ACuteTox: Optimisation and pre-validation of an in vitro test strategy for predicting human acute toxicity

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and the ACuteTox consortium

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Integrated project under the EU Commission 6th framework programme for research

Start: January 2005; extended to July 2010

35 Partners from 13 European countries:
Universities, SME, research institutes, industries, JRC

Aim:
Develop and pre-validate a simple and robust ***in vitro*** **testing strategy for prediction of human acute toxicity** –replace animal tests for regulatory purposes

Background

MEIC-Multicentre Evaluation of in vitro Cytotoxicity tests

- Initiated by: Björn Ekwall 1989-1999
- 100 labs/200 *in vitro* test methods/50 chemicals
- in vitro IC50 vs human LC

EDIT–Evaluation-guided development of in vitro test batteries

- Complement MEIC test battery with *in vitro* tests for kinetics and organ specificity

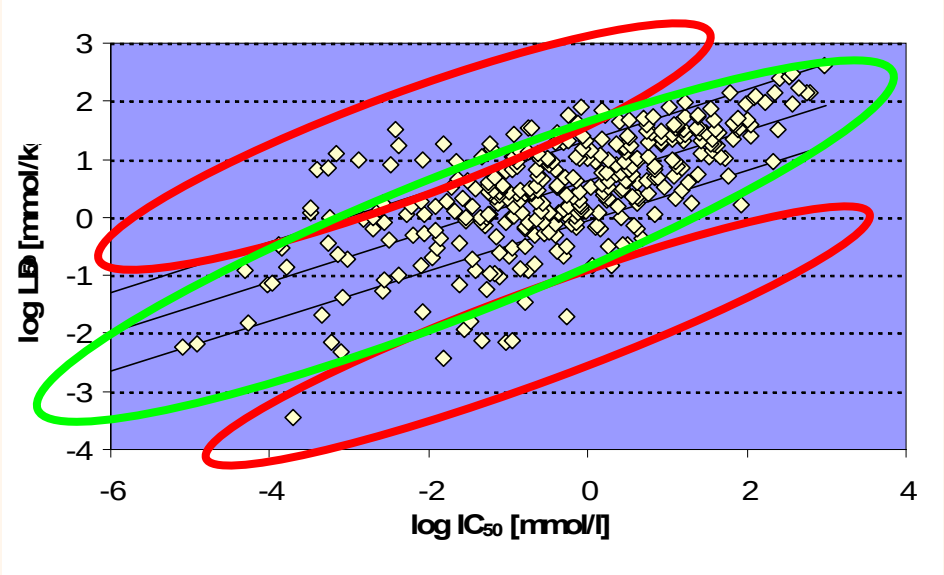
Registry of Cytotoxicity

- Database on LD50 values and IC50 values for ~550 chemicals

ECVAM/ICCVAM validation study of 2 basal cytotoxicity assays

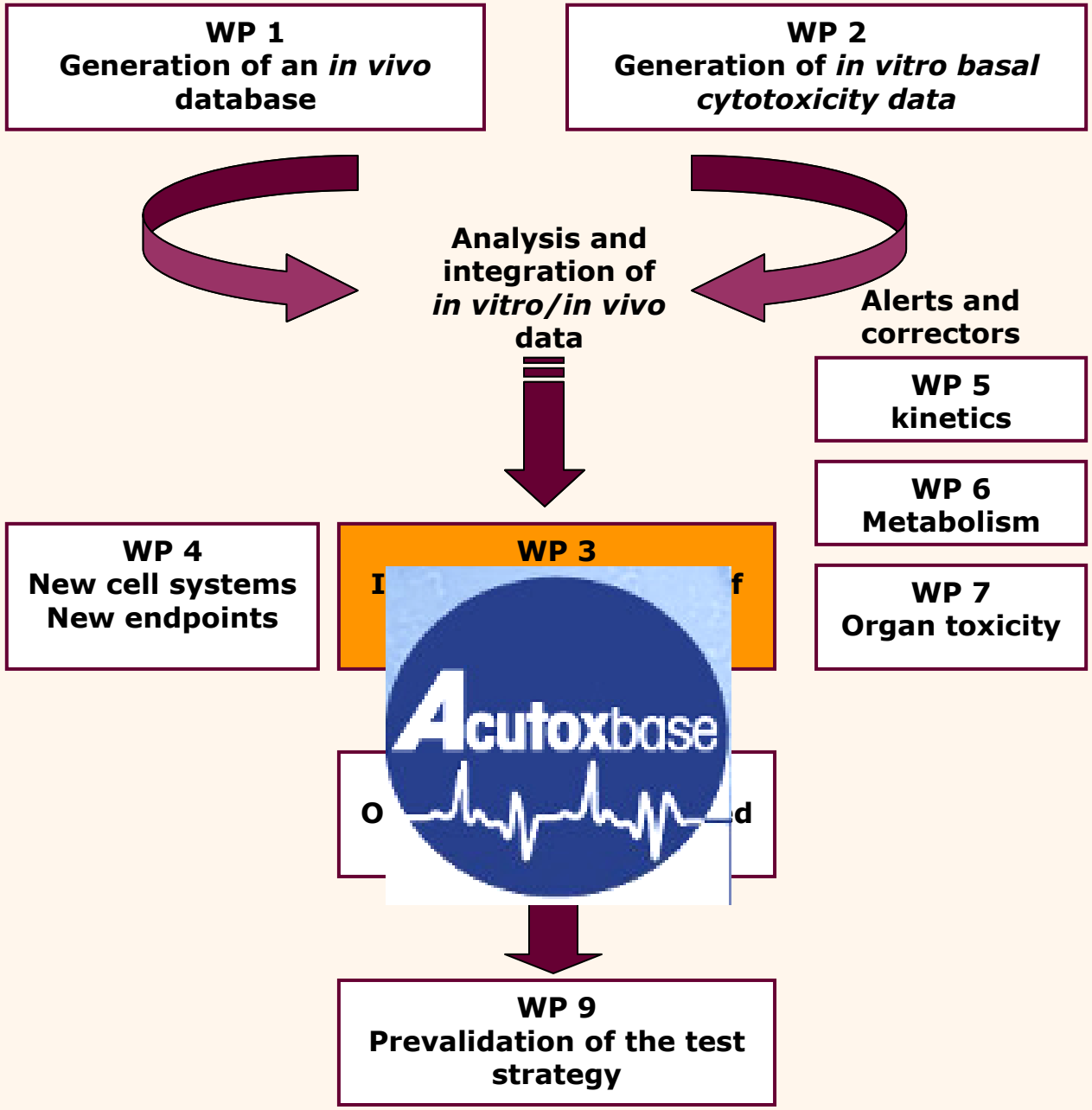
- 72 chemicals
- BALB/c 3T3 and normal human keratinocytes/NR uptake

Background conclusion



- Relatively good prediction (up to 70%)
- Certain number of misclassifications

Aim of ACuteTox: Improve the *in vitro-in vivo* correlation by evaluating existing outliers in order to introduce further parameters (ADE, metabolism, organ specificity) which might improve the correlation.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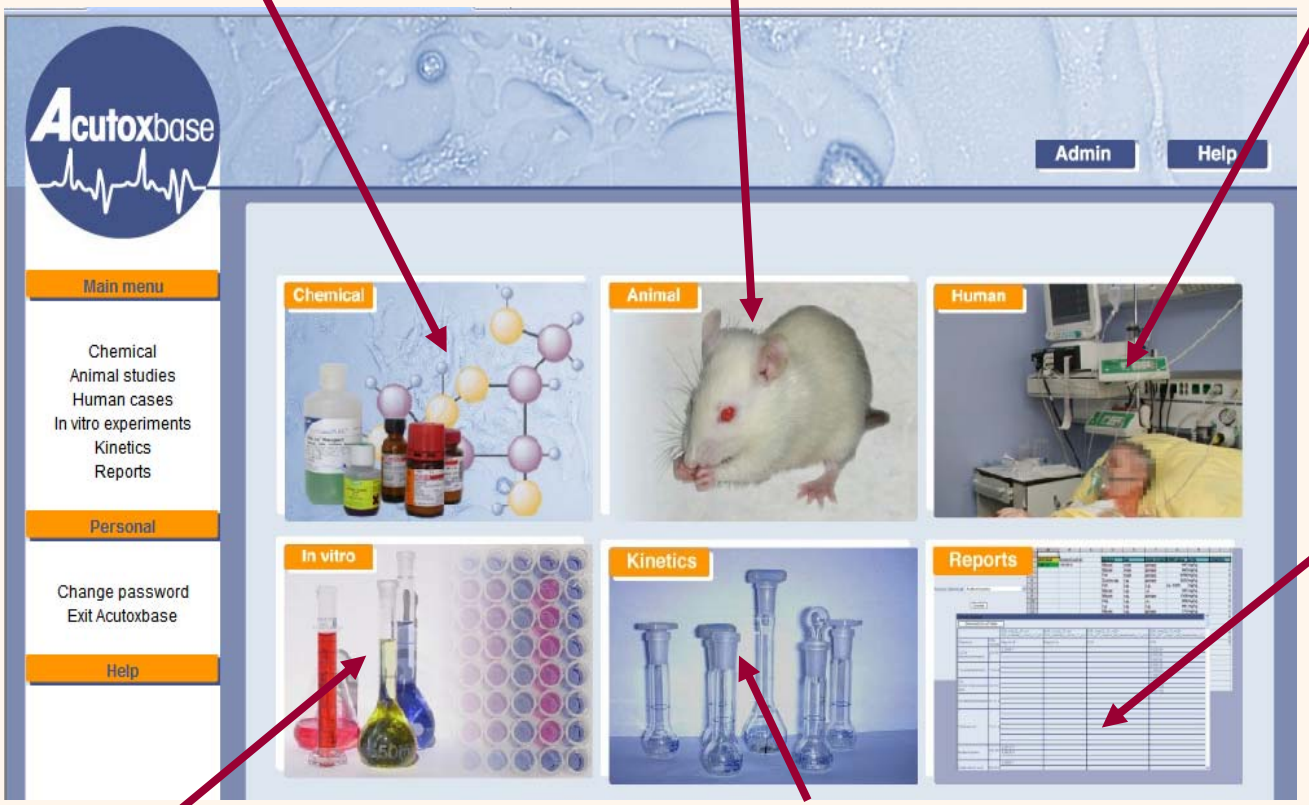
97 reference chemicals

animal in vivo data

human data

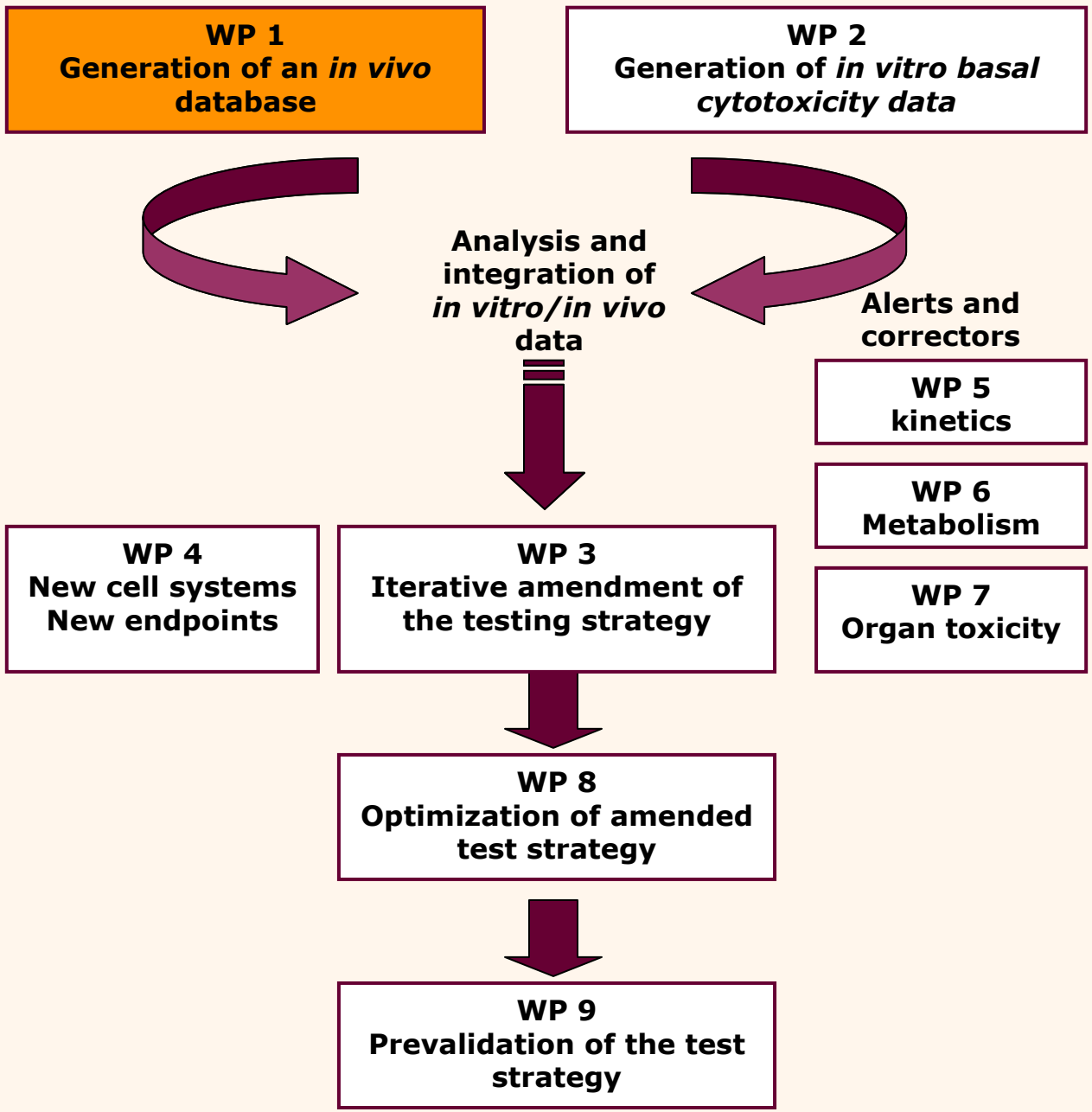


Summary data, > 100 SOPs

in vitro data

biokinetics (in vitro, in silico)

Kinsner-Ovaskainen et al. 2009, Toxicol In Vitro 23: 476-485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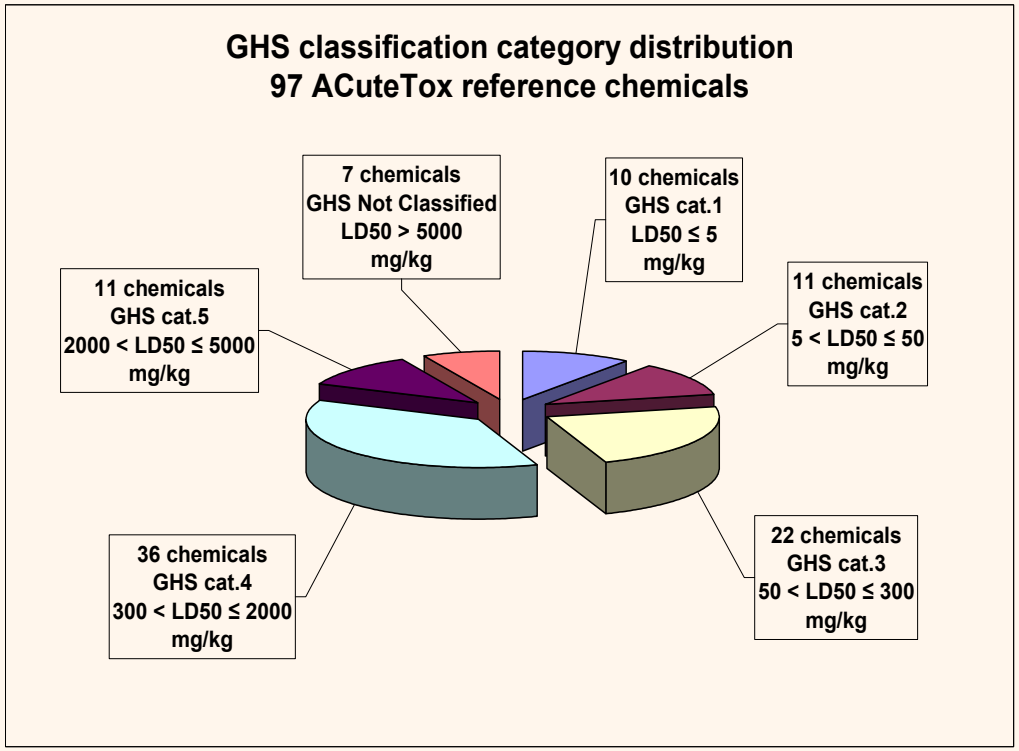
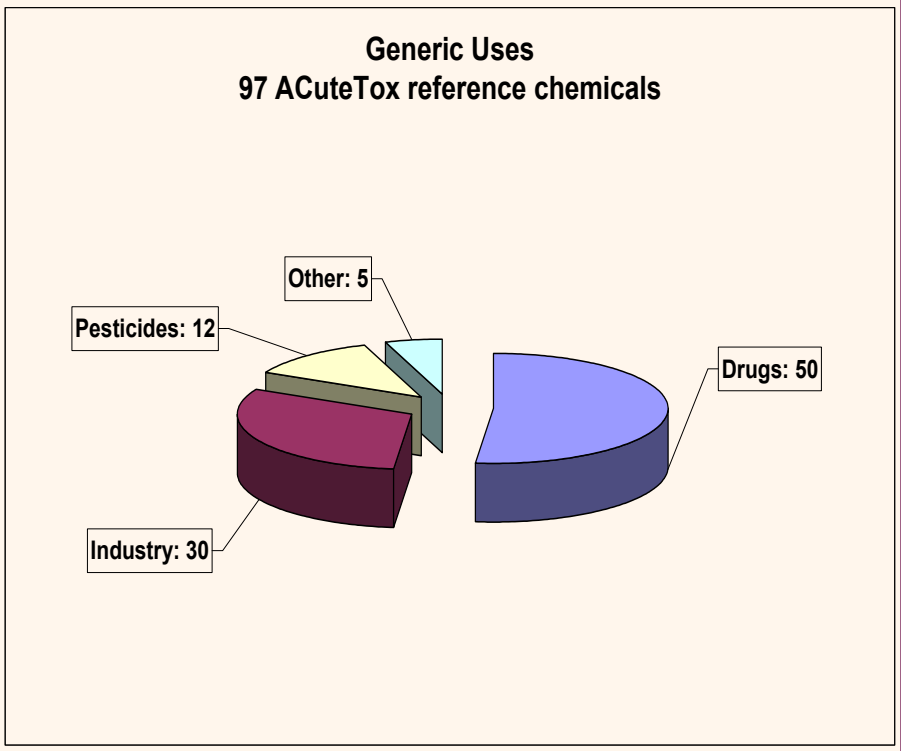
WP1: The In vivo database

- Selection of reference chemicals
- Generation of the *in vivo* database: LD50 values from 2206 animal studies; human data from 2902 cases reports





WP1: LD50 data & Chemicals: criteria for data reduction/selection

- Only LD50 data cited with common unit (mg/kg) selected
- Only LD50 data cited as finite numbers selected
- Of regulatory significance:
  - Focus on rat and mouse data (~40% each, of full dataset)
  - Only oral/gavage dose route analysed
- Chemicals < 3 oral LD50's excluded (unreliable for statistical evaluation)

	rat	*mouse*
Total number of LD50 studies	921	907
Oral studies (total)	601	377
Oral studies (> 2 LD50 values per chemical) (number of eligible chemicals)	**504 (62)**	**300 (51)**

WP1: Evaluation of *in vivo* human data – calc. of LC50 values

View cases

Case type: Sub-lethal acute poisoning (single dose): Clinical observations (time related)
Chemical (CAS): Acetaminophen (103-90-2)

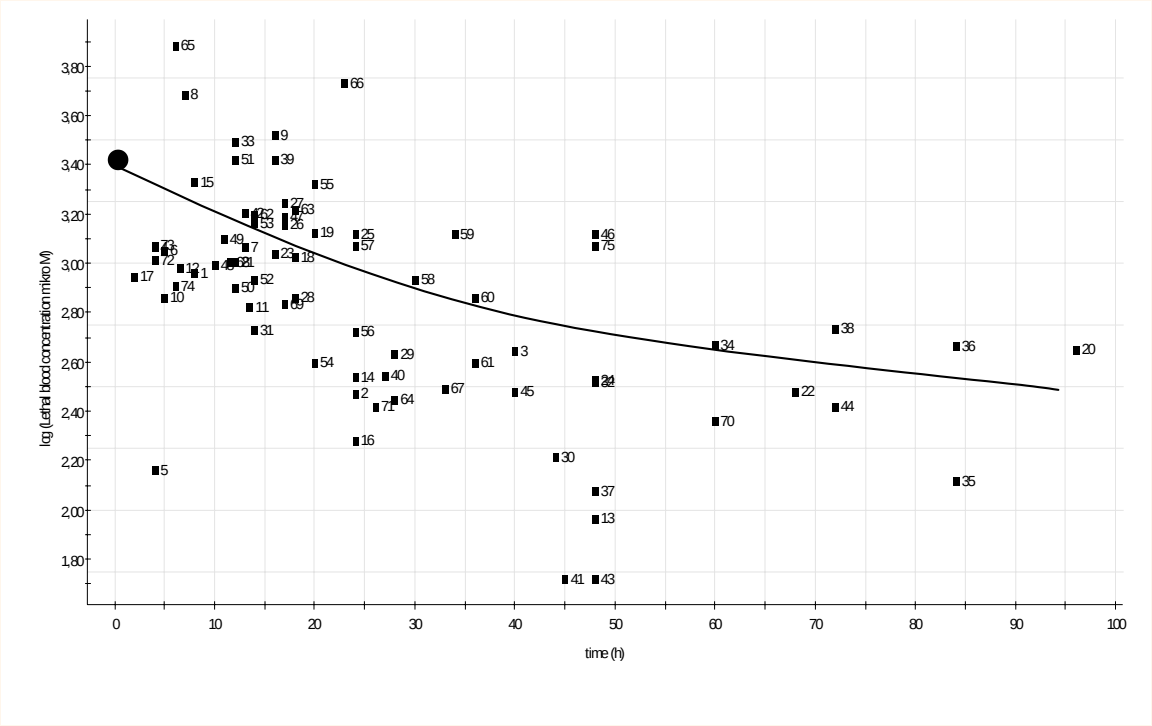
Reference (linked to full source)	Case age/sex	Case category	Dose: g	Notes (case, dose, time)	Time (exposure to sampling): h	Notes (blood sample)	Blood conc.: (mg/l)	Blood conc.: (µM)	Metabolite Blood conc.: (mg/l)	Metabolite Blood conc.: (µM)	Symptoms and signs	Treatment	Time (exposure to recovery): h
SPC 1957	15F	S	20		24		206	1362			0h: C, L	NAC	
SPC 1976:5	17F	S	17.5		4		284	1878			0h: V, MS	MT	
					7		82	542					
SPC 1976:6	24F	S	24		2		484	3200			0h: MS	MT, CA	
					5		150	992					
					9		90	595					
					16		15	99					

The database contains human acute toxicity data from a single poisoning, consisting of:

- **sub-lethal blood concentrations**
- **lethal blood concentrations**
- **post-mortem blood concentrations**

WP1: Estimation of LC50 human

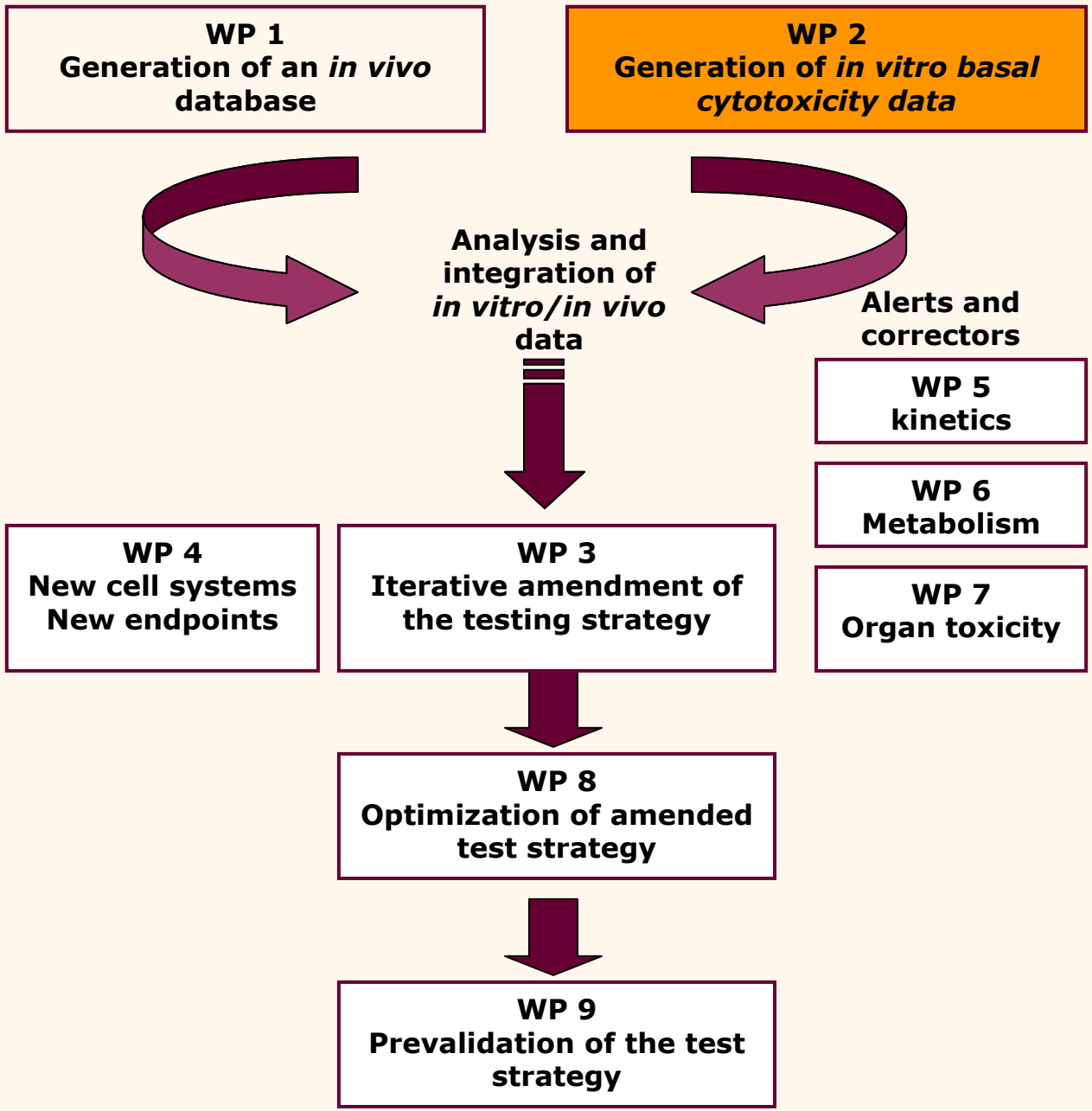
Example: Acetaminophen approximate LC0 and LC100 and LC50



LC100 = 3.40
LC0 = 3.35

LC50 = (3.35+ 3.40)/2= 3.37 in microM
Converted to M LC50=-2.63

Sjöström et al. (2008) Toxicology In Vitro, 22: 1405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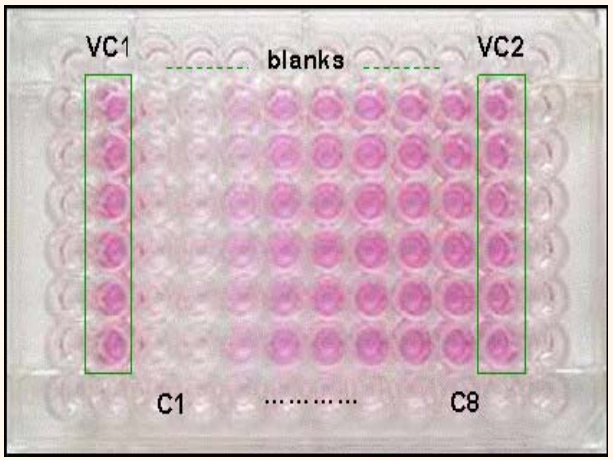
WP 2: Generation of In vitro basal cytotoxicity data

- **Assessment of Basal cytotoxicity on:**

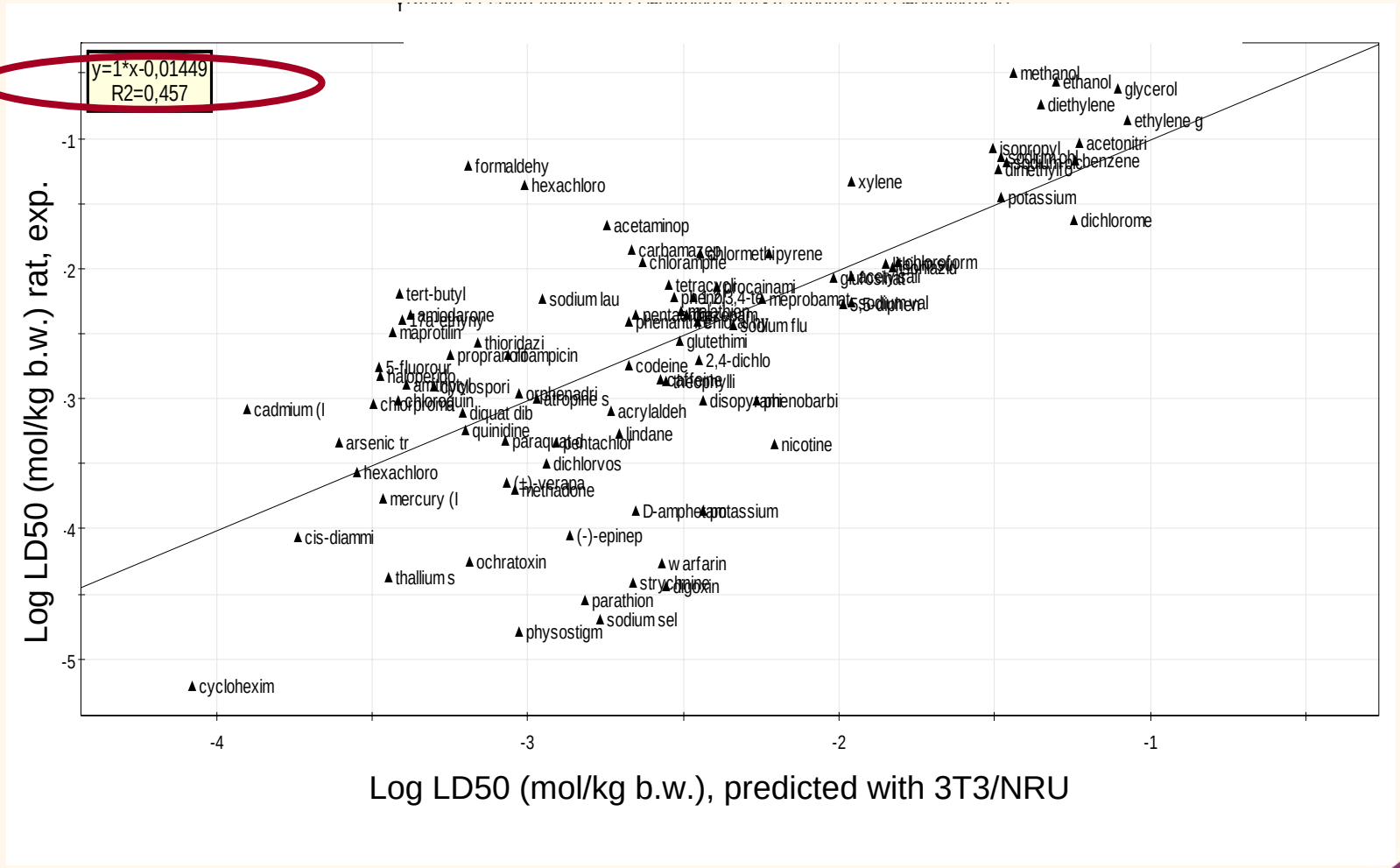
3T3 (NRU)
NHK (NRU)
HL-60 (ATP content)
Fa32 (NRU, total protein)
Hep-G2 (NRU, total protein)



Generation of an *in vitro* database for 97 selected reference chemicals

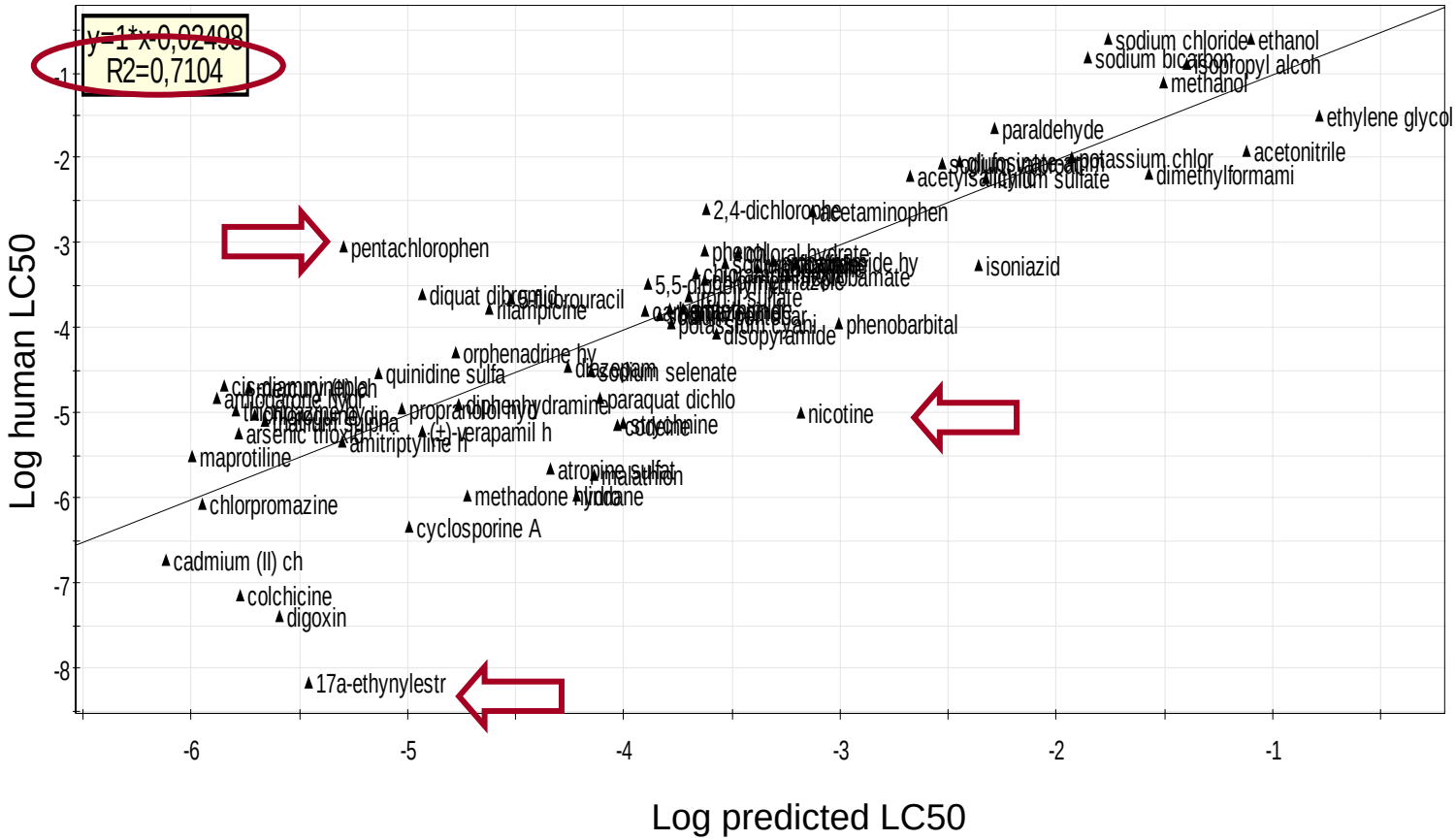
CONCLUSIONS:
All the basal cytotoxicity tests showed similar information i.e. similar ranking; the validated 3T3/NRU seems to be the best candidate

Plot observed rat vs predicted LD50
from in vitro 3T3/NRU, PLS regression analysis



Clothier et al. (2008) ATLA, 36: 503

Plot observed LC50 humans vs predicted *in vitro* variables



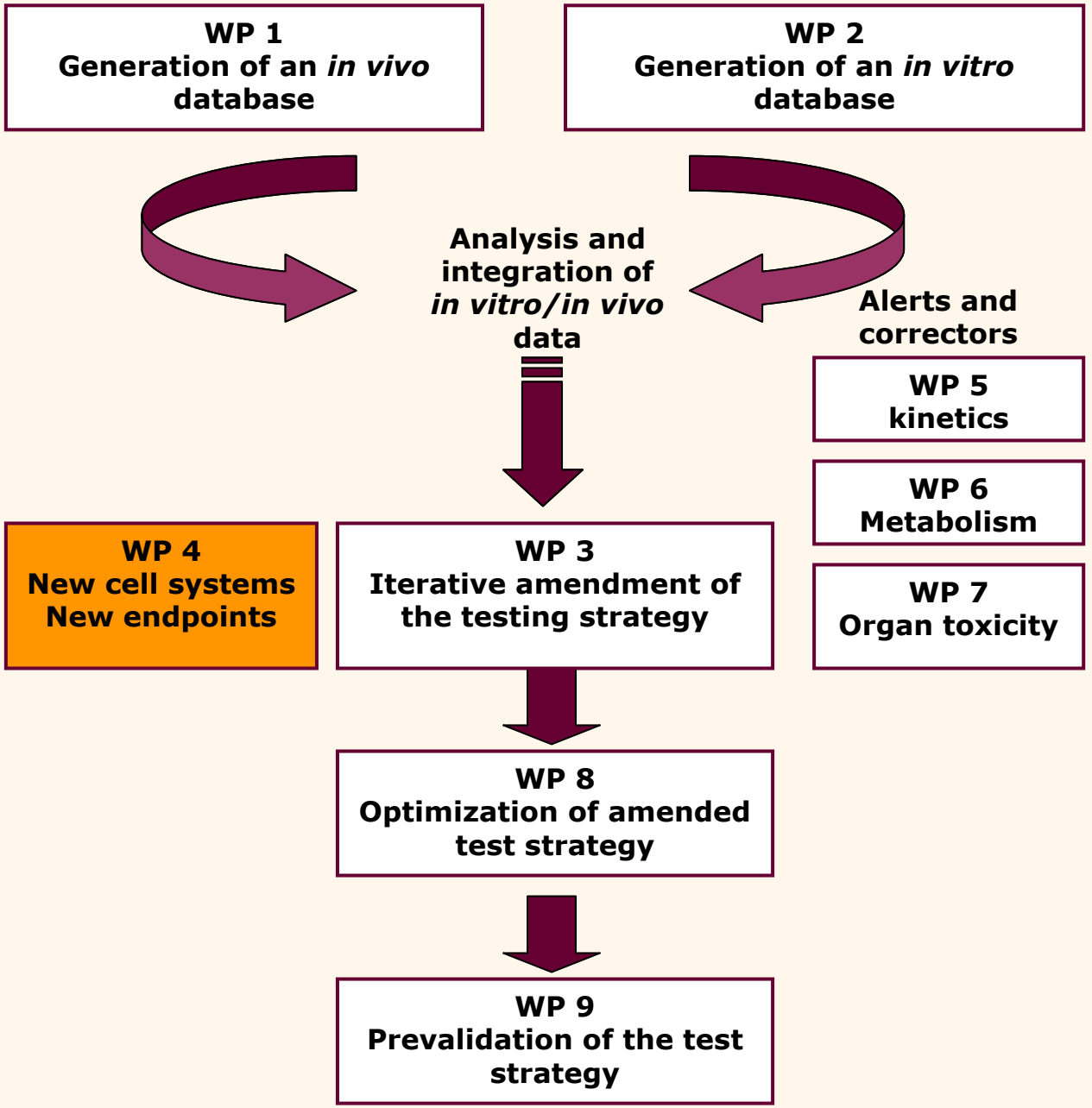
Chemicals with poor human data

03-atropine sulfate monohydrate
08-diazepam
13-pentachlorophenol
14-phenobarbital
21-cadmium chloride
28-amiodarone hydrochloride
30-rifampicine
41-glufosinate ammonium
47-17α-ethynylestradiol
51-dimethylformamide
56-phenol
67-warfarin
89-chlorpromazine hydrochloride
90-paraldehyde
33-nicotine
34-lindane
91-sodium selenate
92-acetonitrile
93-sodium bicarbonate
84-diphenhydramine
85-chlormethiazole
87-procainamide hydrochloride
57-sodium chloride

Summary: Identification outliers

28 outliers identified
16 comparison IC50 3T3 – LD50 rat
17 comparison IC50 3T3 – LC50 human

57 compounds will be tested in WP4-WP7:
28 outliers
29 non-outliers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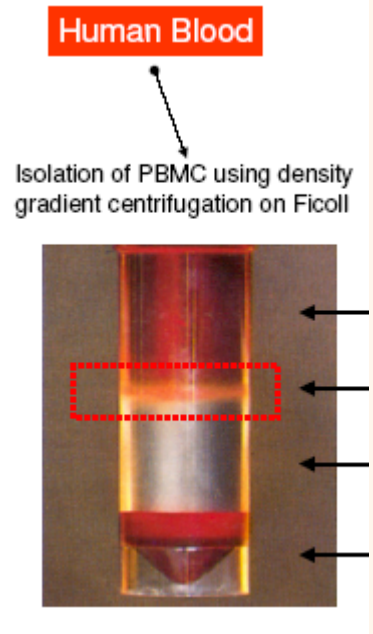
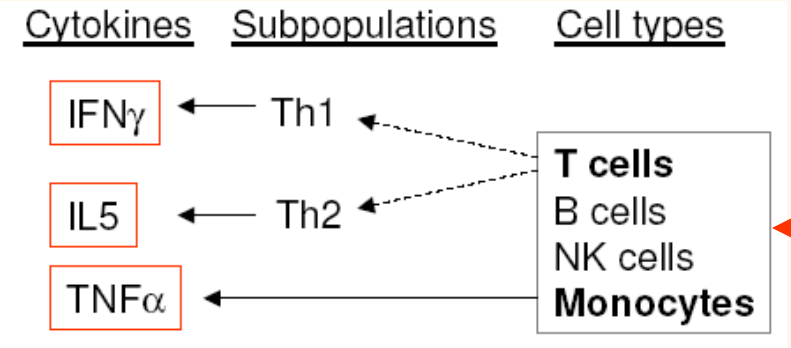
WP4: Cytokine secretion and hematopoiesis



Human cord blood cells

CFU-GM

Colony forming unit-granulocyte/ macrophage



Human peripheral blood mononuclear cells

Good correlation with the rat oral LD50 values (R2 = 0.84 and R2 = 0.86)

WP 4: Other assays showing promissing results

Cytomic panel for cytotoxicity screening including:

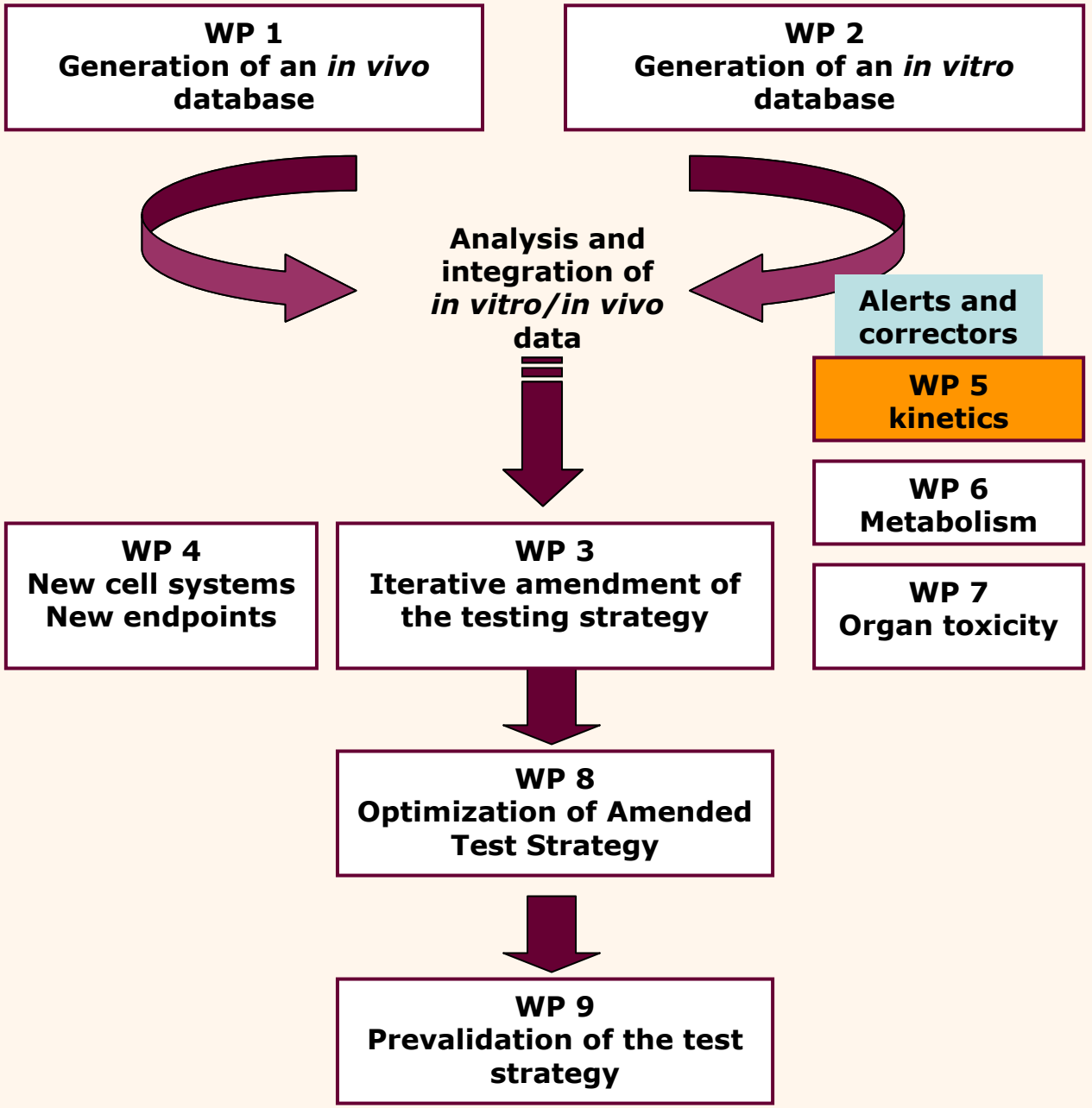
- Intracellular Ca2+ (Fluo-4 probe)
- Mitochondrial membrane potential (rhodamine123)
- Plasma membrane potential (DIBAC probe)
- Intracellular lipid content (BODIPY probe)

Cytomic panel for oxidative stress screening including:

- Intracellular peroxides
- Mitochondrial generation of superoxide
- Intracellular levels of the oxidized DNA base 8-oxo-guanine

Cell lines:

A.704 kidney adenocarcinoma
HepG2 human hepatoma cell line
SH-SY5Y human neuroblastoma cell line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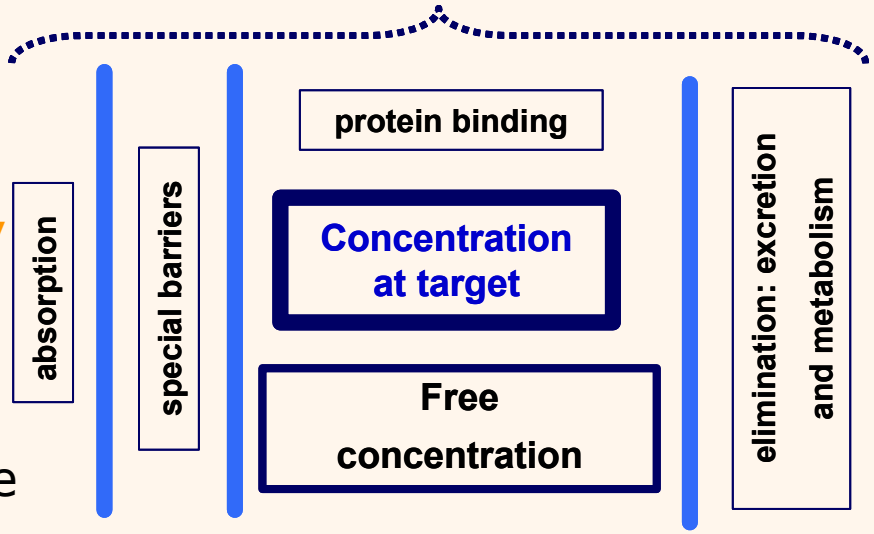
WP5: Role of ADE (in vitro/in silico)

- Measurement of the transport across the **intestinal barrier** and the **blood-brain barrier** using ***in vitro* models** and **neuronal networks**

- Measurement of **protein binding**, **microsomal stability**, **lipophilicity** (n=42)

- Generation **biokinetic model** for the interpretation of *in vitro* toxic concentrations in relation to the *in vivo* acute toxic dose



WP5: Oral absorption

ORAL ABSORPTION MODEL

		HIA		
Chemical	HIApred	Class[a] computer	Class Caco-2	Class Caco-2
Acetaminophen	1.00	H	H	H
Actylsalicylic acid	0.98	H	M	M
Atropine Sulfate	0.71	H	M	M
Caffeine	0.99	H	H	H
Carbamazepine	0.03	P	H	H
Colchicine	1.00	H	P	L/M
Cycloheximide	0.76	H	H	H
Diazepam	0.45	M	H	H
Digoxin	ND	-	M	L/M
Isopropyl alcohol	-0.10	P		
Malathion	0.52	M	H	H
Mercury II Chloride	ND	-		
Pentachlorophenol	1.00	H	H	H
Phenobarbital	0.39	M	H	H
SLS	1.00	H		
Sodium Valproate	1.00	H	H	H

H = High ; HIA > 80 %

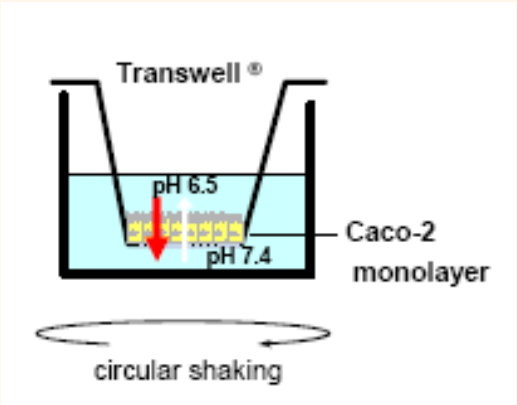
M = Moderate ; HIA < 20-70 %

P = Poor ; HIA < 20 %

Papp 10^{-6}cm/s < 1= Poor (P)

Papp 10^{-6}cm/s< 1 - 10 = Moderate (M)

Papp 10^{-6}cm/s > 10 = High (H)



72% overall accuracy

WP5: Blood-brain barrier

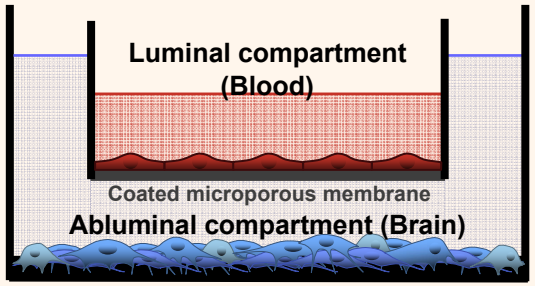
BLOOD-BRAIN BARRIER PASSAGE MODEL

		BBB			
Chemical	LogBBpred	Class[b] P13 computer	Class P35 in vitro	Class P15 in vitro	Exper. Data (logBB)
Acetaminophen	-1.0	P	M	H	-0.31/H
Actylsalicylic acid	-0.6	M	L	M	-0.5/M
Atropine Sulfate	-0.9	P	H	M	
Caffeine	-0.1	H	H	H	
Carbamazepine	0.1	H	H	H	-0.06/H
Colchicine	0.0	H	L	M	0/H
Cycloheximide	-0.9	P	H	M	
Diazepam	-0.5	M	H	M	0.52/H
Digoxin	ND	-	H	-	
Isopropyl alcohol	1.1	H	H	-	-0.15/H
Malathion	-0.2	H	H	M	
Mercury II Chloride	ND	-	H	-	
Pentachlorophenol	-0.1	H	H	M	
Phenobarbital	1.2	H	H	H	0.12/H
SLS	-0.9	P	H		
Sodium Valproate	1.5	H	H	M	-0.22/H

Log BB > -0.7 Poor (P)

-0.7 < Log BB < -0.3 Moderate (M)

Log BB > -0.3 High (H)



73% overall accuracy

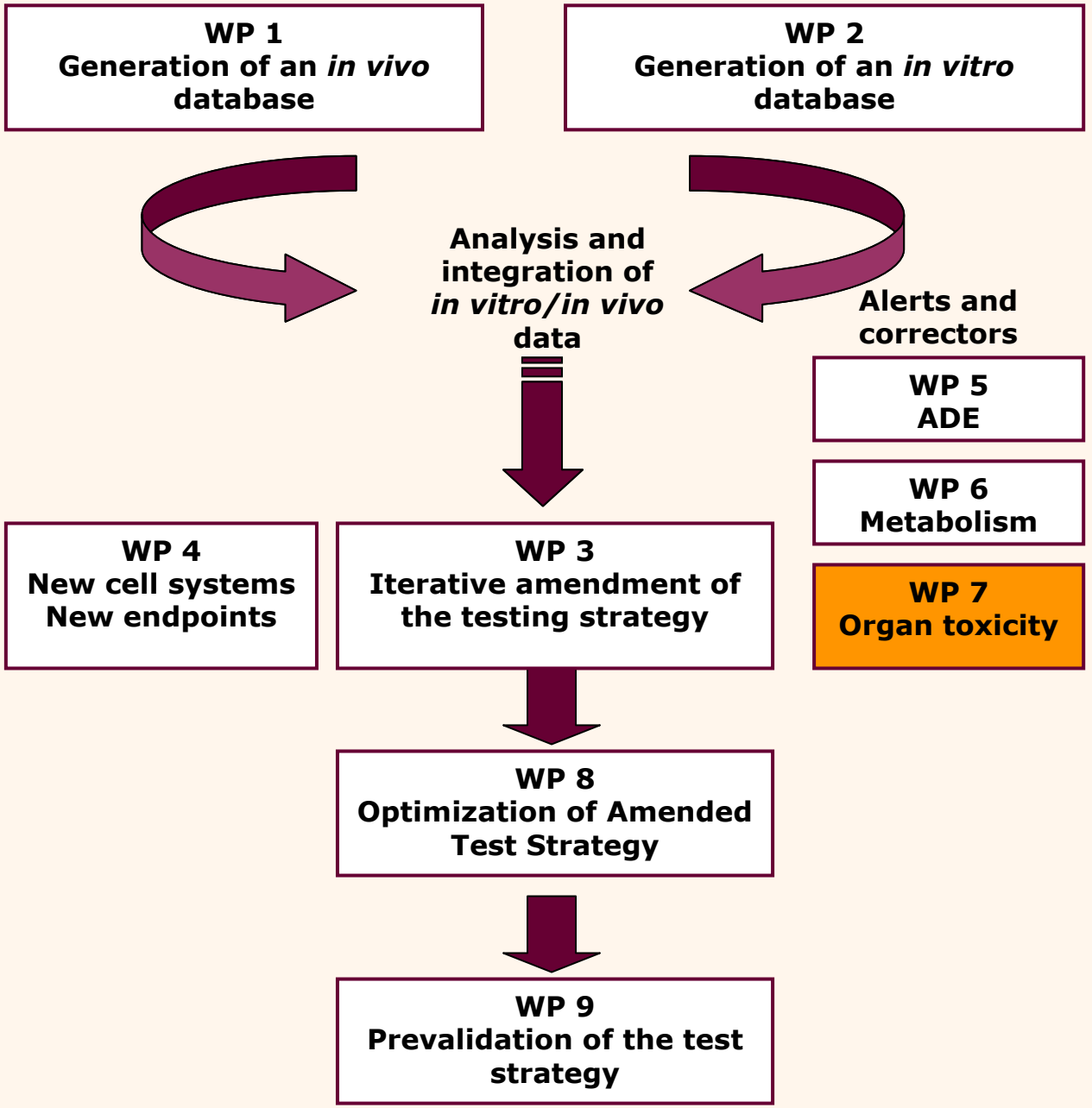
Correction of LD50 values estimated from *in vitro* cytotoxicity by introduction of biokinetics

- Calculation of an **apparent volume of distribution** (Vd), assuming that the total body water volume of a 250 g rat is 170 ml and correcting for 3 factors: lipophilicity, clearance, and protein binding.

- Calculation of the **internal dose** (from IC50 values obtained in 3T3 NRU assay), taking into account the Vd

- Calculation of the **external dose** (estimated LD50) taking into account the oral absorption (calculated from Caco-2 permeability)

The correlation (in mM) improves from **R2 = 0.46 → R2 = 0.63**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WP 7.1: Neurotoxicity

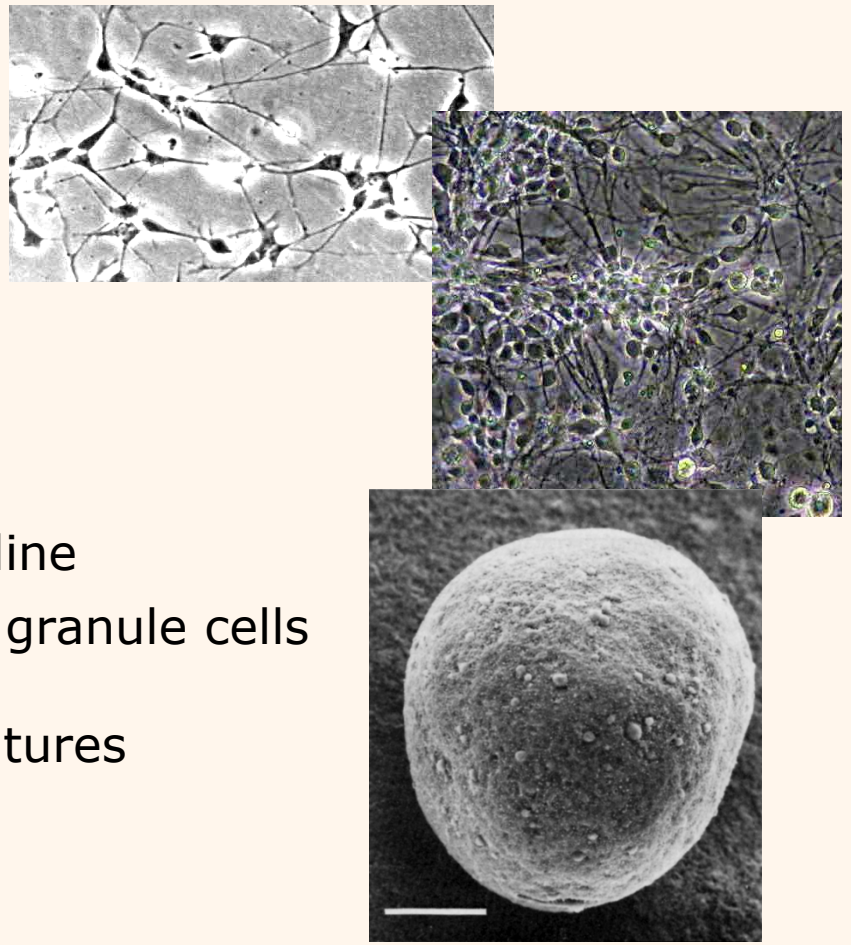
Neurotoxicity test battery (50 endpoints)

- **Basal cytotoxicity**
  Viability (MTT), cell membrane integrity (LDH), total cellular LDH activity

- **General cell physiology**
  energy status, glycolytic activity, Ca2+ homeostasis, cell and mitochondrial membrane potential, oxidative stress (ROS)

- **Neurochemistry**
  Voltage operated ion channels
  Receptor function
  Neurotransmitter synthesis/degradation
  Neurotransmitter uptake
  Neurotransmitter release
  Global electrical activity

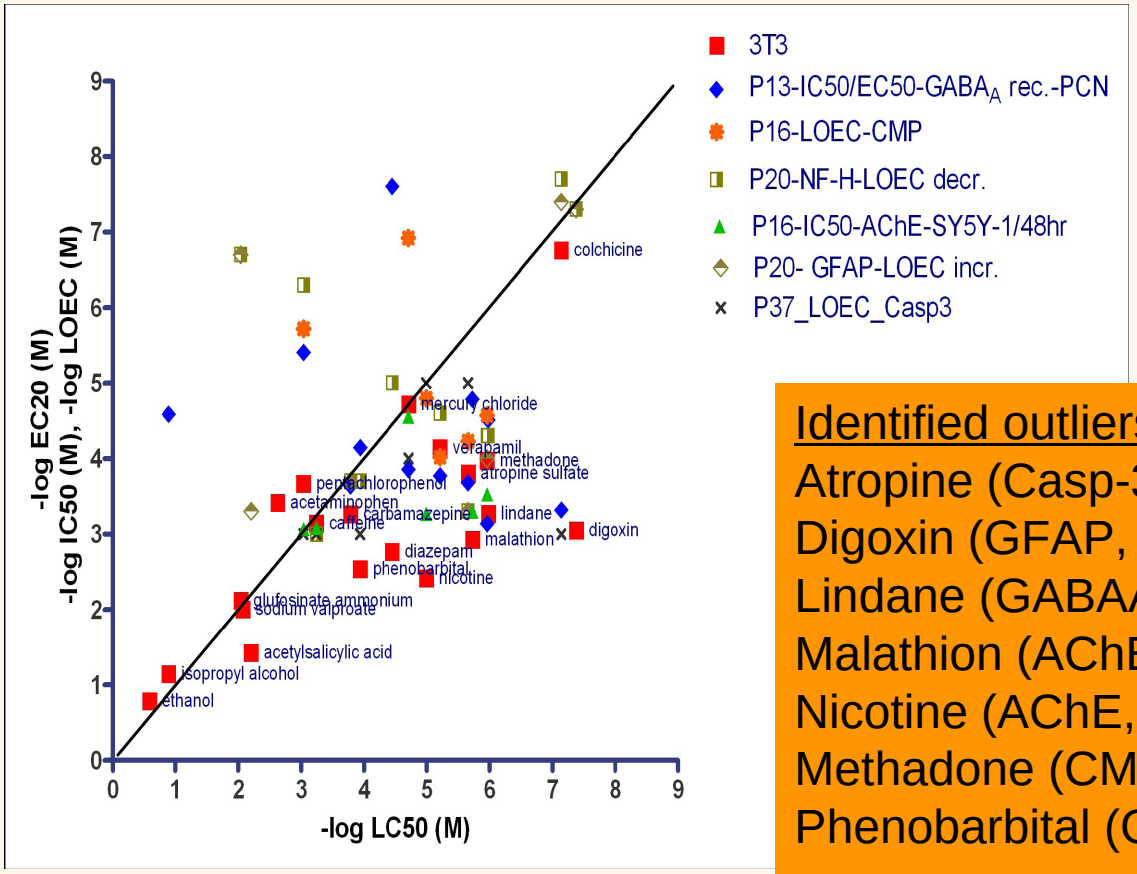
WP 7.1: Neurotoxicity



Modell systems

- Human neuroblastoma SH-SY5Y cell line
- Primary cultures of mouse cerebellar granule cells
- Mixed primary neuronal cultures
- Serum-free aggregating brain cell cultures

Neurotoxicity/3T3 vs. Human LC50



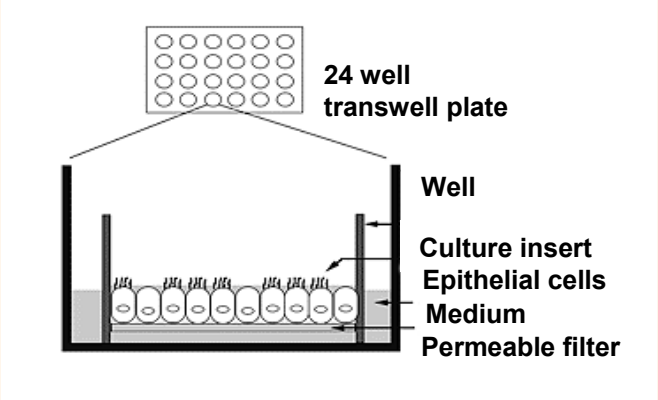
Identified outliers:

Atropine (Casp-3, CMP)
Digoxin (GFAP, NF-H)
Lindane (GABAA-R)
Malathion (AChE, GABAA-R)
Nicotine (AChE, CMP, Casp-3)
Methadone (CMP)
Phenobarbital (GABAA-R)

WP 7.2 Nephrotoxicity

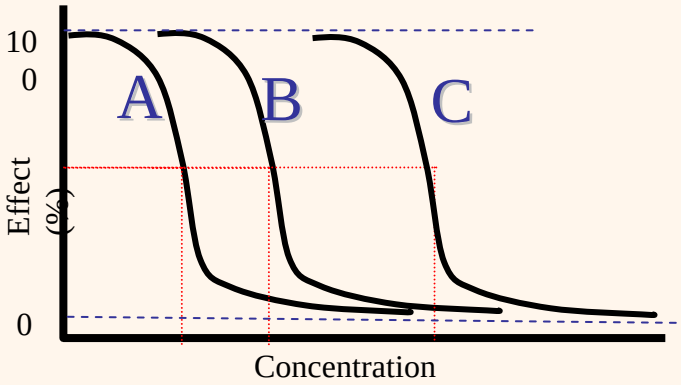
Cells: Renal epithelial cells (LLC-PK_1)
Measurement: Loss of monolayer integrity - Trans epithelial resistance (TER) – compared with Alamar Blue viability test

TER: greater sensitivity for nephrotoxic chemicals. Compounds requiring metabolism (diethylene glycol) did not show toxicity at concentrations used.

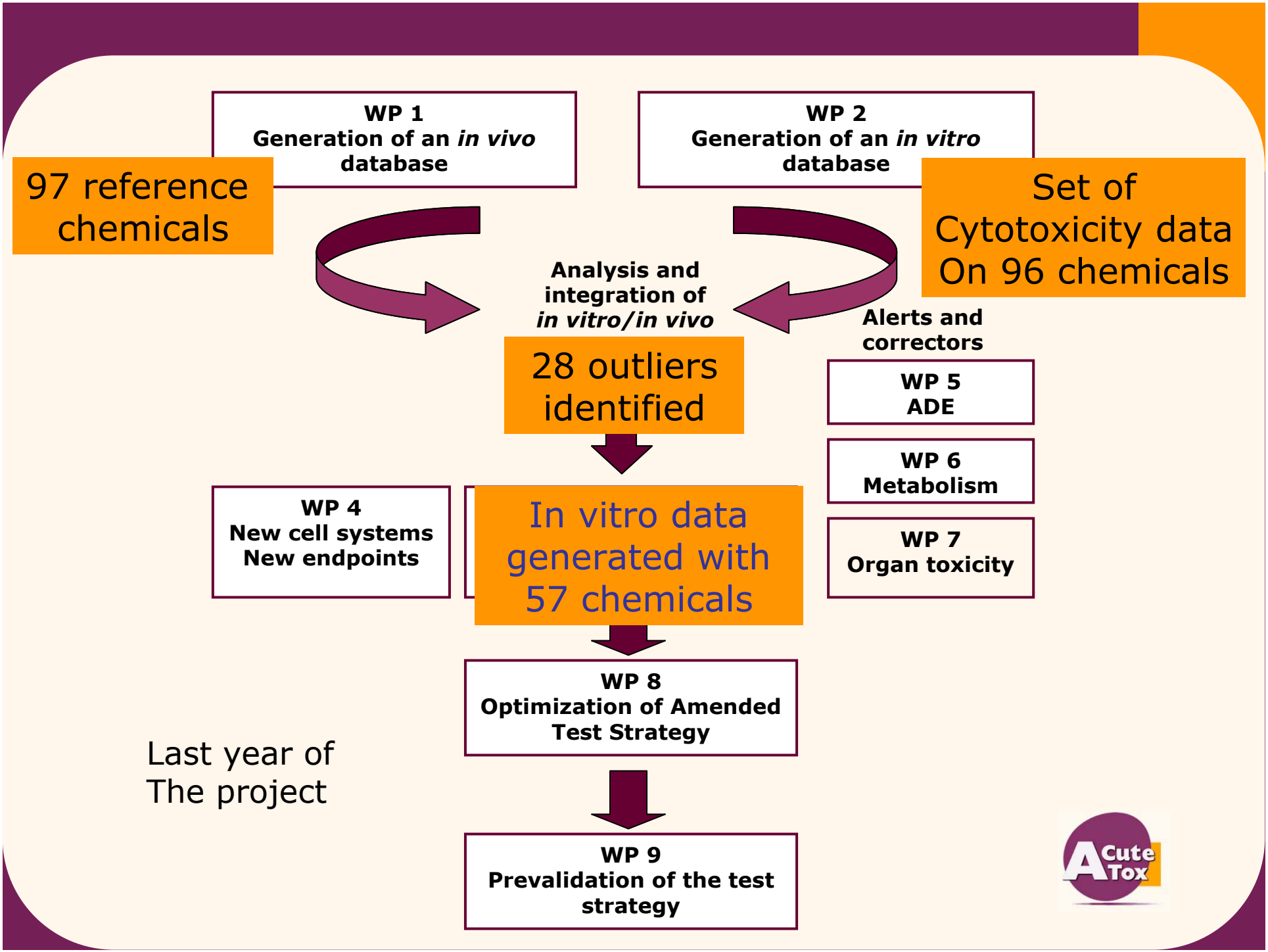


WP6 and 7.3: Role of metabolism and hepatotoxicity

Hepatocyte	Hepatoma	Non-hepatic cell
		
$IC50_A$	$IC50_B$	$IC50_C$



IC50(A) < IC50 (B) ≈ IC50(C):
"hepatotoxic" (bioactivable) → ***alert***
IC50(A) ≈ IC50 (B) < IC50(C):
"hepatotoxic" → ***alert***
IC50(A) ≈ IC50 (B) ≈ IC50(C):
no hepatotoxic → ***no alert***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In vitro data generated with 57 chemicals
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Prevalidation of the test strategy
Last year of
The project

In vivo - in vitro modelling with PLS regression including IC50 values from all assays

Variables	R2	Q2	Most important	Least imp	Excluded
1,2,4,6,55,62,73,75,89-92	0,47	0,45	1,2,4,6,73,74	92	
1,2,4,6,55,62,73,75,89-91	0,49	0,47	1,2,4,6,73,74	89	92
1,2,4,6,55,62,73,75, 90-91	0,51	0,49	1,2,4,6,73,74	62	92,89
1,2,4,6,55,73,75,90-91	0,52	0,50	1,2,4,6,73,74	90	62, 92,89
1,2,4,6,55,73,75,91	0,53	0,52	1,2,4,6,73,74	91	90, 62, 92,89
1,2,4,6,55,73,75	0,55	0,52	1,2,73,74	55	62,73,75,92,89
1,2,4,6,73,75	0,56	0,54	1,2,4,73,75	6	55, 62,73,75,92,89
1,2,4, 73,75	0,57	0,55	1,2,4,75	73	6, 55, 62,73,75,92,89
1,2,4,75	0,58	0,56	1,2,75	4	73, 6, 55,62,73,75,92,89
1,2,75	0,59	0,57	1,2	75	4, 73, 6, 55,62,73,75,92,89
1,2	057	0,56	1	2	75, 4, 73, 6, 55,62,73,75,92,89
1	0,52	0,52			
2	0,48	0,48			
4	0,49	0,47			
75	0,49	0,47			

1 (NHK/NRU)
2 (3T3/NRU)
75 (gene expression, uridine incorporation and 2-deoxyglucose uptake in brain aggregates)

R^2=0.59

2 (3T3/NRU)

R^2=0.46

Subcontractor: Tasks for Statistical Analysis

1. Dose-response analysis: recalculate 57 × 71 *in vitro* data matrix
   - Raw data extraction
   - Statistical dose-response analysis strategy
   - Assessment of assay variability
   - Correlation between assays

2. Predict GHS class by use of *in vitro* data matrix:

   a) regression approach
   b) classification approach

3. Select 6-10 *in vitro* assays promising for prediction of GHS class.

Statistical Dose-Response Analysis Strategy

Model fitting using a 4-parameter log-logistic model

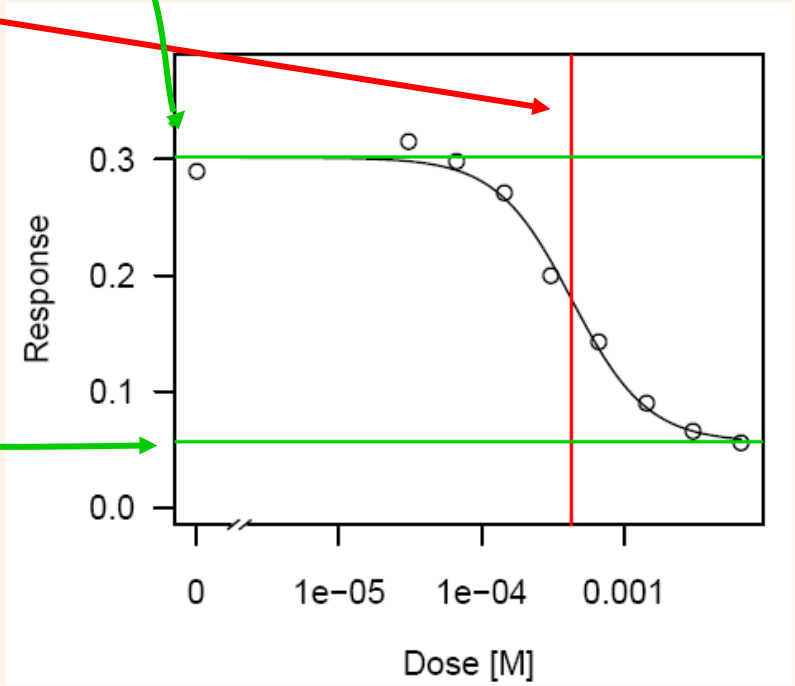
$$f(\text{conc}) = c + \frac{d - c}{1 + e^{b(\log(\text{conc}) - e)}}$$

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

c: lower asymptote

d: upper asymptote

e: log(EC50)



- Advantage of using modeling approach:
  Estimation procedure provides estimate + 95%-Confidence Interval
- Often: Response values normalized, i.e. response value divided by mean control response. Nevertheless fit 4-parameter log-logistic model

Candidate assays for prevalidation

The following assays have been selected on the basis of the statistical analysis:

1. Neutral Red Uptake in 3T3 mouse fibroblasts (general cytotoxicity)
2. Cytokine release (IL-1, TNFa, IL-6) in human whole blood (immunotoxicity)
3. Gene expression (GFAP, NF, Hsp-32, MBP) in rat brain aggregates (neurotoxcity)
4. Uridine and methionine uptake in rat brain aggregates (neurotoxcity)
5. CFU-GM assay (hematotoxicity)
6. Cytomic panel (incl. endpoints for oxidative stress, Ca uptake, mitochondrial and plasma membrane potential) in A704, HepG2, SH-SY5Y cells
7. MTT assay in rat hepatocytes (metabolism)

Candidate assays for prevalidation

In addition, the inclusion of algorithms for:

- The estimation of the oral dose from the effective concentration observed *in vitro* (by including kinetic parameters such as Vd, protein binding, clearance, oral absorption)
- The estimation of compound passage through the BBB using neuronal networks (for neurotoxicity assays) will be considered.

Probably not all of the tests listed above will be included in the final testing strategy.
After the testing of additional 33 compounds under blind conditions is completed, the results obtained will be used retrospectively to validate the preliminary TS.

Classification of chemicals based on *in vitro* assays

Performance of classification algorithm measured by correct classification rate
Statistical method used: **Classification and Regression Tree (CART)**
Exemplary analysis including preliminary EC50 data of 34 assays:

		True GHS class				
		1	**2**	**3**	**4**	**5**
Predicted GHS class	**1**	**0**	0	0	0	0
	2	0	**8**	3	**2**	**3**
	3	**3**	2	**7**	1	**1**
	4	0	0	2	**14**	2
	5	0	0	0	0	**8**

Correctly classified:	37/56 = 66%	PLS analysis:	25 /55 = 45%
Underpredicted toxicity:	1 class: 4 /56 2 classes: 3 /56		10 /55 4 / 55
Overpredicted toxicity:	1 class: 6 /56 2 classes: 3 / 56 3 classes: 3 /56		13 /55 2 /55 0 / 55

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