Progress Report: ACuteTox

Annette Kopp-Schneider [(1)]
Pilar Prieto [(2)]
Agnieszka Kinsner-Ovaskainen [(2)]
Sven Stanzel [(1)]

[(1)] **Div. Biostatistics, German Cancer Research Center Heidelberg, Germany**

and

[(2)] **In Vitro Methods Unit/ECVAM, IHCP, European Commission Joint Research Centre, Ispra, Italy**

- Title: Optimisation and prevalidation of an *in vitro* test strategy for predicting human acute oral toxicity

- Integrated Project of the 6th Framework Programme of the European Commission – **www.acutetox.eu**

- 35 Partners from 13 European states: Universities, SME, Research Institutes, Industries, Foundations, JRC

- Start: January 2005; End: June 2010

Objectives

- Improving *in vitro* science: to develop and prevalidate an *in vitro/in silico* testing strategy to predict human acute oral toxicity (identification of alerts as indicators for specific organ toxicity)

- Applied objective: to use the proposed testing strategy to classify chemicals into official acute oral toxicity categories.

Basic Concepts

- Rat toxicity as a surrogate for human toxicity

- Acute rat toxicity assessed by rat oral LD50,

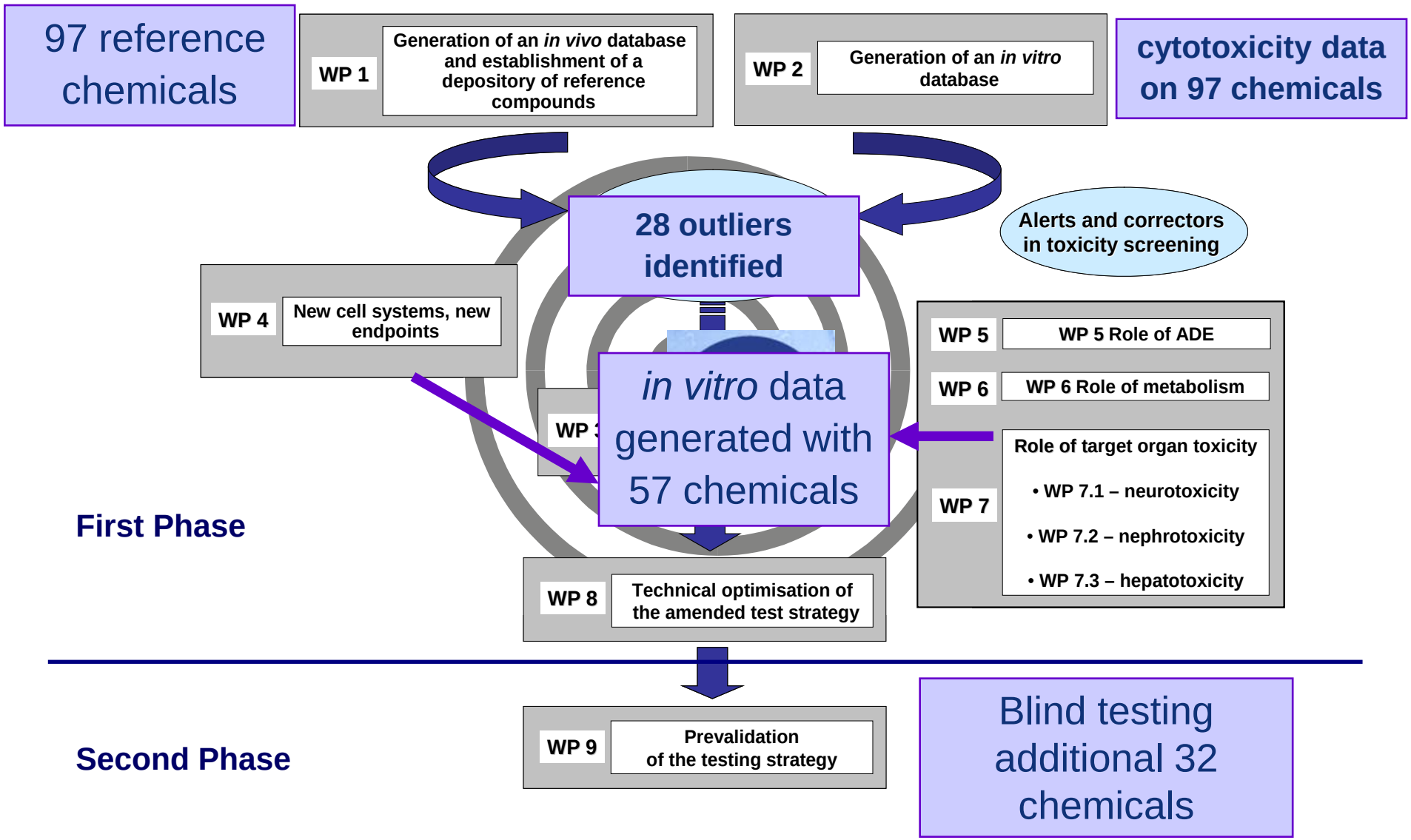
  use of acute oral toxicity categories

  EU CLP 1: LD50 ≤ 5 mg/kg
  EU CLP 2: 5 mg/kg < LD50 ≤ 50 mg/kg
  EU CLP 3: 50 mg/kg < LD50 ≤ 300 mg/kg
  EU CLP 4: 300 mg/kg < LD50 ≤ 2000 mg/kg
  Not classified under the EU CLP system (NC): LD50 > 2000 mg/kg

- Concentration-response experiments performed in *in vitro* assays

- Characteristic value for compound in assay endpoint reflected by e.g. IC/EC50

Project Phases



Organizational setup

First Phase: Optimization

Selection of test methods considered as candidates for building blocks of final testing strategy

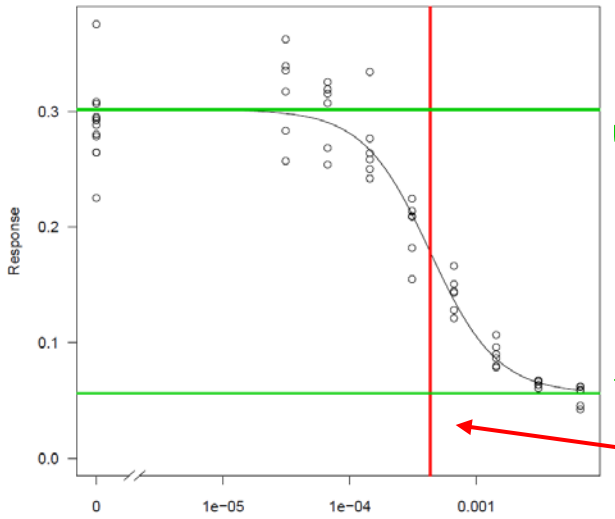
- 26 *in vitro* assays including 71 endpoints
- 57 compounds tested
- January 2005 - December 2009

Statistical Concentration-Response Analysis Strategy

Step 1: Assessment of relevance of effect
If sample size is large enough then use ANOVA,
else use other criterion to assess relevance of effect. Stop if no effect.

Step 2: Model fitting using a 4-parameter log-logistic model
Determine characteristic value: IC/EC50 or LOEC

$$f(\text{conc}) = c + \frac{d - c}{1 + e^{b(\log(\text{conc}) - e)}}$$



b: Hill slope
(relates to slope of curve in EC50)

d: upper asymptote

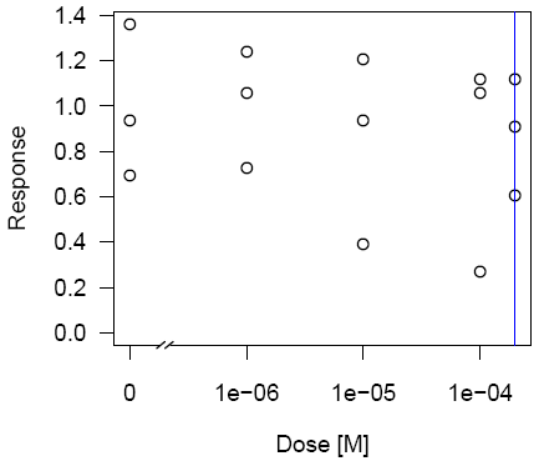
c: lower asymptote

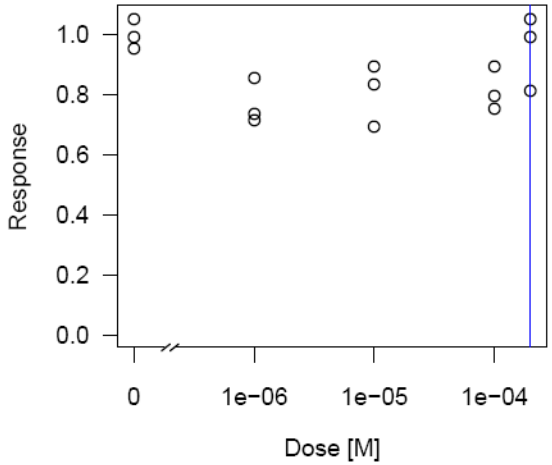
e: log(EC50)

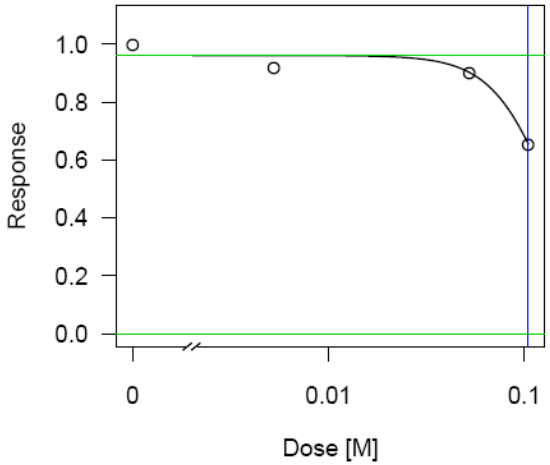
Statistical Concentration-Response Analysis Strategy

If no effect observed: Report characteristic value **> maximum concentration**

in statistical terminology: characteristic value is right-censored







Data matrix for statistical classification analysis

Result from concentration-response analysis

For 57 chemicals and 71 endpoints:

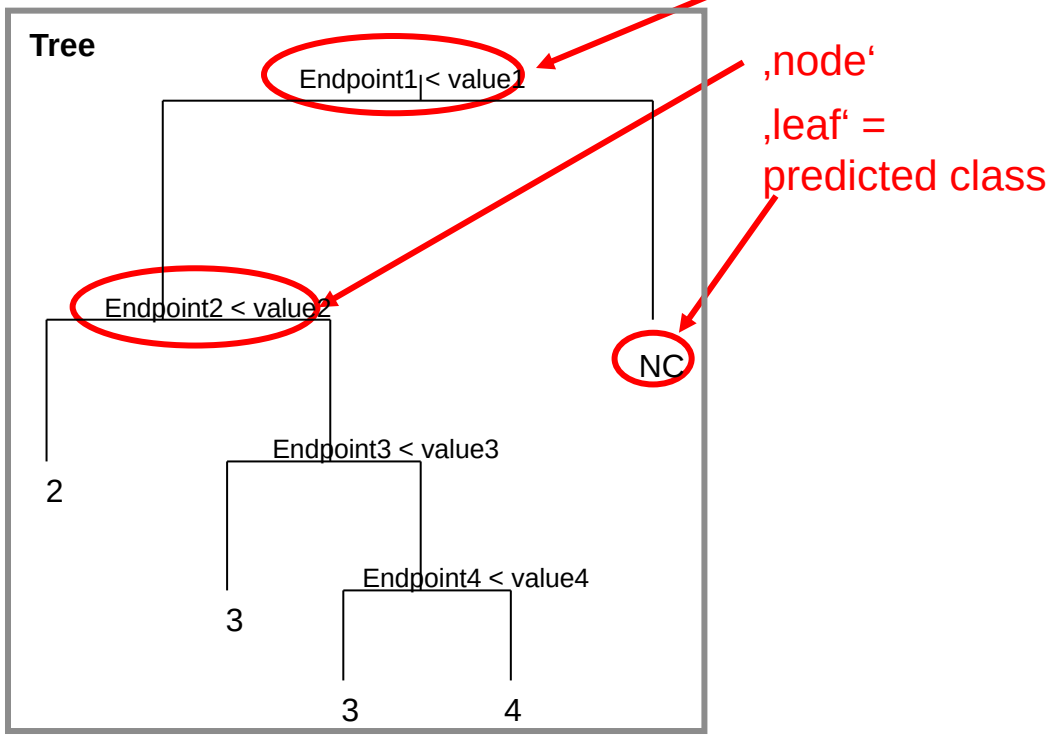
IC/EC50 or LOEC estimate summarized from all experiments of chemical x endpoint combination

5			Mean 3T3/NRU		SH-SY5Y/8-oxoG nuc. EC20 RMFI		SH-SY5Y/8-oxoG mit. EC20 RMFI	
6	Chemical	GHSclass (EU)	Preval1	cens1	Preval2	cens2	Preval3	cens3
7	17a-ethynylestradic	4	2.055017e-05	0	1.5e-04	1	1.5e-04	1
8	2.4-dichlorophenox	4	1.21649e-03	0	1.477767e-01	0	2e-01	99
9	5-fluorouracil	3	9.075736e-07	0	6.3e-04	1	6.3e-04	1
10	Acetaminophen	5	2.980966e-04	0	2.25e-02	1	2.25e-02	1
11	Acetonitrile	5	1.764001e-01	0	1.24516e-03	2	1.2e-02	99
12	Acetylsalicylic_aci	4	2.985553e-03	0	4.067141e-05	0	1e-03	1
13	Acrylaldehyde	2	3.677934e-04	0	1.275375e-07	2	1e-05	1
14	Amiodarone_hydroc	5	2.493740e-05	0	3.360453e-05	2	1e-06	99
15	Amitriptyline_hydro	4	2.2051e-05	0	3e-08	99	1e-05	1

Statistical classification of chemicals based on *in vitro* assays: Classification and Regression Trees (CART)

- Nonparametric statistical method for classification into EU CLP acute oral toxicity classes
- Recursive method
- In every node: identification of best endpoint /split-point regarding classification
- Stop when no improvement is obtained

Outcome:



Confusion Table

	True toxicity class				
Predicted toxicity class	1	2	3	4	NC
1	0	0	0	0	0
2	0	8	3	2	3
3	3	2	7	1	1
4	0	0	2	14	2
NC	0	0	0	0	8

Statistical classification of chemicals based on *in vitro* assays: Random Forests

- Collection of CART trees, e.g. 10 000 CART trees
- Every tree is built for a bootstrap sample (= sample with replacement) of compounds
- Every node in a tree is built using a random subset of endpoints
- Prediction for compound: Get predicted class for every tree, take majority vote
- Outcome:

  - ‚Endpoint Importance Plot‘: For every endpoint determine how many trees contain this endpoint
  - ‚Black box‘ containing 10 000 trees used for prediction
  - Confusion table

Assessing the performance of classifiers

- Classifier:

    1. classification method (e.g. CART, Random Forests,…)

        and

    2. endpoints included in the classification model

- Classifier built on basis of training set of compounds (n=57)
- Confusion table: predicted vs. true class → correct classification rate (CCR)
- Caution: CCR based on training set are much too optimistic!
- Use bootstrap techniques to estimate CCR (training set)

Selection of *in vitro* assays for Second Phase by exclusion criteria

Level 1: Exclusion from classification analysis

- Poor quality of raw data reporting
- Many missing chemicals

Level 2 : Exclusion from prevalidation study

- Poor performance in Univariate CART analysis
- Multivariate CART results not affected by removing the assay
- Too many censored values

Organizational setup

Second Phase: Prevalidation

Assess the predictive capacity of proposed testing strategies

- 10 *in vitro* assays including 36 selected endpoints:

  (1) Neutral Red Uptake (NRU) cytotoxicity assay in Balb/3T3 cells

  (2) Cytomic Panel for: Oxidative Stress and Cytotoxicity Screening in HepG2, SH-SY5Y, A704

  (3) Whole blood assay of inflammatory cytokine secretion (IL-1, TNF-α and IL-6)

  (4) CFU-GM Assay from Human Cord Blood Cells

  (5) MTT assay in primary rat hepatocytes

  (6) Gene expression (GFAP, HSP-32, MBP, HF-H) and uridine incorporation in primary rat brain aggregates

  (7)) Estimation of blood-brain barrier passage using neuronal networks

  (8) Human Plasma Protein Binding

  (9) Metabolic stability (human and rat)

  (10) Caco-2 permeability (intestinal absorption)

- Blind testing of 32 compounds
- January 2010 - June 2010

Assessing the performance of classifiers (2)

ACuteTox test set in existence (n=32 compounds from prevalidation phase)

- Develop classifier on training set (57 chemicals used in the optimisation phase)

- Choose best classifier on basis of training set and estimated CCR

- Evaluate performance of classifier on test set

Set-up for classifier selection

- 36 endpoints from 10 assays identified in optimisation phase

- Reduce number of official acute oral toxicity classes:

  EU CLP toxicity cat. 1-3: $LD_{50} \leq 300$ mg/kg

  EU CLP toxicity cat. 4: $300 \text{ mg/kg} < LD_{50} \leq 2000$ mg/kg

  EU CLP Not Classified (NC): $LD_{50} > 2000$ mg/kg

- IC/EC50, LOEC in μg/ml

- Results shown for compounds with logP ≤ 5

  54 of 57 in training set

  27 of 32 in test set

Random Forests Model with 9 endpoints

Endpoints preselected on basis of statistical and toxicological considerations:

- CFU-GM assay
- NRU assay in 3T3 cells
- Lowest gene expression in primary rat brain aggregates
- HSP-32 mRNA expression in rat brain aggregates
- NF-H mRNA expression in rat brain aggregates
- Lowest EC in cytomic panel performed in SH-SY5Y cells
- MMP measurement in HepG2 cells
- MTT assay in primary rat hepatocytes
- IL-1β release in freshly isolated human whole blood

Confusion table for test compounds:

	True toxicity class		
Predicted toxicity class	**1-3** < 300 mg/kg	**4** 300 - 2000 mg/kg	**NC** > 2000 mg/kg
1-3	**3**	**4**	**4**
4	**2**	**4**	**4**
NC	**0**	**0**	**6**

Underpredicted toxicity:

Brucine, Paraquat

Summary and final remarks

- Toolbox of *in vitro* methods with associated optimised protocols
- Complementing the 3T3/NRU assay with specific *in vitro* assays is not improving significantly the classification of compounds in acute oral toxicity categories 1 to 4
- Rodent LD_{50} subject to large variations, i.e. toxicity class may not be the ‚truth'
- The estimation of the oral dose by including kinetic parameters needs to be further evaluated
- Several *in vitro* assays have proved to be useful to identify alerts for tissue specific toxicities → Statistical classification analysis for tissue-specific toxicity