

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



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and the ACuteTox consortium



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

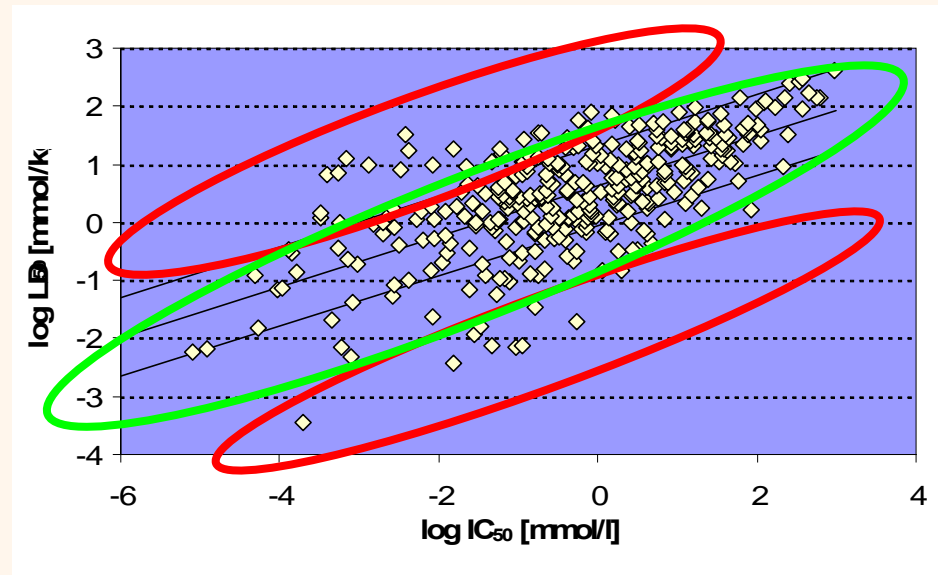
- Database on LD50 values and IC50 values for ~550 chemicals

ECVAM/ICCVAM validation study of 2 basal cytotoxicity assays

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



Background conclusion

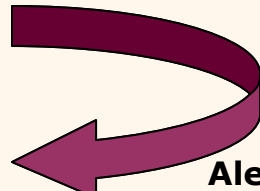
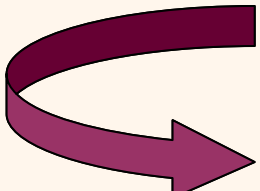


- Relatively good prediction (up to 70%)
- Certain number of misclassifications

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.

WP 1
Generation of an *in vivo*
database

WP 2
Generation of *in vitro basal*
cytotoxicity data



**Analysis and
integration of
in vitro/in vivo
data**

**Alerts and
correctors**

WP 5
kinetics

WP 6
Metabolism

WP 7
Organ toxicity

WP 4
New cell systems
New endpoints

WP 3
I f



WP 9
Prevalidation of the test
strategy



97 reference chemicals

animal in vivo data

human data

Acutoxbase

Admin Help

Main menu

- Chemical
- Animal studies
- Human cases
- In vitro experiments
- Kinetics
- Reports

Personal

- Change password
- Exit Acutoxbase

Help

Chemical

Animal

Human

In vitro

Kinetics

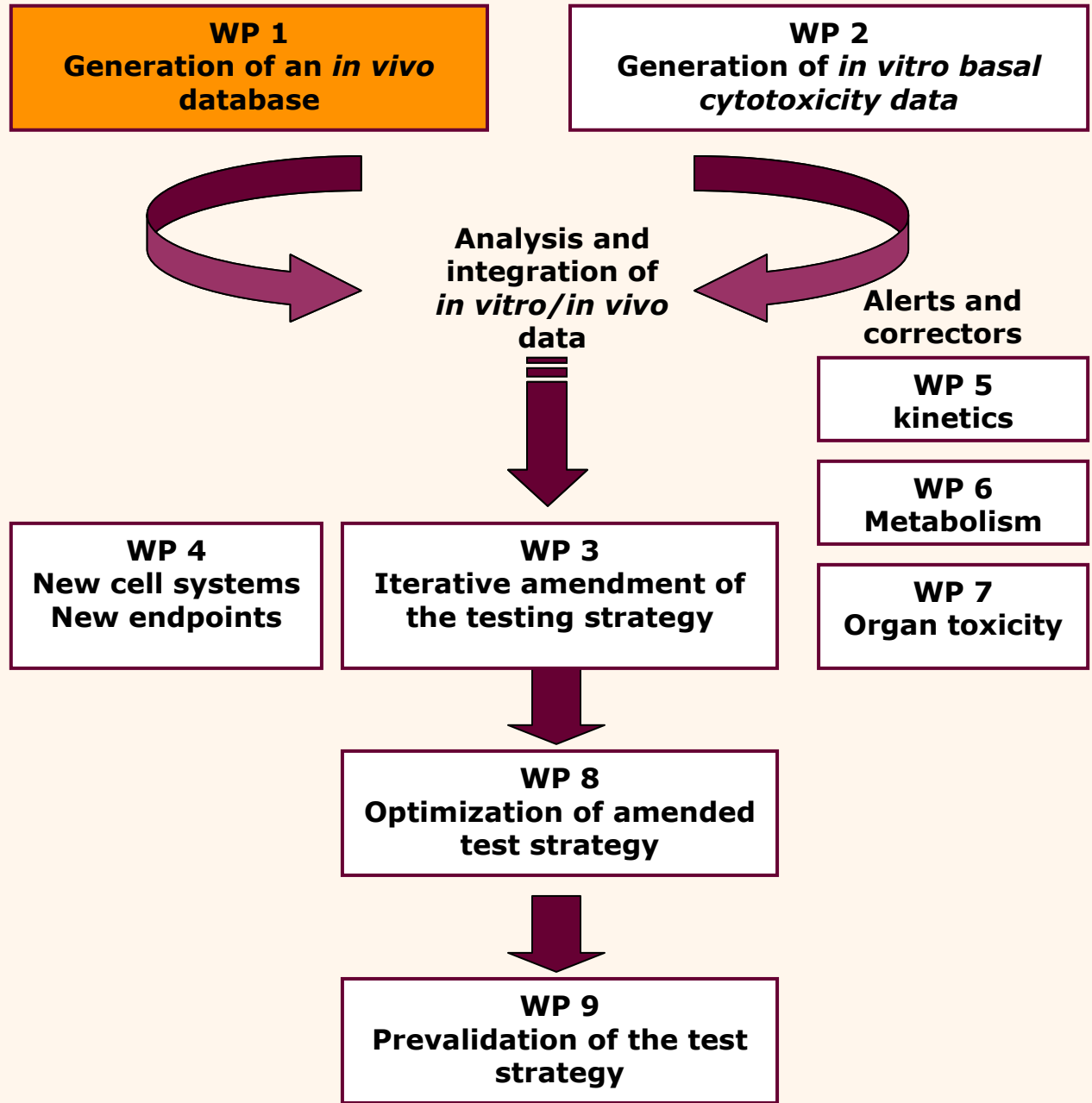
Reports

Summary data,
> 100 SOPs

in vitro data

biokinetics
(in vitro, in silico)

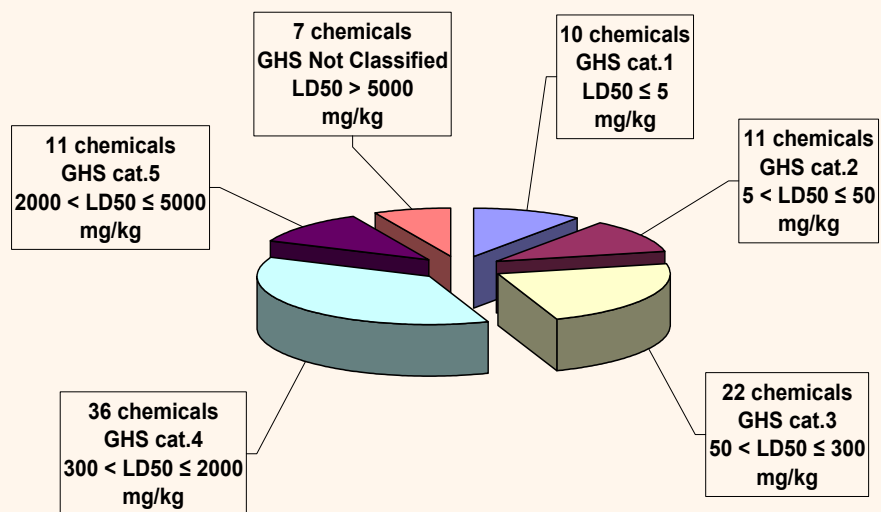
Kinsner-Ovaskainen et al. 2009, Toxicol In Vitro 23: 476-485



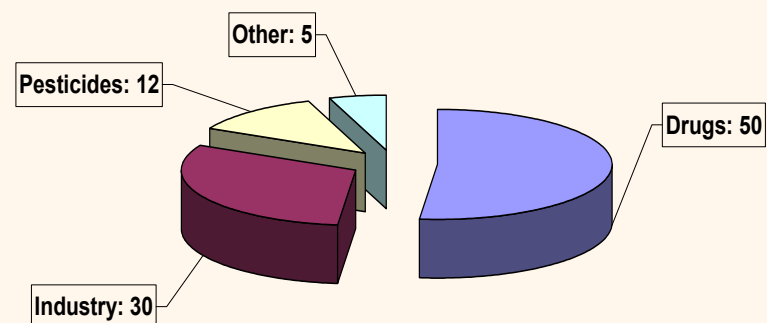
WP1: The In vivo database

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

GHS classification category distribution
97 ACuteTox reference chemicals



Generic Uses
97 ACuteTox reference chemicals



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)

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

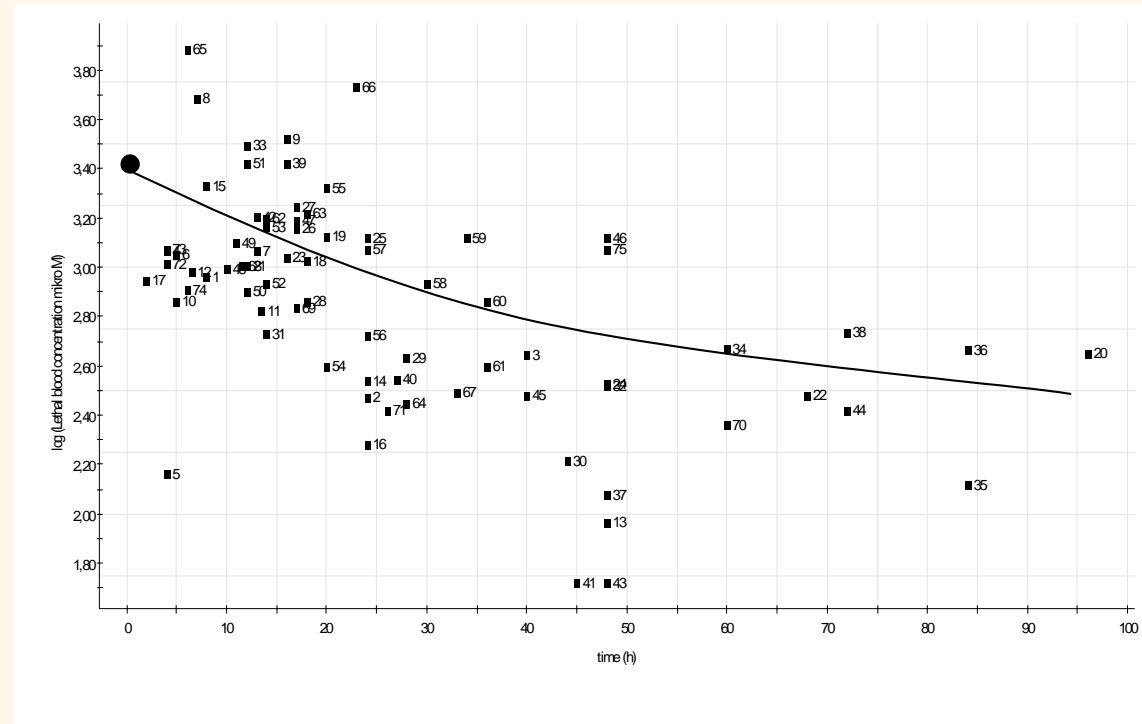
The database contains **human acute toxicity data from a single poisoning**, consisting of:

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



WP1: Estimation of LC50 human

Example: Acetaminophen approximate LC0 and LC100 and LC50



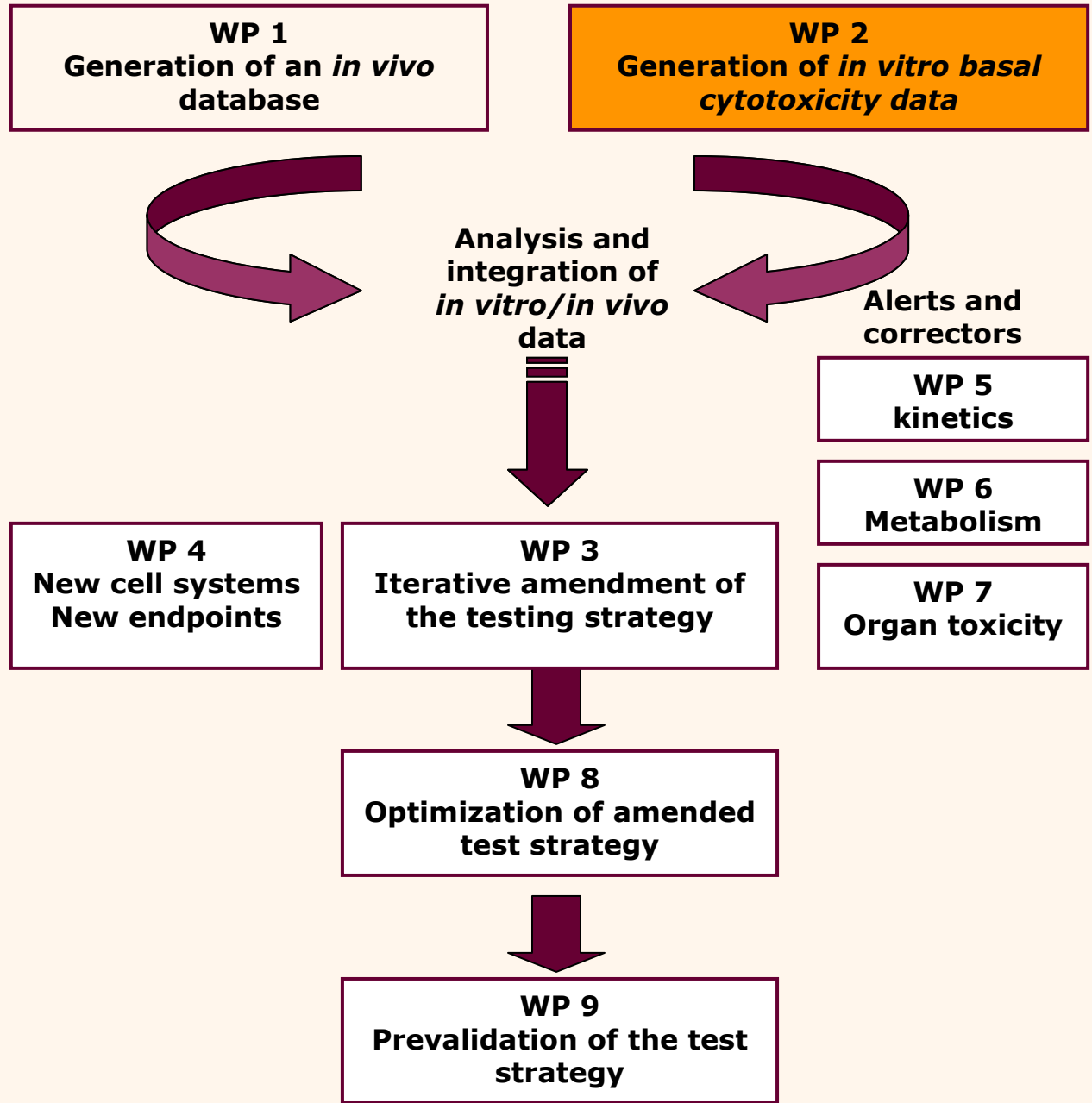
$$\text{LC100} = 3.40$$
$$\text{LC0} = 3.35$$

$$\text{LC50} = (3.35 + 3.40) / 2 = 3.37 \text{ in microM}$$

Converted to M LC50 = -2.63

Sjöström et al. (2008) Toxicology In Vitro, 22: 1405





WP 2: Generation of In vitro basal cytotoxicity data

•Assessment of Basal cytotoxicity on:

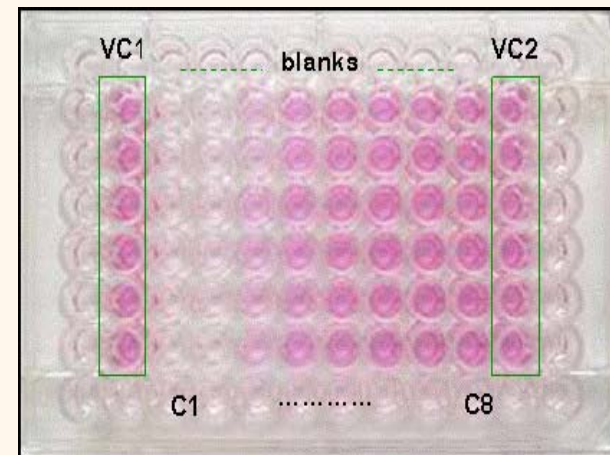
3T3 (NRU)

NHK (NRU)

HL-60 (ATP content)

Fa32 (NRU, total protein)

Hep-G2 (NRU, total protein)

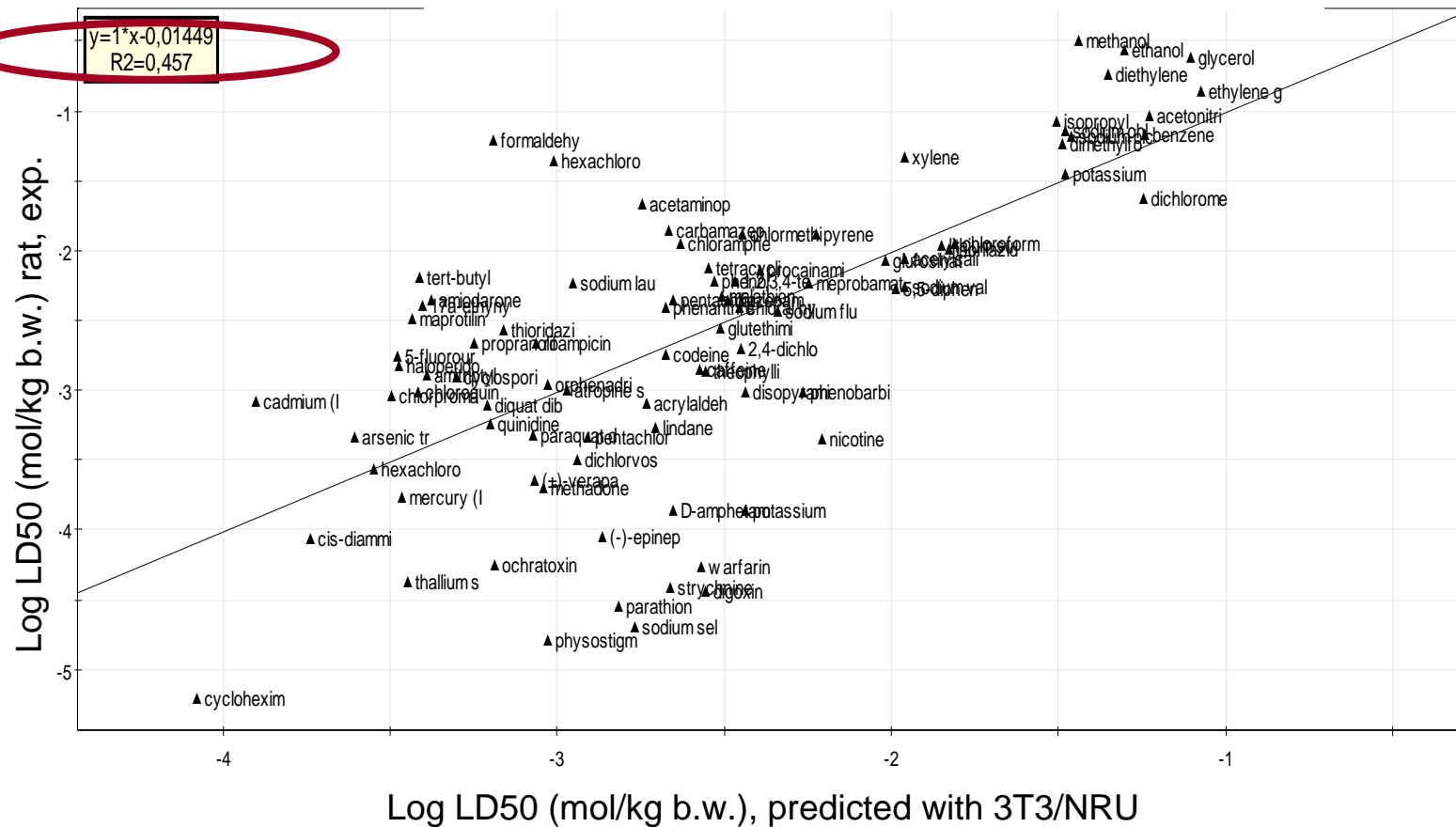


Generation of an *in vitro* database for 97 selected reference chemicals

CONCLUSIONS:

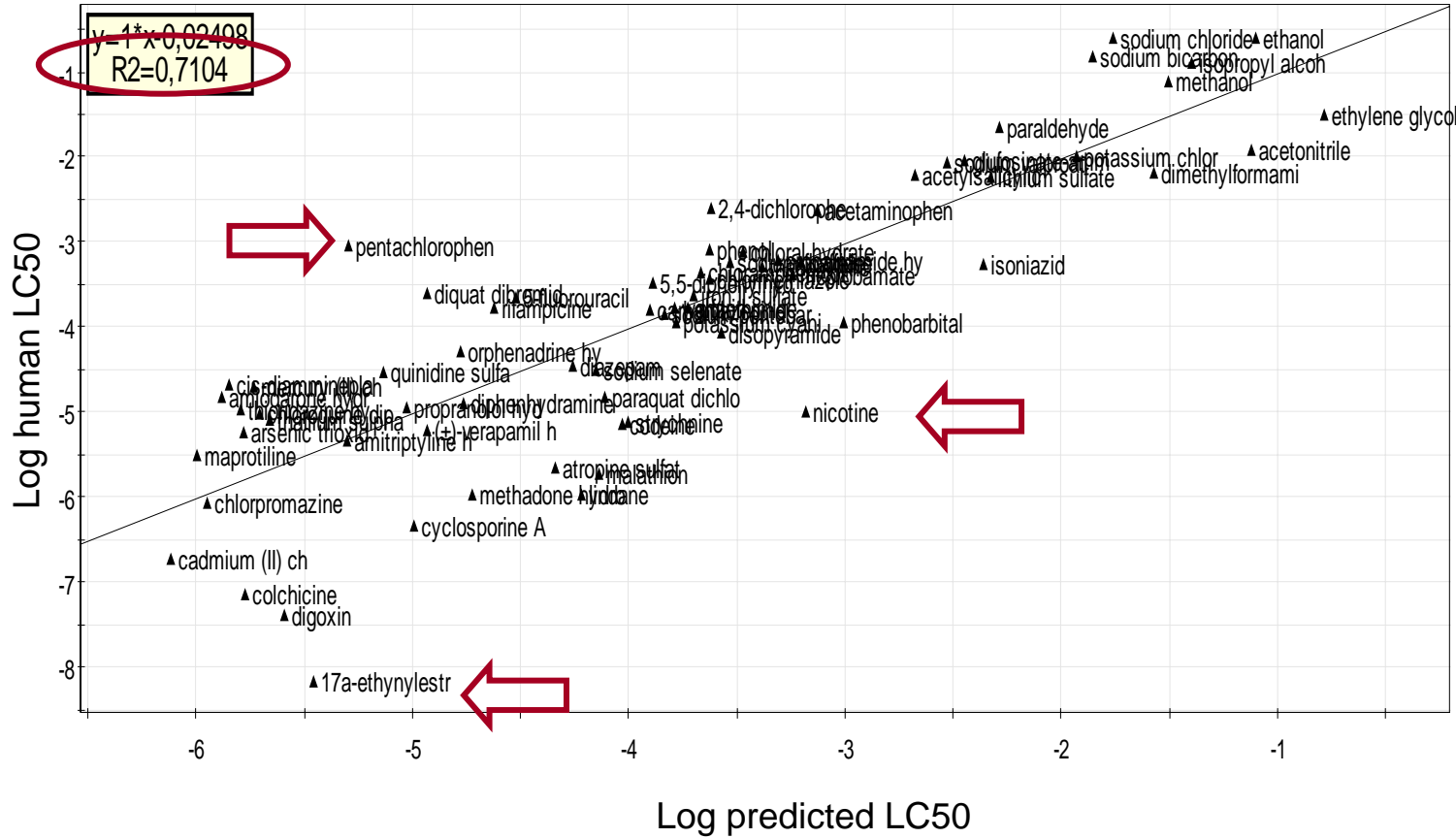
All the basal cytotoxicity tests showed similar information i.e. similar ranking; the validated 3T3/NRU seems to be the best candidate

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



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
- 47-17a-ethynylestradiol**
- 51-dimethylformamide
- 56-phenol
- 67-w arfarin
- 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:

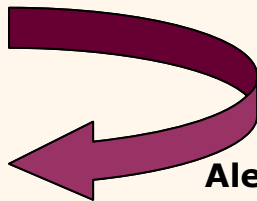
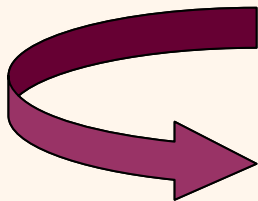
28 outliers

29 non-outliers



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New endpoints

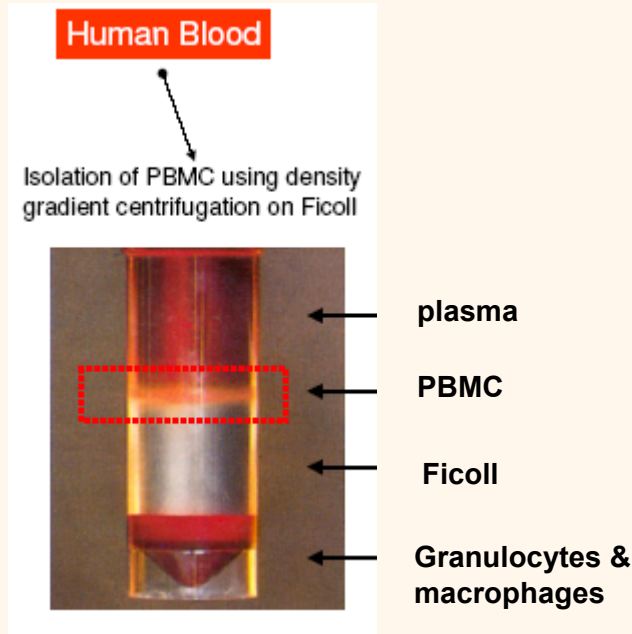
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WP 8
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WP4: Cytokine secretion and hematopoiesis

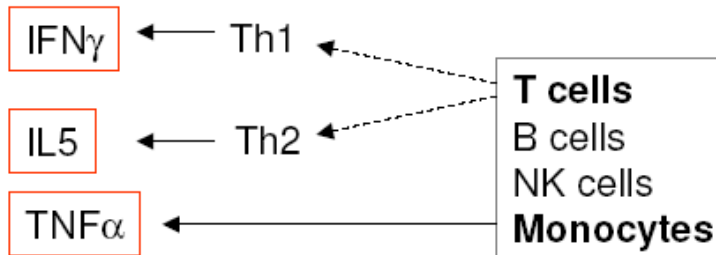


Human cord blood cells



CFU-GM
Colony forming unit-granulocyte/macrophage

Cytokines Subpopulations Cell types



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 promising 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

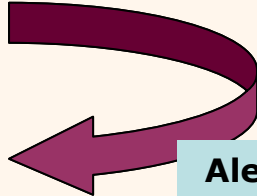
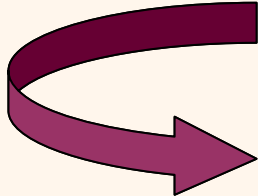
HepG2 human hepatoma cell line

SH-SY5Y human neuroblastoma cell line



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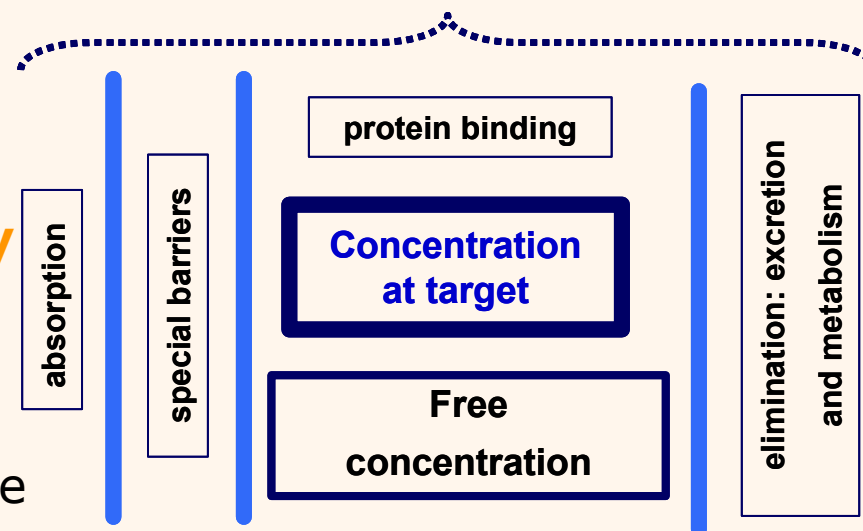
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WP5: Role of ADE (in vitro/in silico)

- 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	HIA _{pred}	Class ^a computer	Class Caco-2	Class Caco-2
Acetaminophen	1.00	H	H	H
Acetylsalicylic 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 %

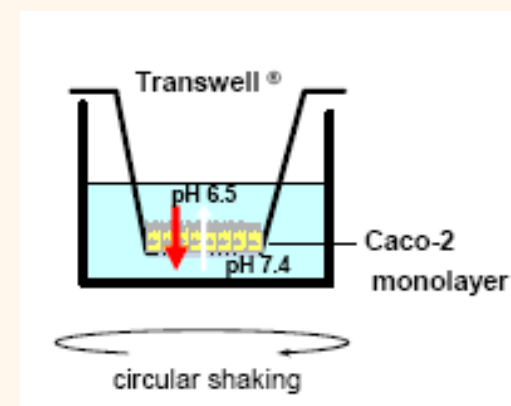
M = Moderate ; HIA < 20-70 %

P = Poor ; HIA < 20 %

Papp 10⁻⁶cm/s < 1 = Poor (P)

Papp 10⁻⁶cm/s < 1 - 10 = Moderate (M)

Papp 10⁻⁶cm/s > 10 = High (H)



72% overall accuracy



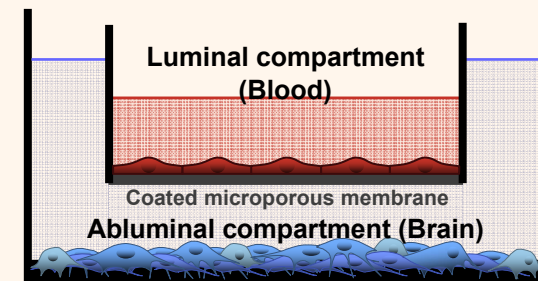
WP5: Blood-brain barrier

BLOOD-BRAIN BARRIER PASSAGE MODEL					
Chemical	LogBB _{pred}	BBB			Exper. Data (logBB)
		Class ^b n13 computer	Class D25 in vitro	Class n15 in vitro	
Acetaminophen	-1.0	P	M	H	-0.31/H
Acetylsalicylic 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

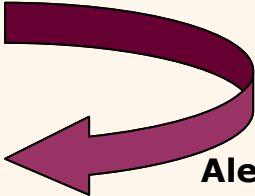
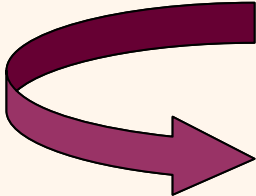
- Calculation of an **apparent volume of distribution** (Vd), assuming that the total body water volume of a 250 g rat is 170 ml and correcting for 3 factors: lipophilicity, clearance, and protein binding.
- Calculation of the **internal dose** (from IC50 values obtained in 3T3 NRU assay), taking into 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 **$R^2 = 0.46 \rightarrow R^2 = 0.63$**



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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

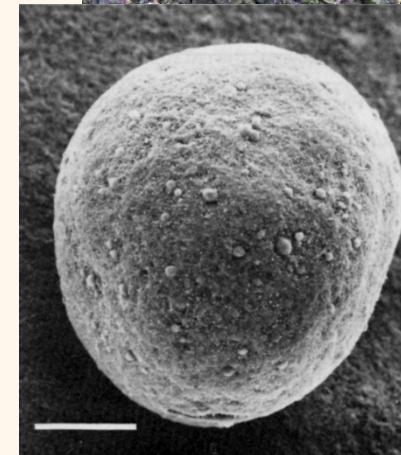
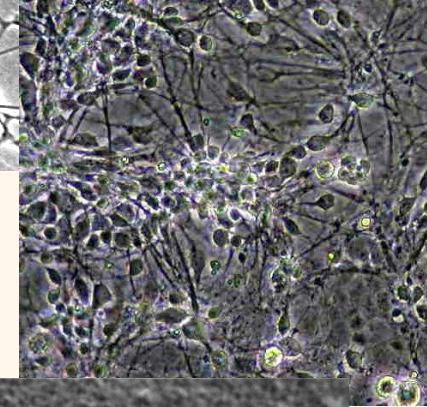
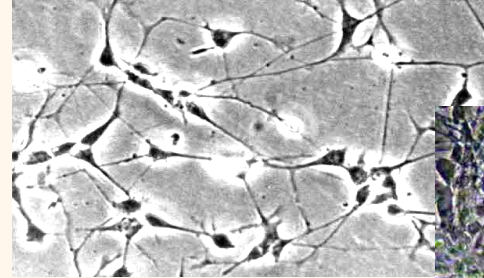
Global electrical activity



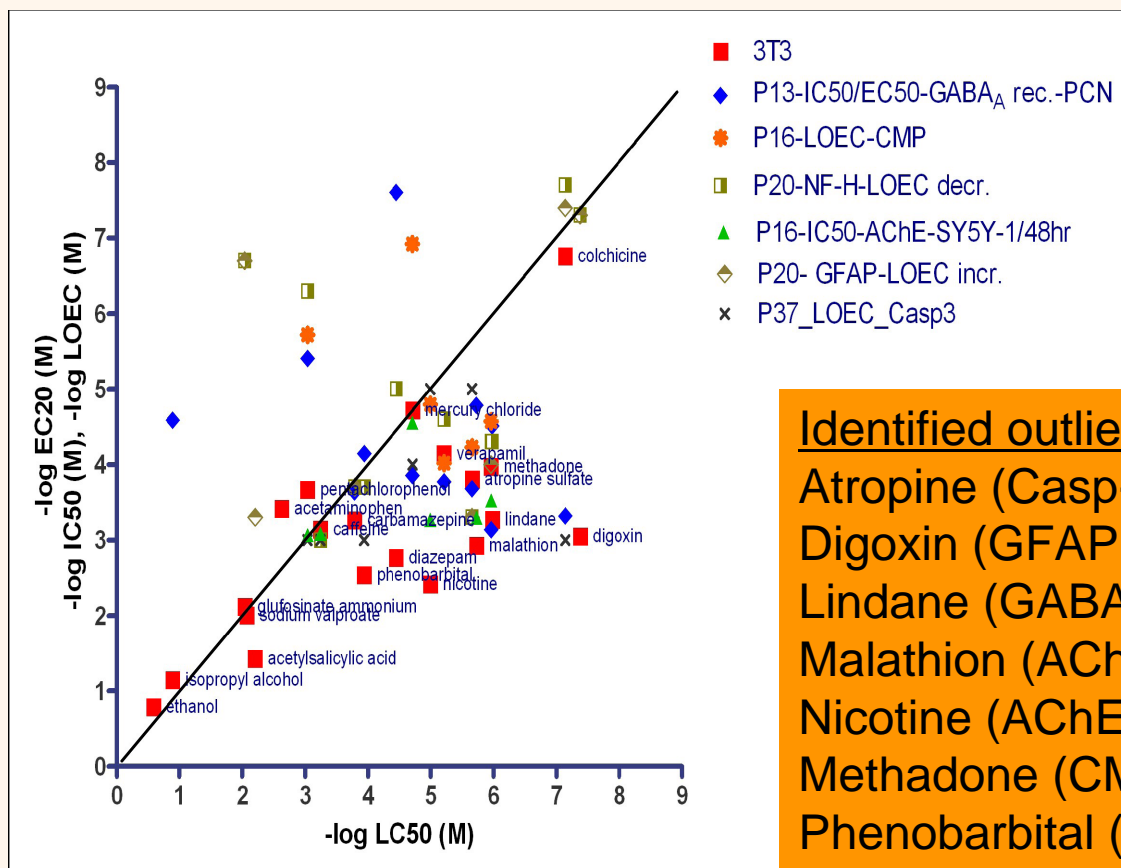
WP 7.1: Neurotoxicity

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:

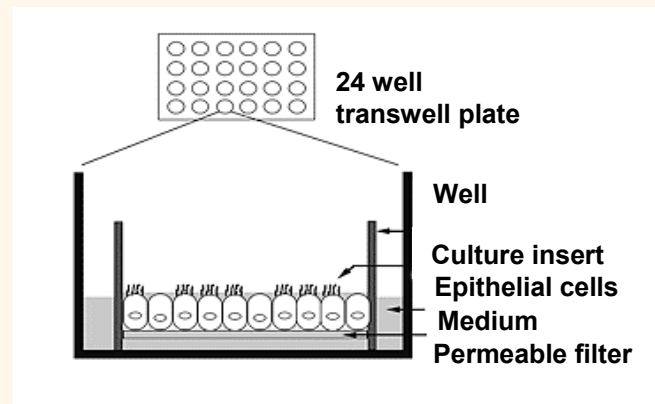
Atropine (Casp-3, CMP)
 Digoxin (GFAP, NF-H)
 Lindane (GABAA-R)
 Malathion (AChE, GABAA-R)
 Nicotine (AChE, CMP, Casp-3)
 Methadone (CMP)
 Phenobarbital (GABAA-R)

WP 7.2 Nephrotoxicity

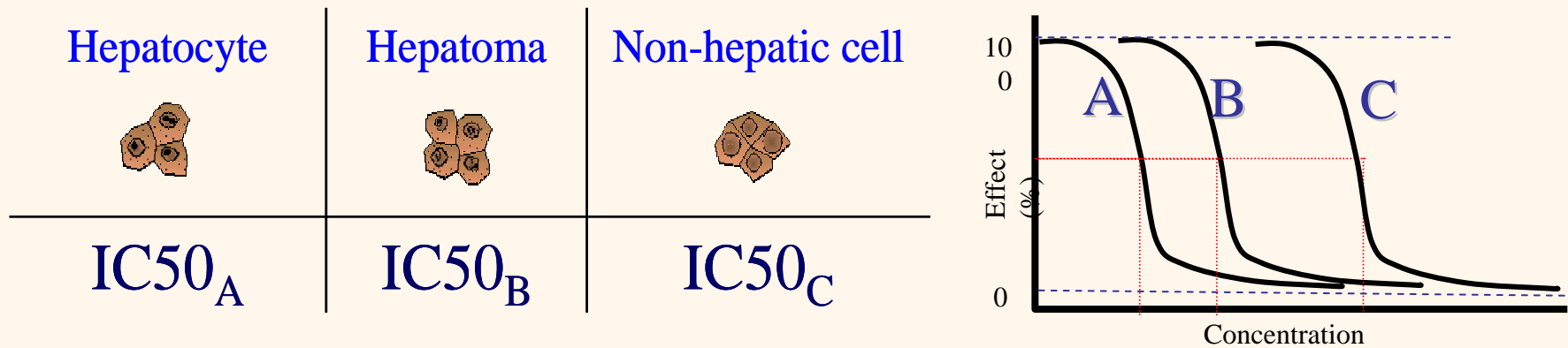
Cells: Renal epithelial cells (LLC-PK₁)

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.



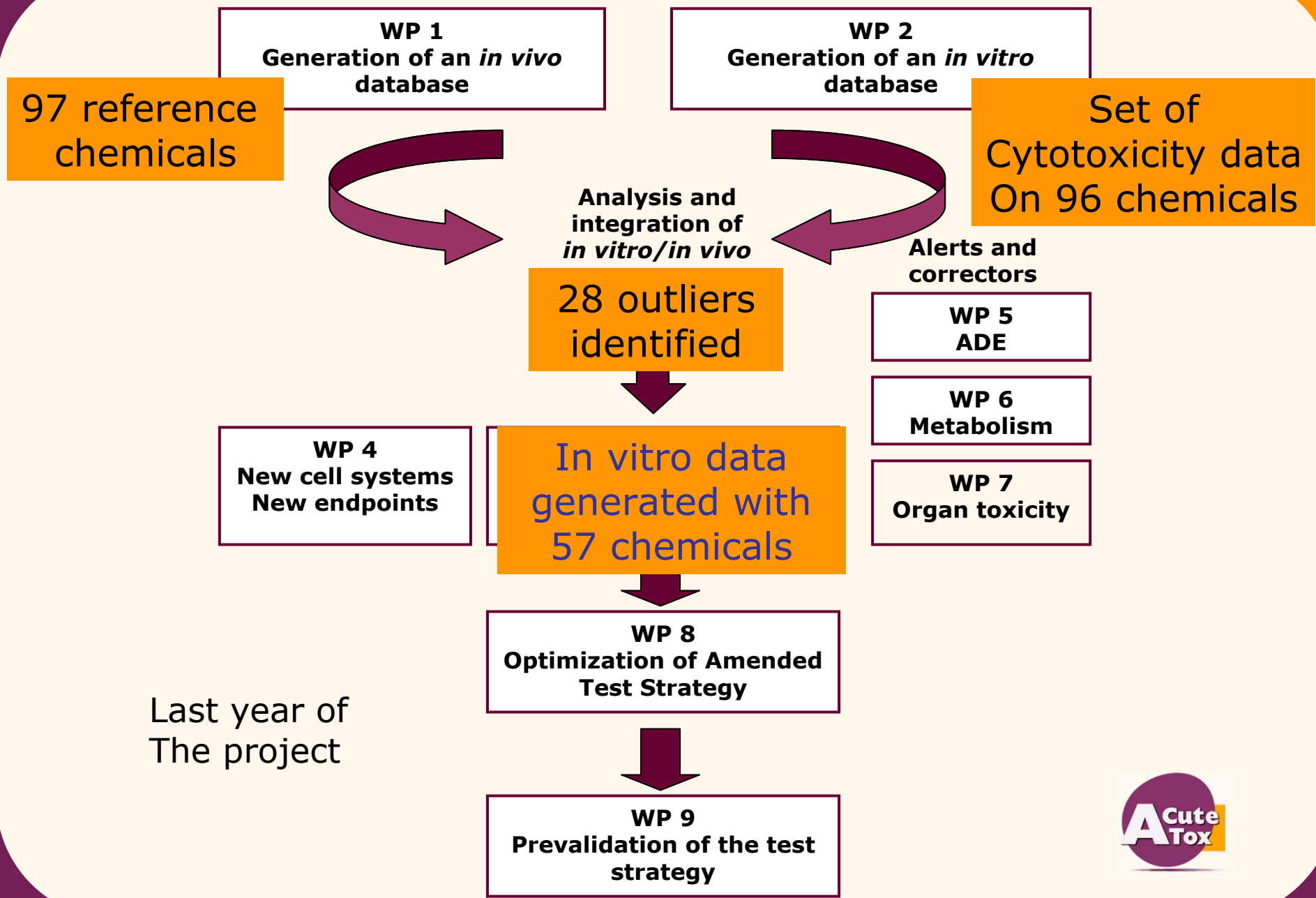
WP6 and 7.3: Role of metabolism and hepatotoxicity



$IC50(A) < IC50(B) \approx IC50(C)$:
 "hepatotoxic" (bioactivable) → **alert**

$IC50(A) \approx IC50(B) < IC50(C)$:
 "hepatotoxic" → **alert**

$IC50(A) \approx IC50(B) \approx IC50(C)$:
 no hepatotoxic → **no alert**



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	0,57	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

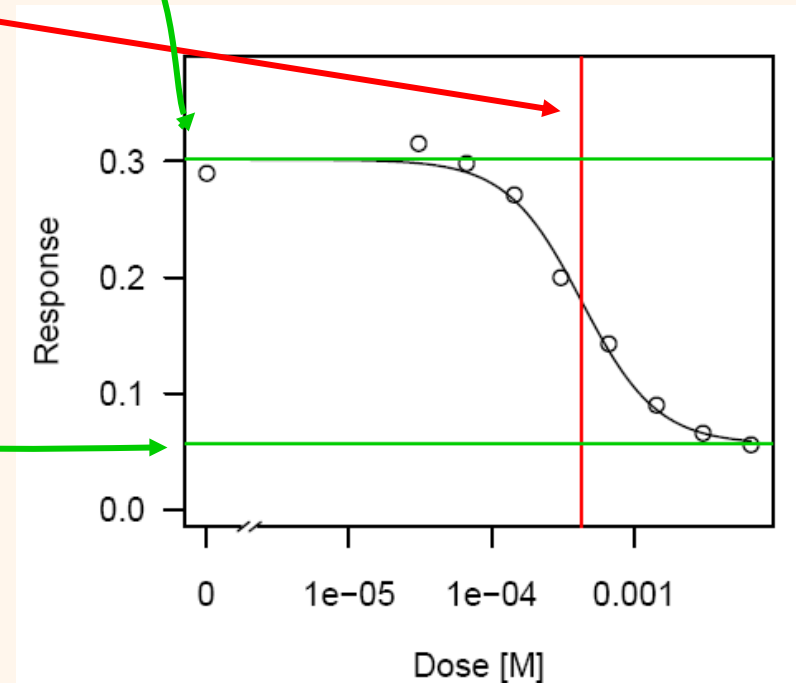
$$f(\text{conc}) = c + \frac{d - c}{1 + e^{b(\log(\text{conc}) - e)}}$$

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

c: lower asymptote

d: upper asymptote

e: $\log(\text{EC50})$



- 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, TNF α , IL-6) in human whole blood (immunotoxicity)
3. Gene expression (GFAP, NF, Hsp-32, MBP) in rat brain aggregates (neurotoxicity)
4. Uridine and methionine uptake in rat brain aggregates (neurotoxicity)
5. CFU-GM assay (hematotoxicity)
6. Cytotoxic 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%

Underpredicted toxicity:
 1 class: 4 /56
 2 classes: 3 /56

Overpredicted toxicity:
 1 class: 6 /56
 2 classes: 3 / 56
 3 classes: 3 /56

PLS analysis: 25 /55 = 45%

10 /55
 4 / 55

13 /55
 2 /55
 0 / 55

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