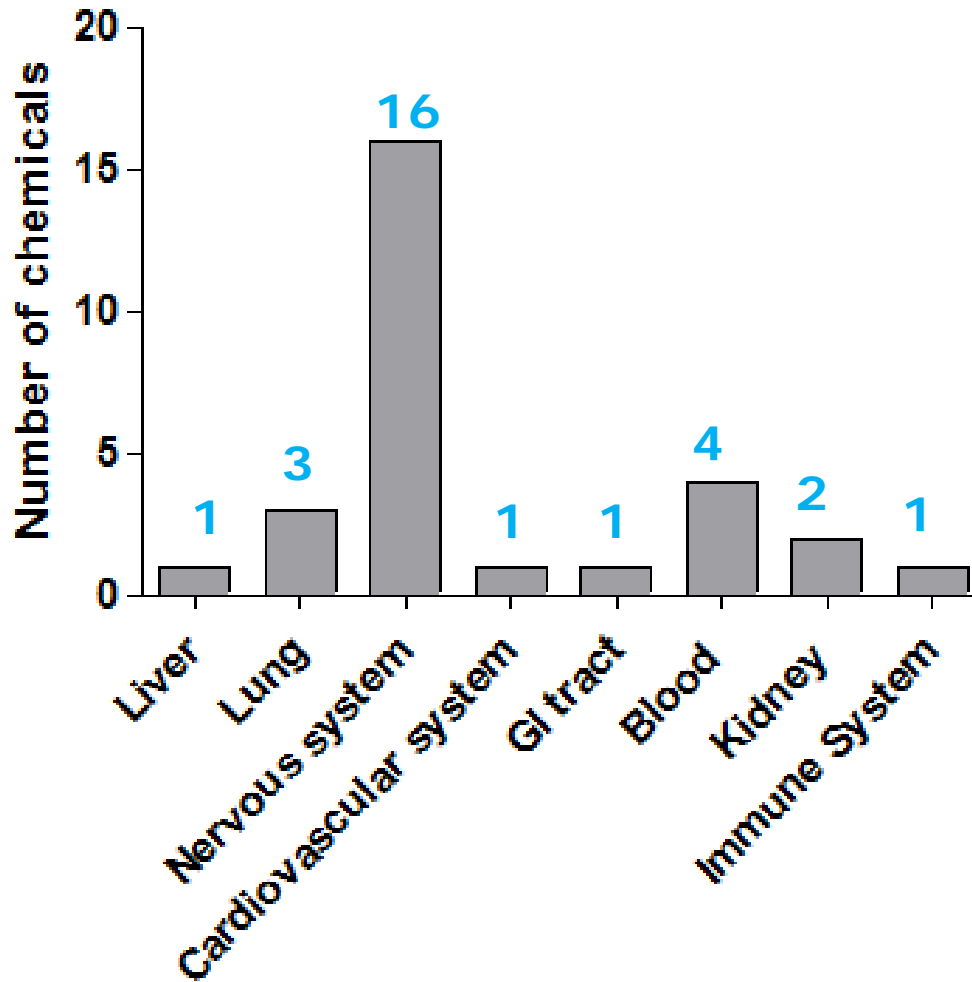
- Lack of mechanistic knowledge for acute systemic toxicity
- **Data rich** set of **181** chemicals
 - IC₅₀ values; oral LD₅₀ values & functional information
 - 99 industrial chemicals & 82 "others" ie biocides, pharmaceutical
 - 66 non-classified & 115 "toxic"
- 8 target organs:



- Aim: Complement 3T3 NRU results with mechanistically relevant information

3T3 NRU dataset - mechanism mapping

How often are the 8 organs the SINGLE targets of toxicity?



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

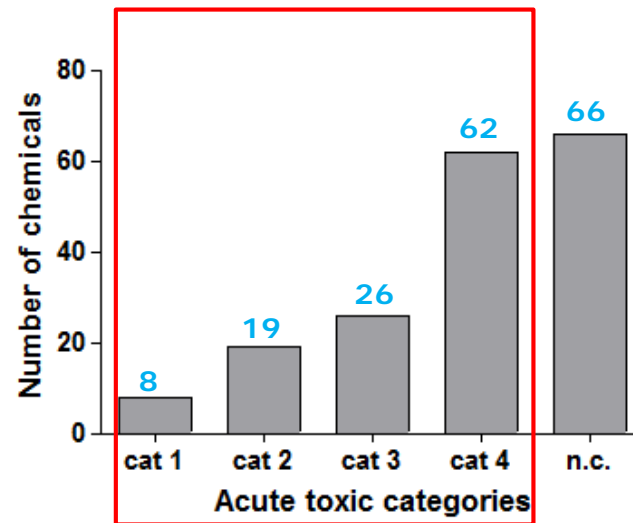
Conclusions & summary

- *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

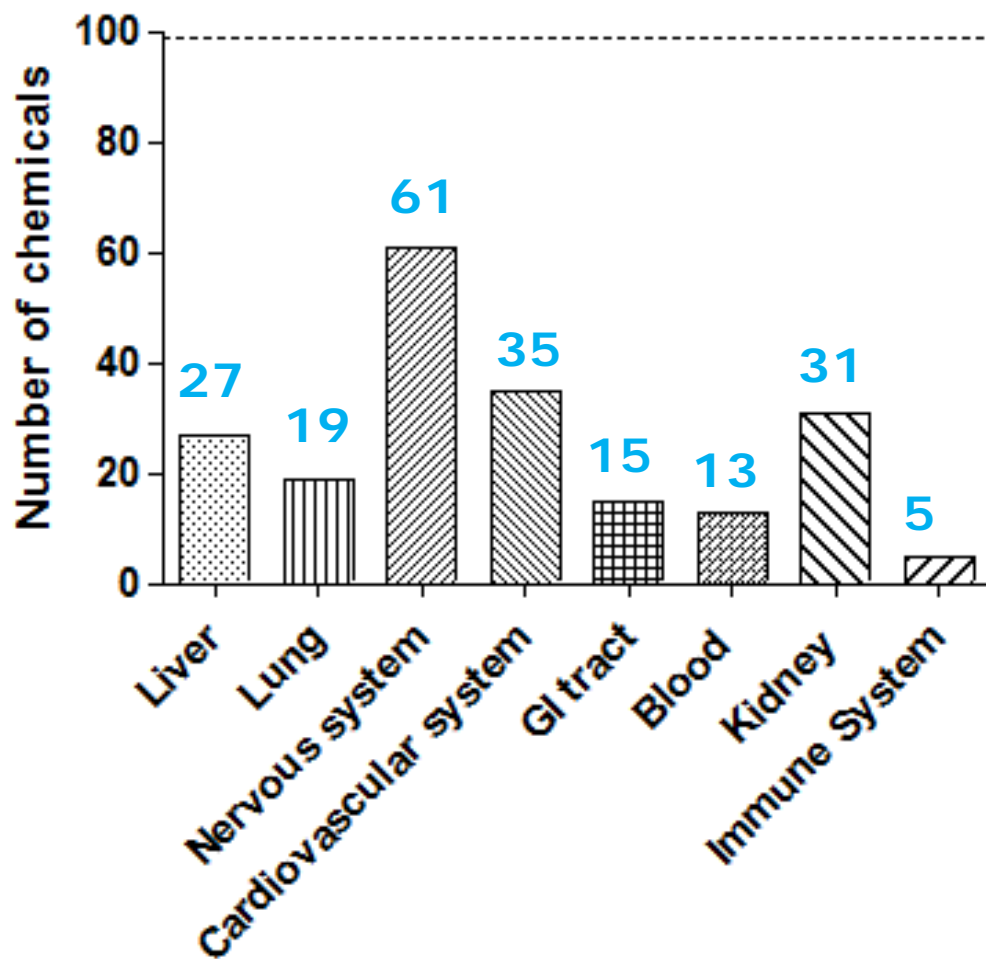
Thanks



3T3 NRU dataset - mechanism mapping



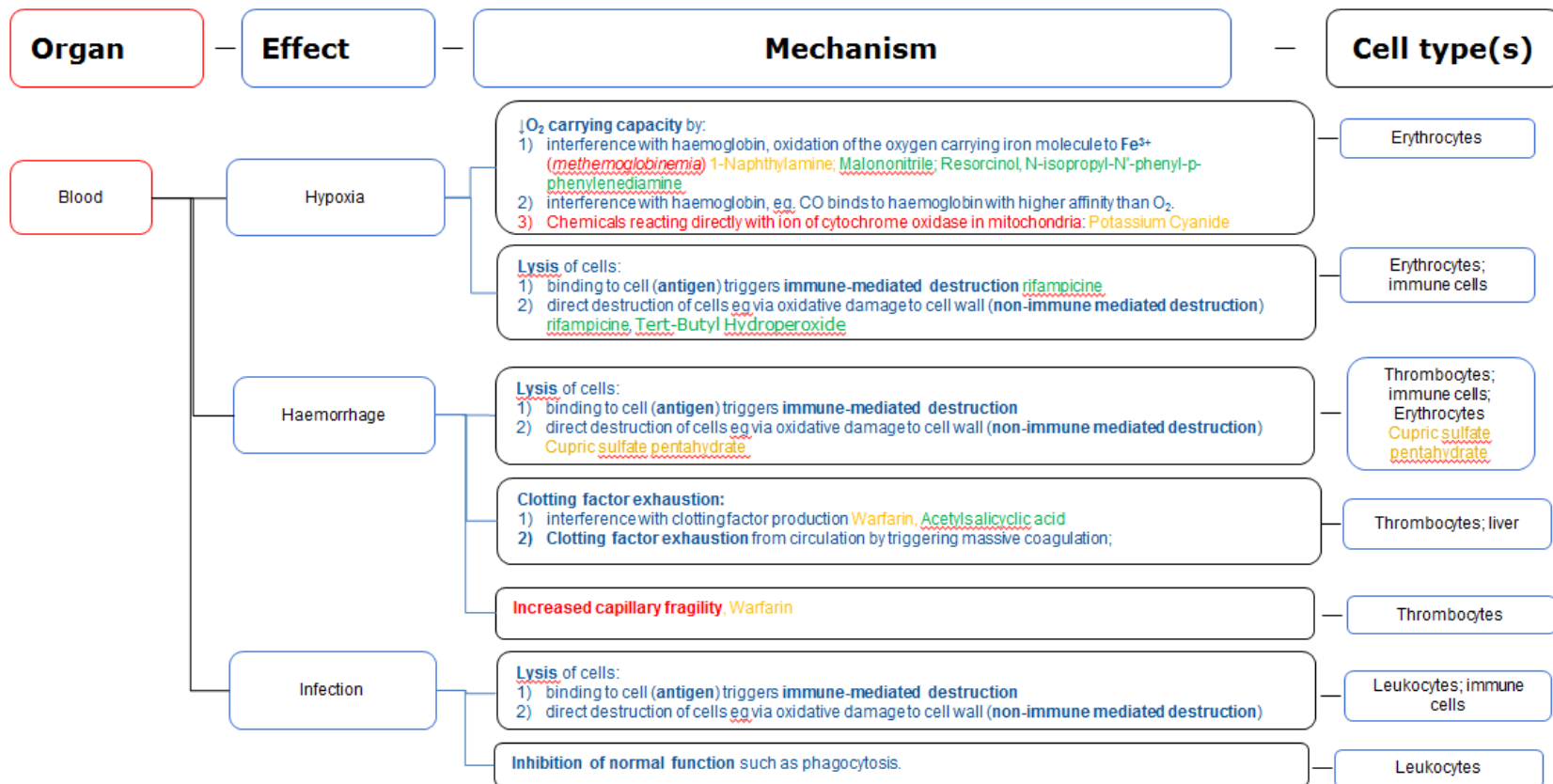
3T3 NRU dataset - mechanism mapping



26 of these chemicals were identified to have a mechanism of general cytotoxicity.

3T3 NRU dataset - mechanism mapping

Blood



Increasing confidence - 3T3 NRU + QSAR methods

Simulations for all 19 true negatives

| Name | CAS N° | N° of unique metabolites | Min predicted LD ₅₀ (mg/kg) | Max predicted LD ₅₀ (mg/kg) | Avg predicted LD ₅₀ (mg/kg) |
|------------------------------|------------|--------------------------|--|--|--|
| Dichloromethane | 75-09-2 | 4 | 106 | 567 | 289 |
| *1,2-Dichlorobenzene | 95-50-1 | 4 | 248 | 578 | 413 |
| Gibberellic acid | 77-06-5 | 5 | 264 | 553 | 418 |
| 1,1,1-Trichloroethane | 71-55-6 | 5 | 263 | 906 | 469 |
| Benzene | 71-43-2 | 5 | 536 | 1219 | 994 |
| *Ethylene glycol | 107-21-1 | 3 | 585 | 2065 | 1185 |
| *2,6-Diethylaniline | 579-66-8 | 8 | 1311 | 2624 | 1804 |
| 2-Ethylhexyl acrylate | 103-11-7 | 4 | 685 | 4200 | 2586 |
| Tris(nonylphenyl)phosphite | 26523-78-4 | 13 | 374 | 3699 | 2950 |
| Glycerol | 56-81-5 | 3 | 2705 | 4634 | 3436 |
| Glycerol triacetate | 102-76-1 | 8 | 752 | 5720 | 3744 |
| Tripotassium Citrate | 866-84-2 | 1 | 3837 | 3837 | 3837 |
| 1,2-Benzenedicarboxylic Acid | 68515-48-0 | 6 | 2715 | 5620 | 3901 |
| Di-"isodecyl" phthalate | 26761-40-0 | 6 | 2976 | 6145 | 4226 |
| Tween 20 | 9005-64-5 | 16 | 752 | 8970 | 4759 |
| 2-(2-Butoxyethoxy)ethanol | 112-34-5 | 15 | 752 | 7389 | 4854 |
| Triethanolamine | 102-71-6 | 1 | 9307 | 9307 | 9307 |
| Sodium bicarbonate | 144-55-8 | 0 | NA | NA | NA |
| Urea | 57-13-6 | 0 | NA | NA | NA |

*Officially classified as Acute Tox. 4/H302 – harmful if swallowed in Annex VI of the Regulation (EC) No. 1336/2008 (EU, 2008a). OECD's QSAR Toolbox is the metabolism simulator (rat liver S9 metabolism profiler).