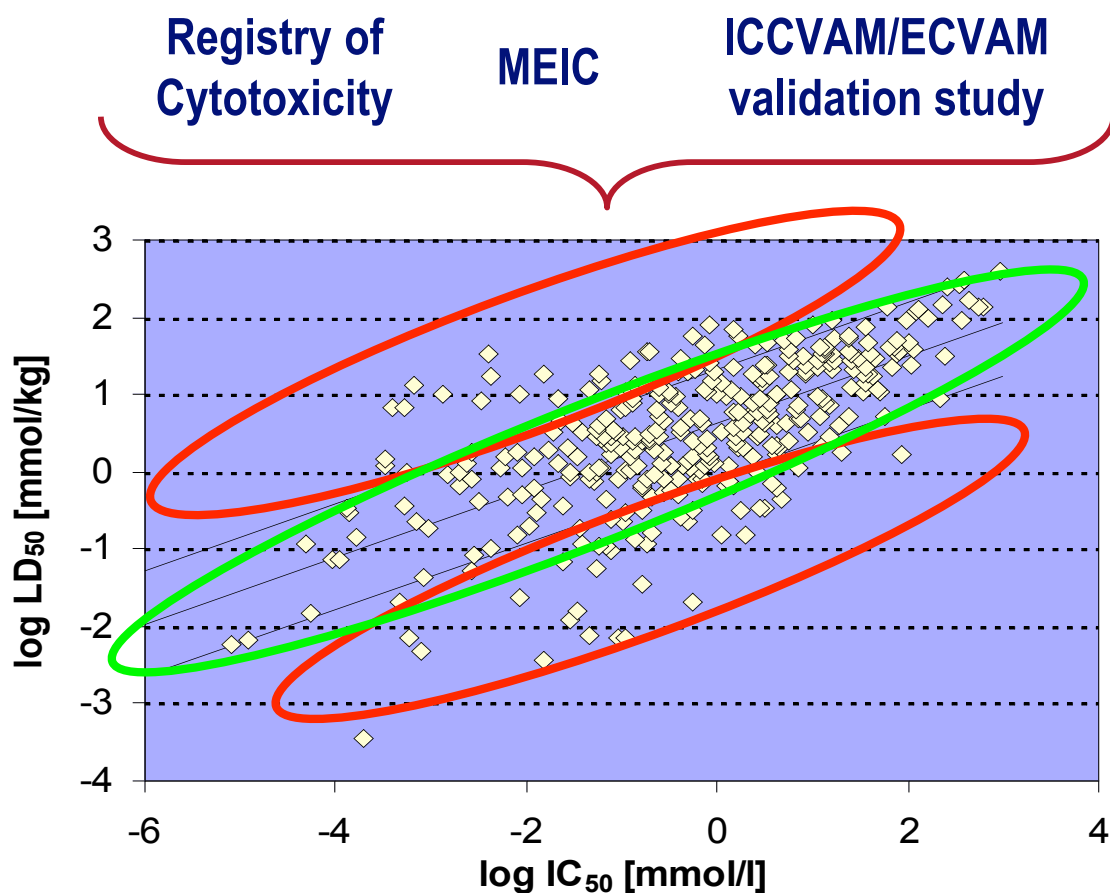# The A-Cute-Tox Project: Optimization and Prevalidation of an *in vitro* Test Strategy for Predicting Human Toxicity

Thomas Hartung, Agnieszka Kinsner, Sandra Coecke, Pilar Prieto

Strategy to Replace Acute Toxicity Testing

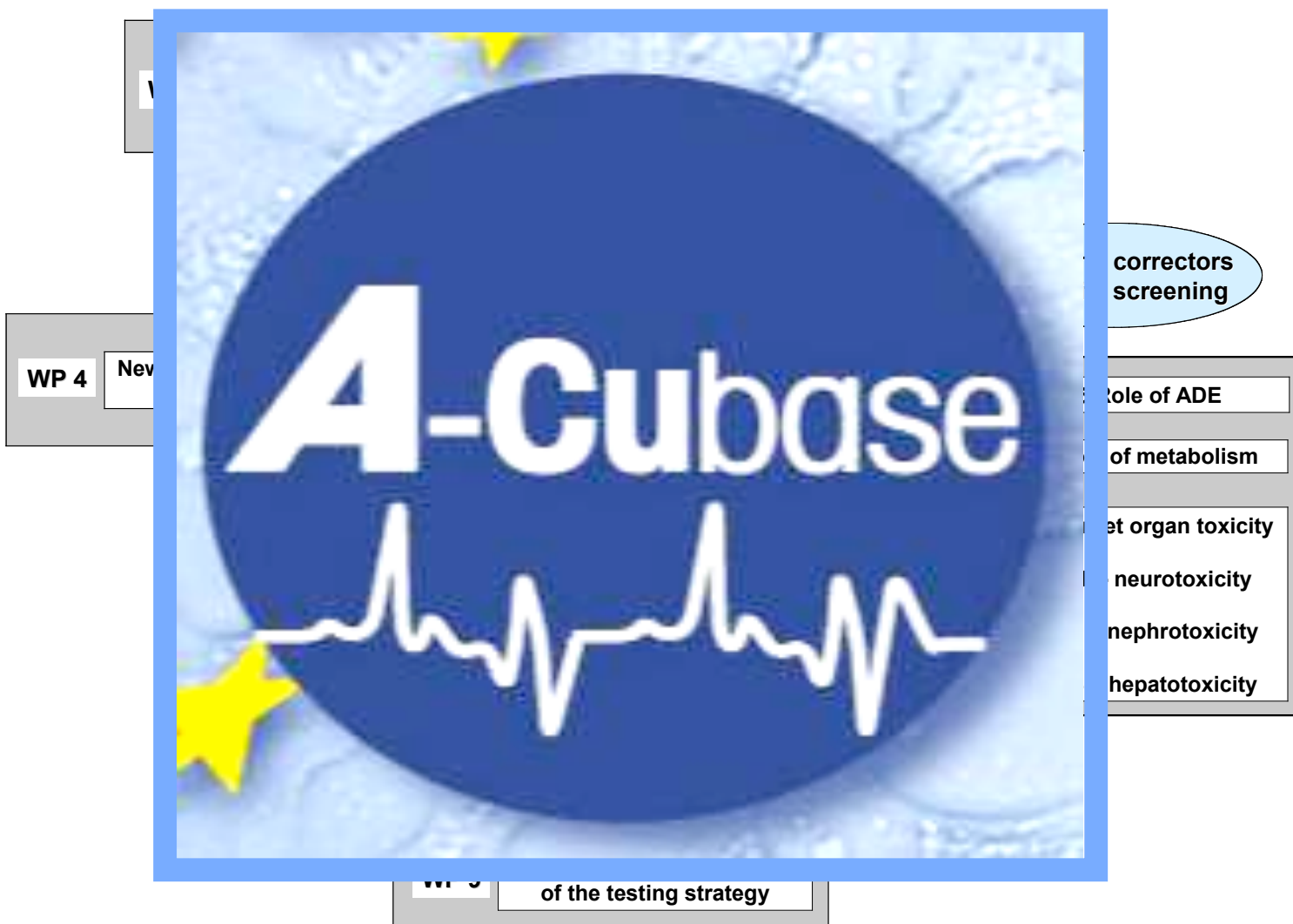Registry of Cytotoxicity   MEIC   ICCVAM/ECVAM validation study
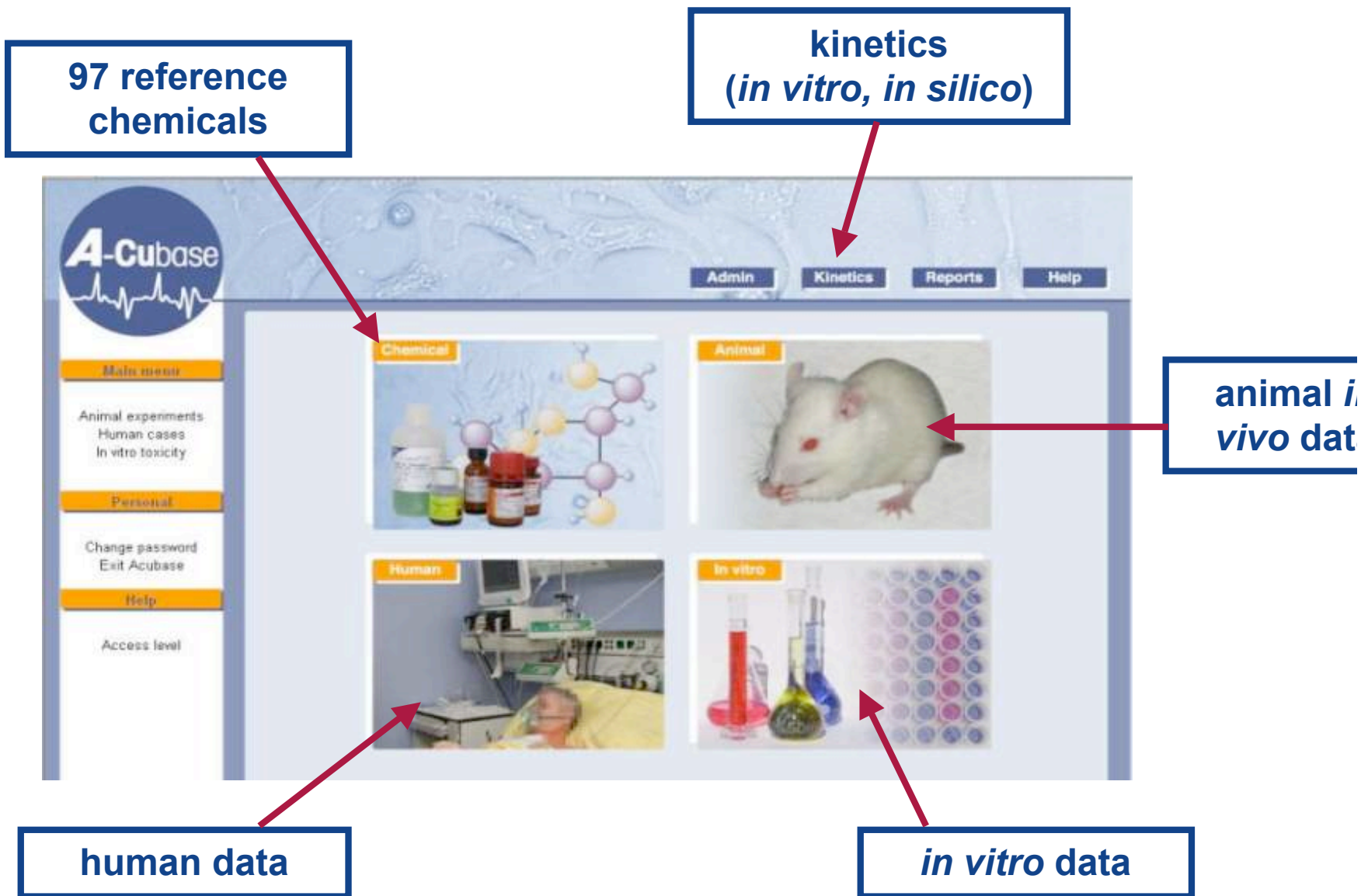

In vitro cytotoxicity test:

Relatively good correlation (~70%)

Certain number of misclassifications

Further needs:

Improve the *in vitro - in vivo* correlation by evaluating existing outliers in order to introduce further parameters (ADE, metabolism, organ specificity).

WP 4   New

WP 3   of the testing strategy

correctors
screening

Role of ADE

of metabolism

et organ toxicity

neurotoxicity

nephrotoxicity

hepatotoxicity

97 reference chemicals

kinetics (*in vitro, in silico*)

animal *in vivo* data
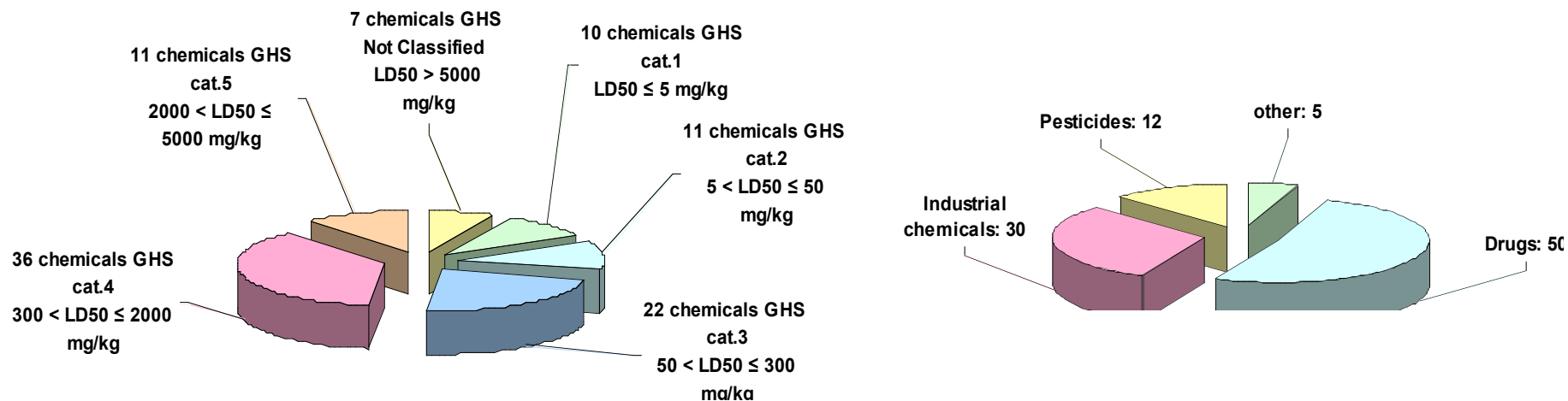
human data

***in vitro* data**

WP1: Selection of reference chemicals and collection

of *in vivo* data

- **97 reference chemicals** were selected within a wide range of acute toxicity and generic u

11 chemicals GHS
cat.5
2000 < LD50 ≤
5000 mg/kg

7 chemicals GHS
Not Classified
LD50 > 5000
mg/kg

10 chemicals GHS
cat.1
LD50 ≤ 5 mg/kg

11 chemicals GHS
cat.2
5 < LD50 ≤ 50
mg/kg

36 chemicals GHS
cat.4
300 < LD50 ≤ 2000
mg/kg

22 chemicals GHS
cat.3
50 < LD50 ≤ 300
mg/kg

Pesticides: 12

other: 5

Industrial
chemicals: 30

Drugs: 5(

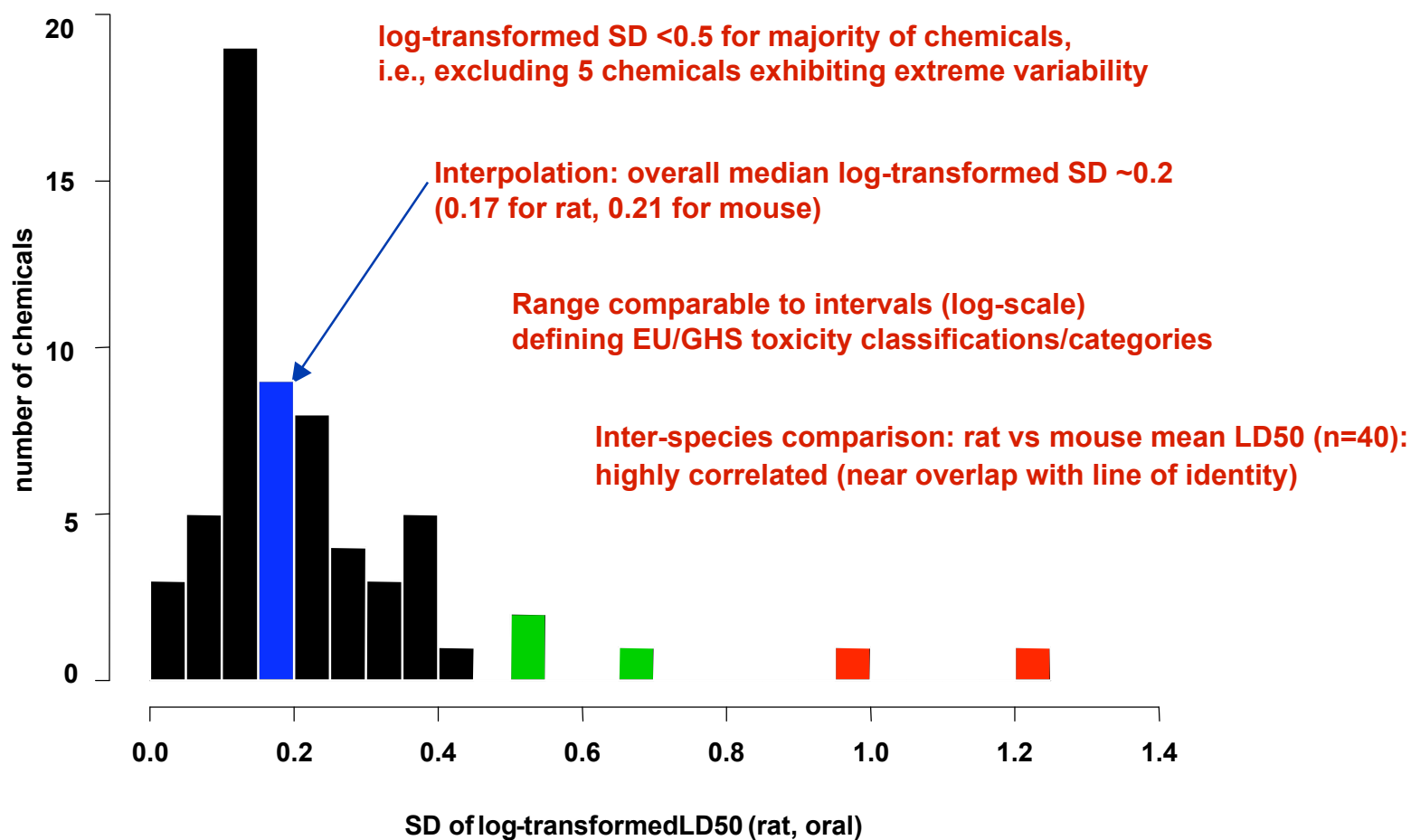- Generation of the *in vivo* database (animal and human)

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)	504	300
(number of eligible chemicals)	(62)	(51)

WP1: Evaluation of *in vivo* animal data – variability

Distribution of SD for log-transformed LD50 (rat oral studies: 62 chemicals)

log-transformed SD <0.5 for majority of chemicals,
i.e., excluding 5 chemicals exhibiting extreme variability

Interpolation: overall median log-transformed SD ~0.2
(0.17 for rat, 0.21 for mouse)

Range comparable to intervals (log-scale)
defining EU/GHS toxicity classifications/categories

Inter-species comparison: rat vs mouse mean LD50 (n=40):
highly correlated (near overlap with line of identity)

number of chemicals

SD of log-transformedLD50 (rat, oral)

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**
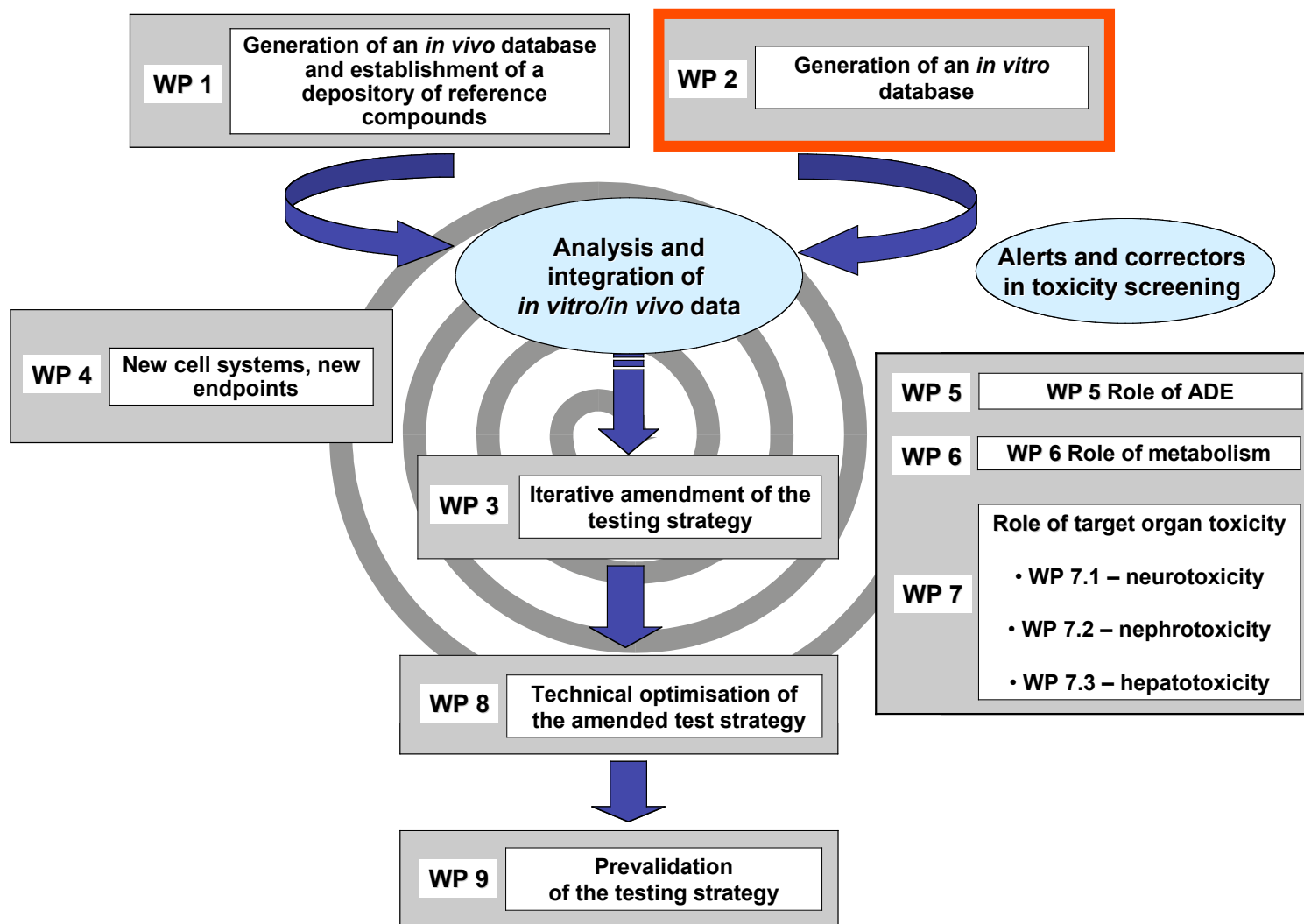- **post-mortem blood concentrations**

WP1: Estimation of LC50 human

Example: Acetaminophen approximate LC0 and LC100 and LC50
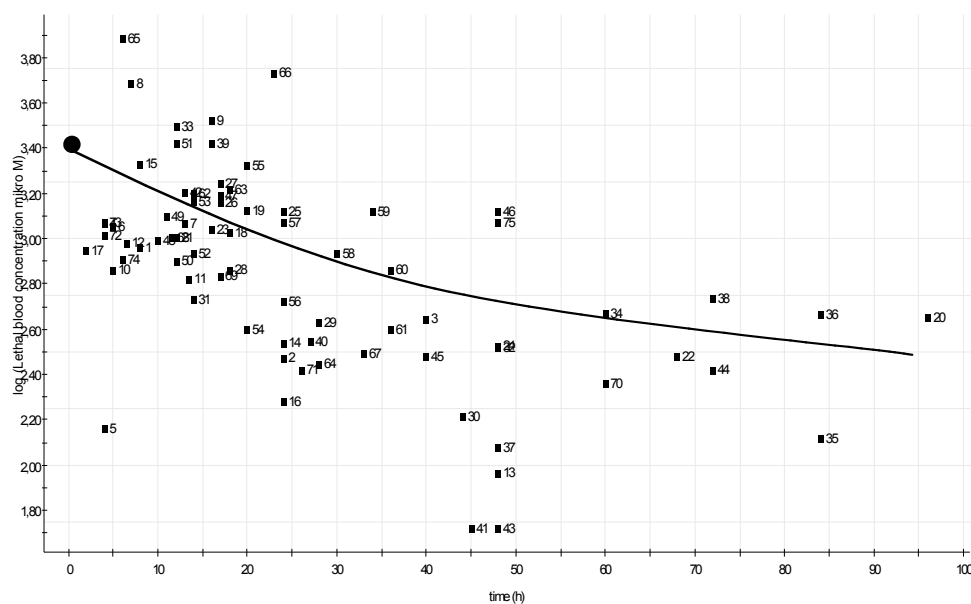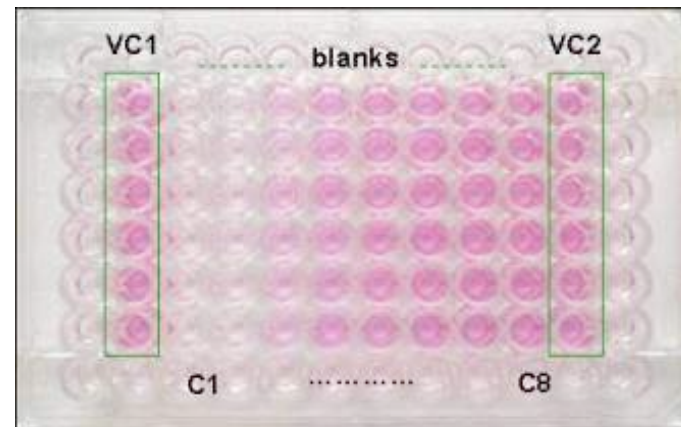

LC100 = 3.40   LC50 = (3.35+ 3.40)/2= 3.37 in microM

LC0 = 3.35   Converted to M LC50=-2.63

WP2: Generation of *in vitro* basal cytotoxicity data

- **Assessment of basal cytotoxicity in:**
  - **BALB/3T3 (NRU)**
  - **NHK (NRU)**
  - **HL-60 (ATP)**
  - **HepG2 (NRU, total protein)**
  - **Fa32 (NRU, total protein)**

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

Automation
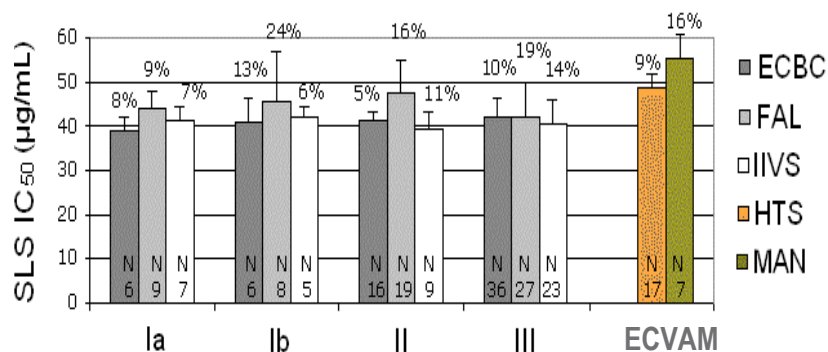
ECVAM/NMI Pilot Test Platform

3T3 NRU *validated protocol*

ECVAM Unit, IHCP, JRC

NMI Unit, IHCP, JRC

University Nottingham, UK

21 chemicals from the ACuteTox list tested

Positive Control chemical SLS

AUTOM. (HTS) IC
CV = 9%

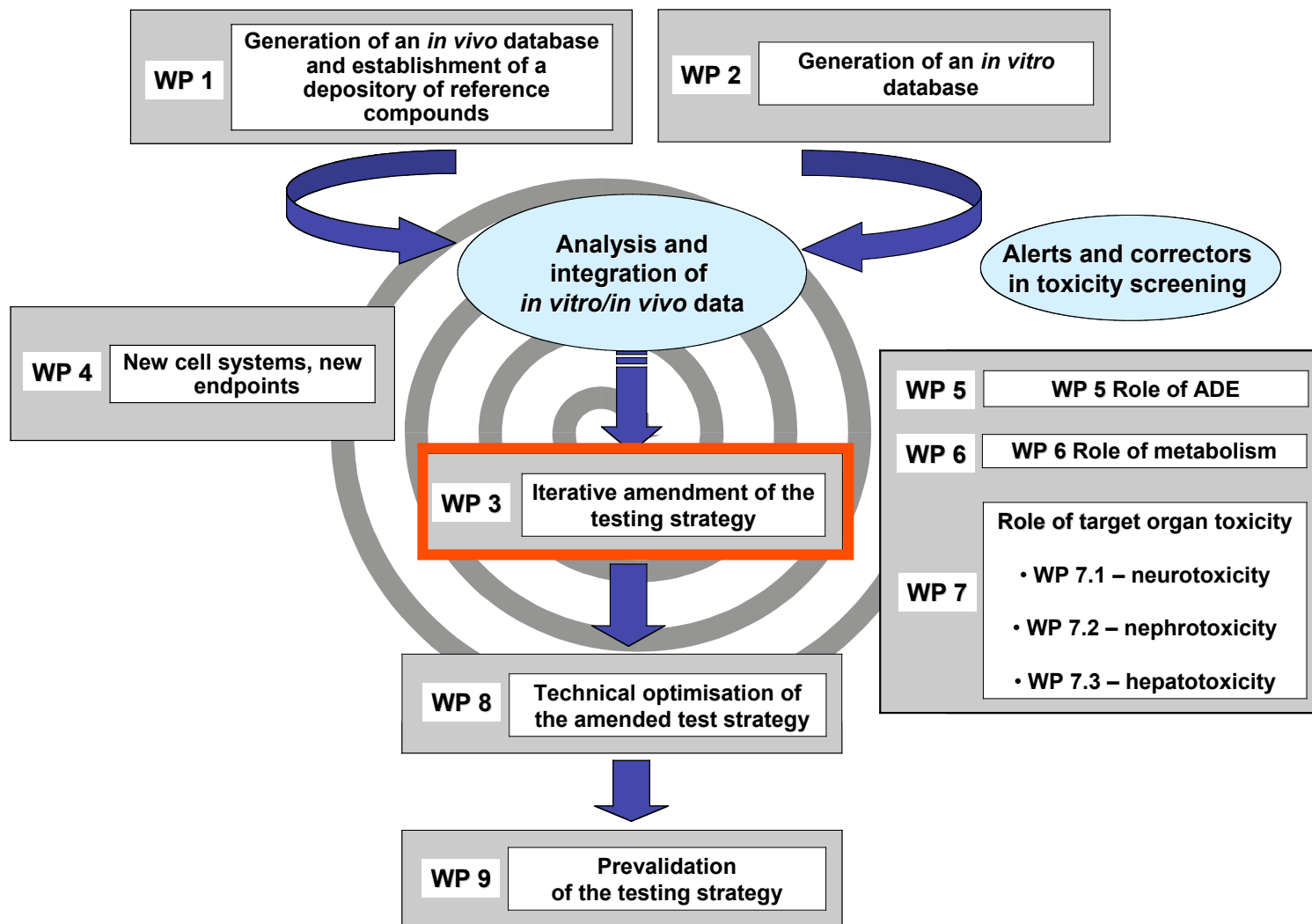**MANUAL (MAN)**
CV = 16%

LDH protocol on *NeoHep*

ECVAM/NMI Units, IHCP, JRC

University of Konstanz, Germany

TU Munich, Germany

University of Valencia, Spain

University of Oulu, Finland

11 chemicals tested from the ACuteTox list: 6 reported metabolism-mediated effect

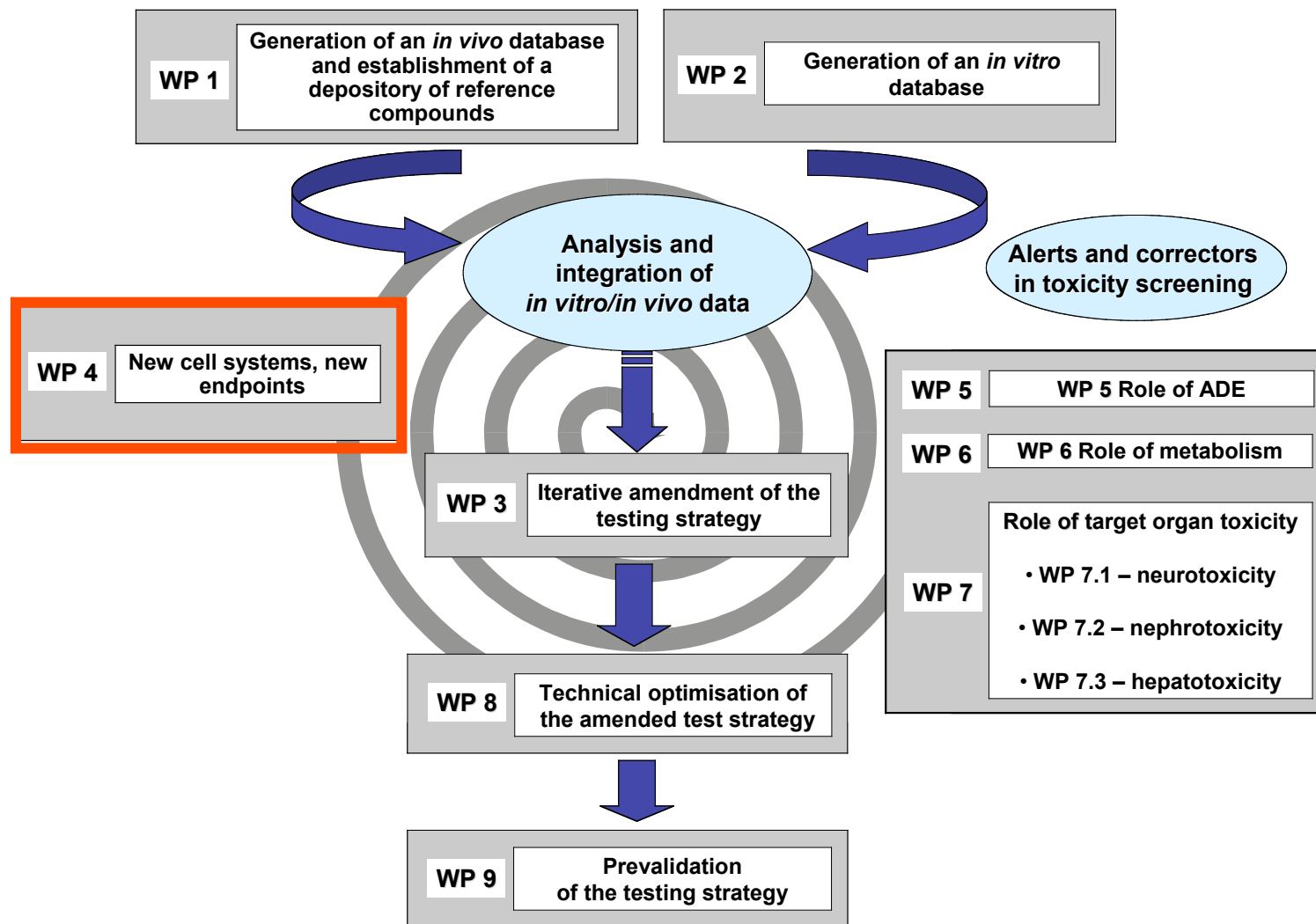# WP3: Evaluation of in vitro cytotoxicity data

- **6 basal cytotoxicity tests: similar information i.e. similar ranking**

- **The validated 3T3/NRU seems to be the best candidate**

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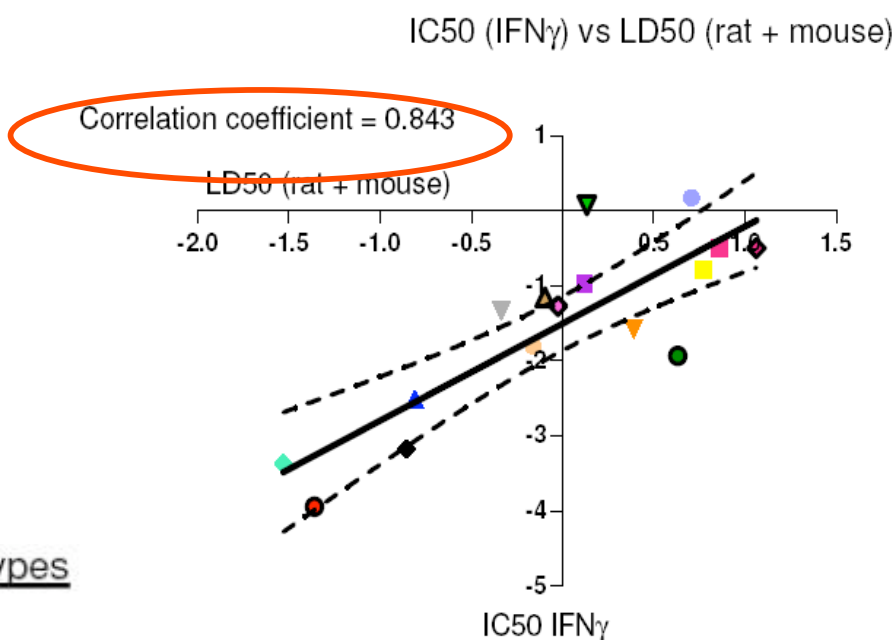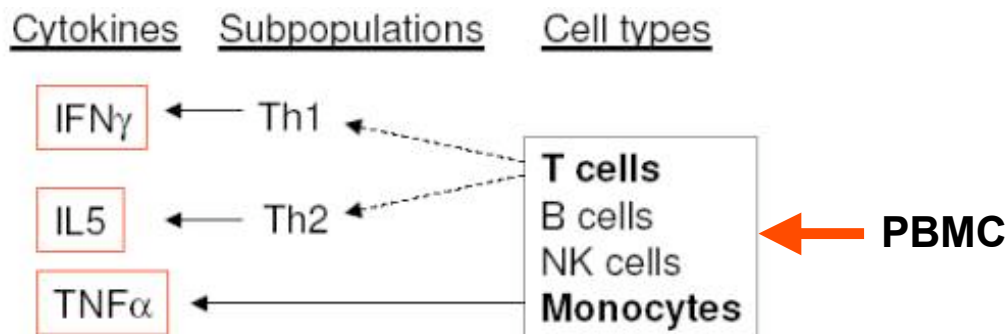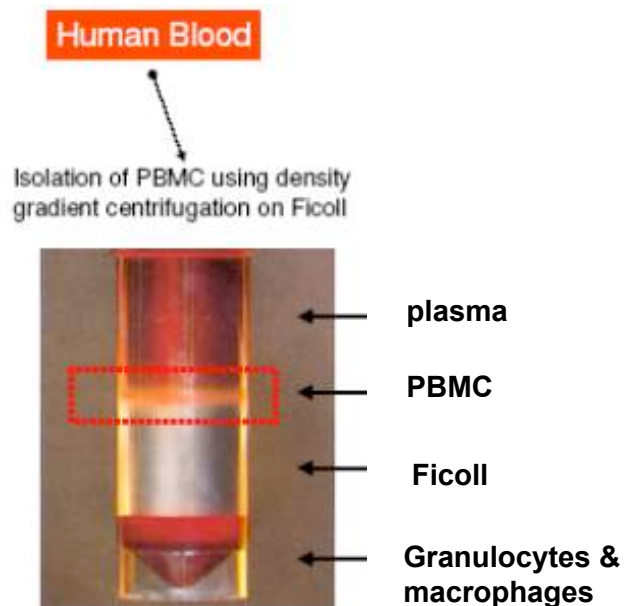## *From in vitro 3T3/NRU, PLS regression analysis*

Plot observed LC50 humans vs predicted from *in vitro*
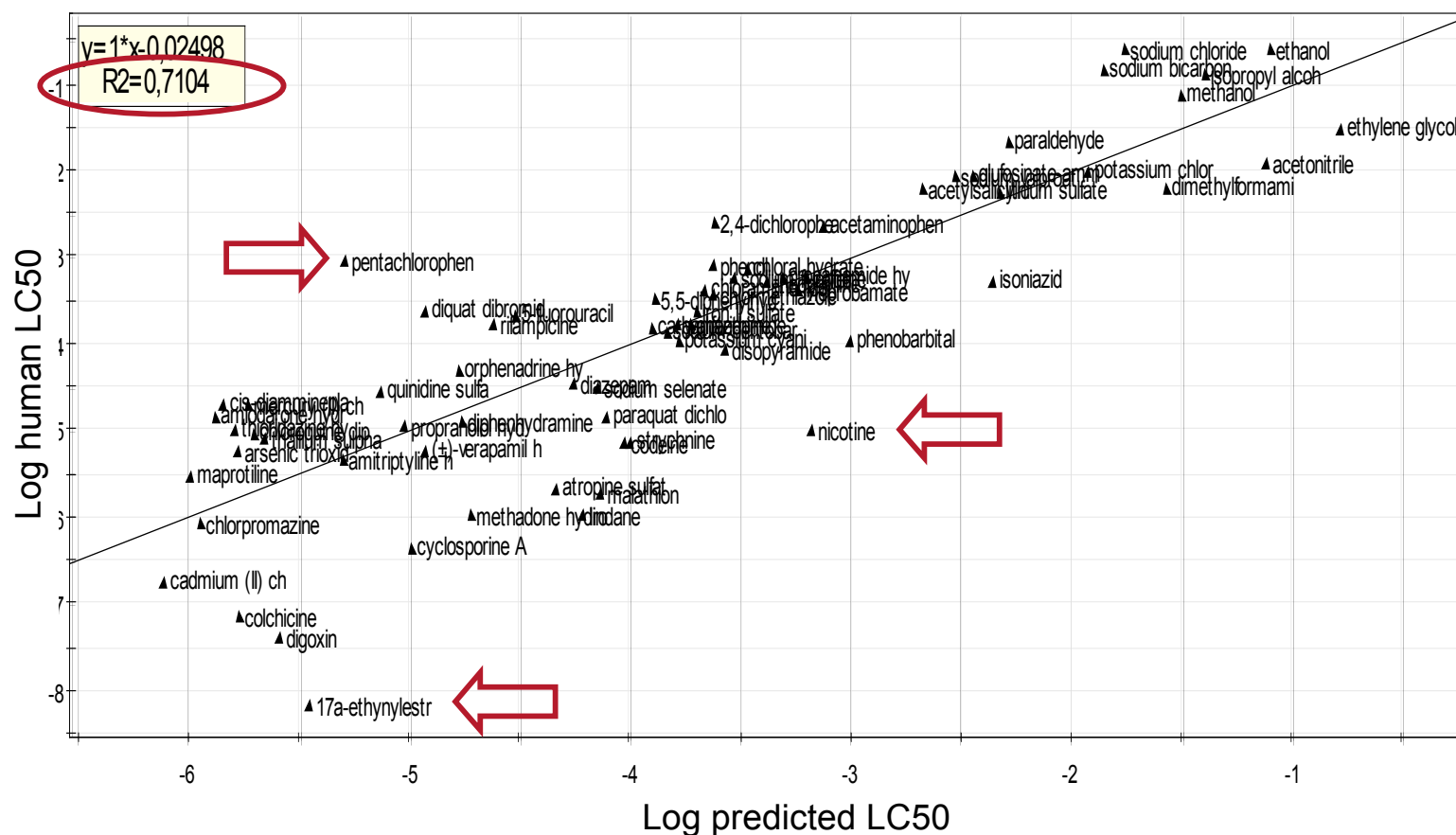
Chemicals wit...
poor human da...

03-atropine sulfate monohydrate
08-diazepam
13-pentachlorophenol
14-phenobarbital
21-cadmium chloride
28-amiodarone hydrochloride
30-rifampicine
41-glufosinate ammonium
47-17_-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

WP4: Cytokine secretion

Human Blood

Isolation of PBMC using density gradient centrifugation on Ficoll

plasma

PBMC

Ficoll

Granulocytes & macrophages

IC50 (IFNγ) vs LD50 (rat + mouse)

Correlation coefficient = 0.843

LD50 (rat + mouse)

IC50 IFNγ

Cytokines	Subpopulations	Cell types

IFNγ ← Th1

IL5 ← Th2

TNFα

T cells
B cells
NK cells
Monocytes
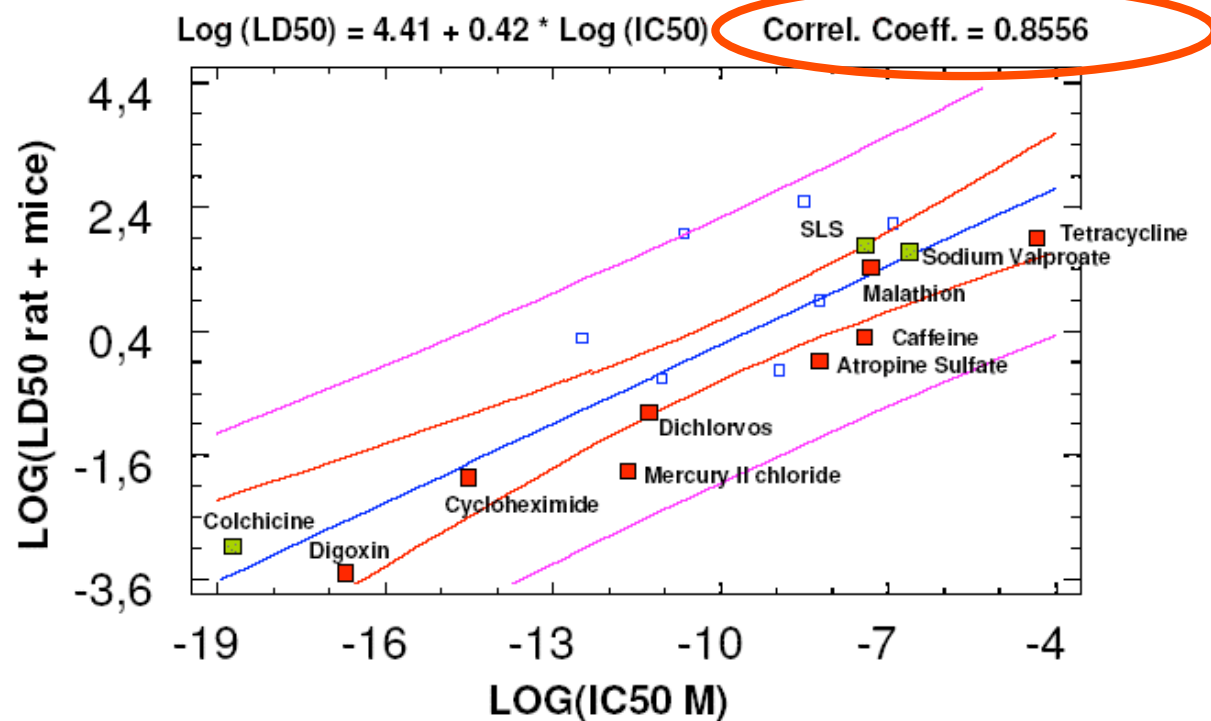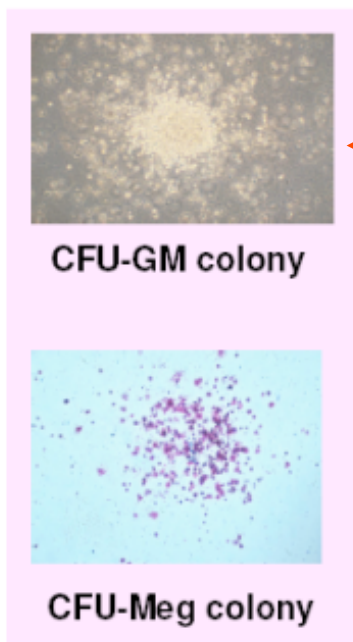
PBMC

- 5-fluorouracil
- acrylaldehyde
- atropine sulfate monohy
- cadmium (II) chloride
- caffeine
- carbamazepine
- colchicine
- cycloheximide
- diazepam
- digoxin
- malathion
- mercury (II) chloride
- pentachlorophenol
- sodium lauryl sulfate
- sodium valproate
- tert-butyl hydroperoxide

WP4: Haematopoiesis

CFU-GM colony

CFU-Meg colony

Log (LD50) = 4.41 + 0.42 * Log (IC50)   Correl. Coeff. = 0.8556

LOG(LD50 rat + mice) vs LOG(IC50 M)

Labeled points: SLS, Tetracycline, Sodium Valproate, Malathion, Caffeine, Atropine Sulfate, Dichlorvos, Mercury II chloride, Cycloheximide, Colchicine, Digoxin

Legend	
Confidence limit (95%)	Outliers in MEIC or RC studies
Prediction limits	Not studied in MEIC project
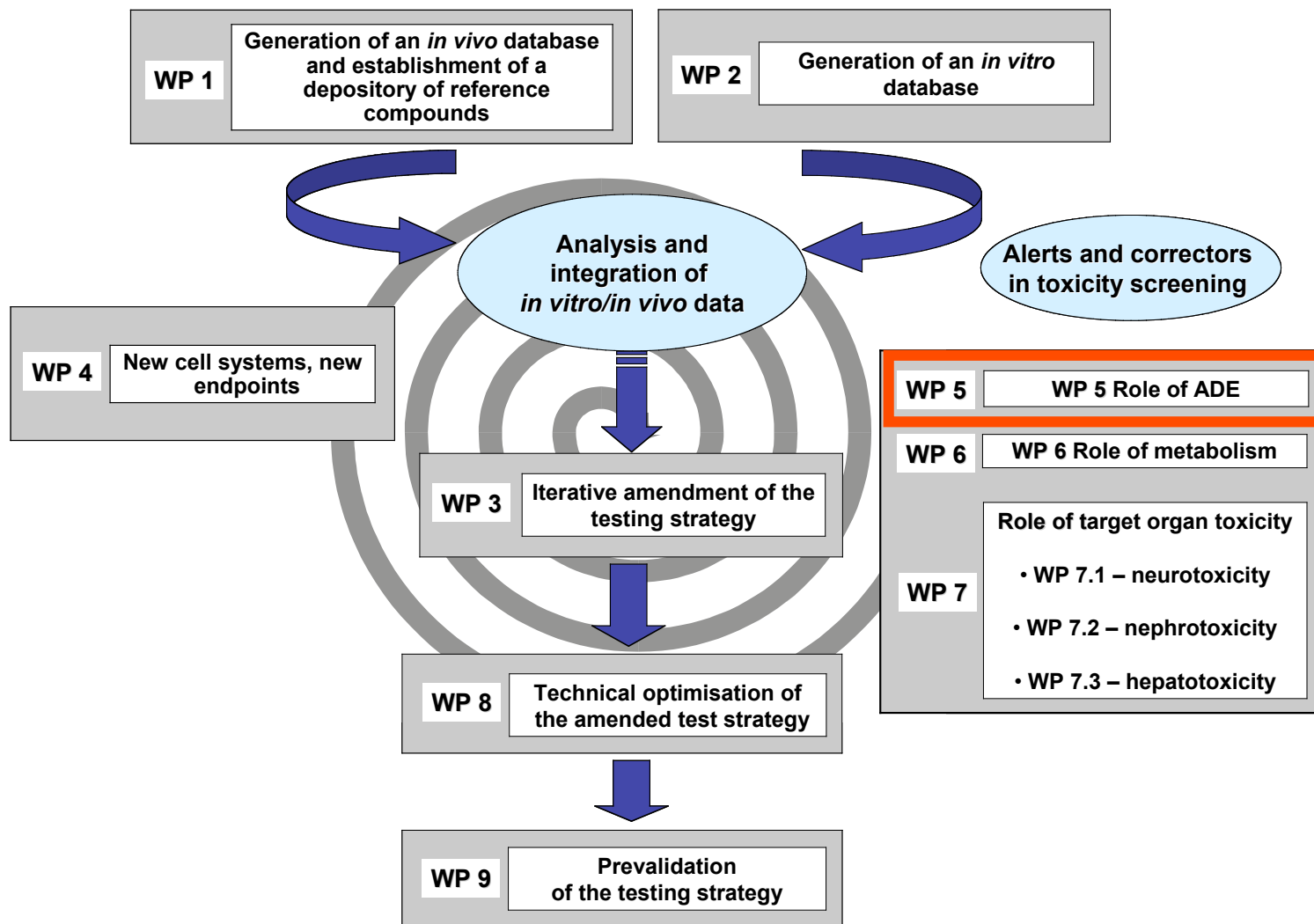
WP5: Role of ADE (in vitro/in silico)

- Measurement of the transport across the **intestinal barrier** and the **blood-brain barrier** using *in vitro models* and **neuronal networks** (n=21)

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

- Measurement (n=3) and modelling of free concentration of compounds in the *in vitro* systems.

- Generic biokinetic model for the interpretation of *in vitro* toxic concentrations in relation to the *in vivo* acute toxic dose – under development
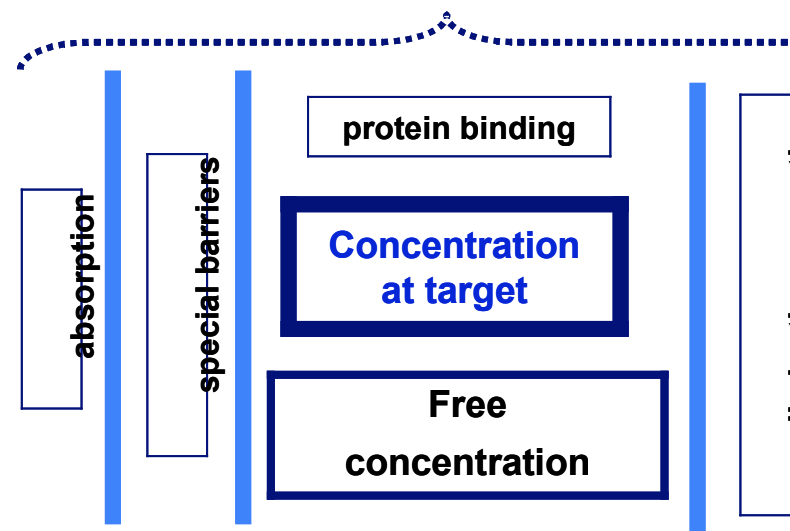
WP5: Oral absorption

ORAL ABSORPTION MODEL

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

HIA

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)

Transwell ®

pH 6.5

Caco-2 monolayer

pH 7.4

circular shaking
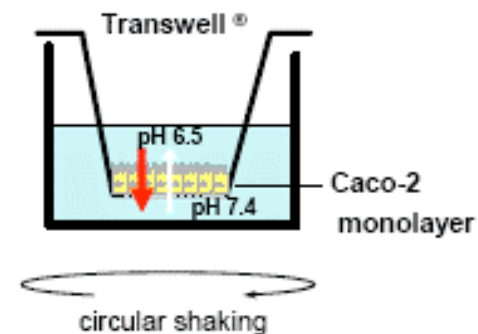
72% overall accuracy

WP5: Blood-brain barrier

BLOOD-BRAIN BARRIER PASSAGE MODEL

Chemical	LogBBpred	Class[b] P13	Class P35	Class P15	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)

Luminal compartment (Blood)

Coated microporous membrane

Abluminal compartment (Brain)
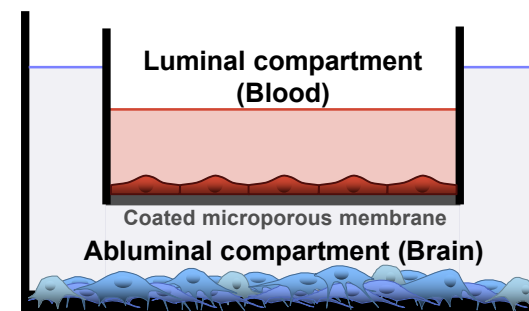
73% overall accuracy

WP6: Role of metabolism

Hepatocyte | Hepatoma

$IC50_A$ | $IC50_B$

Bioactivated
$IC50_A < IC50_B$

A B

Not Bioactivated
$IC50_A = IC50_B$
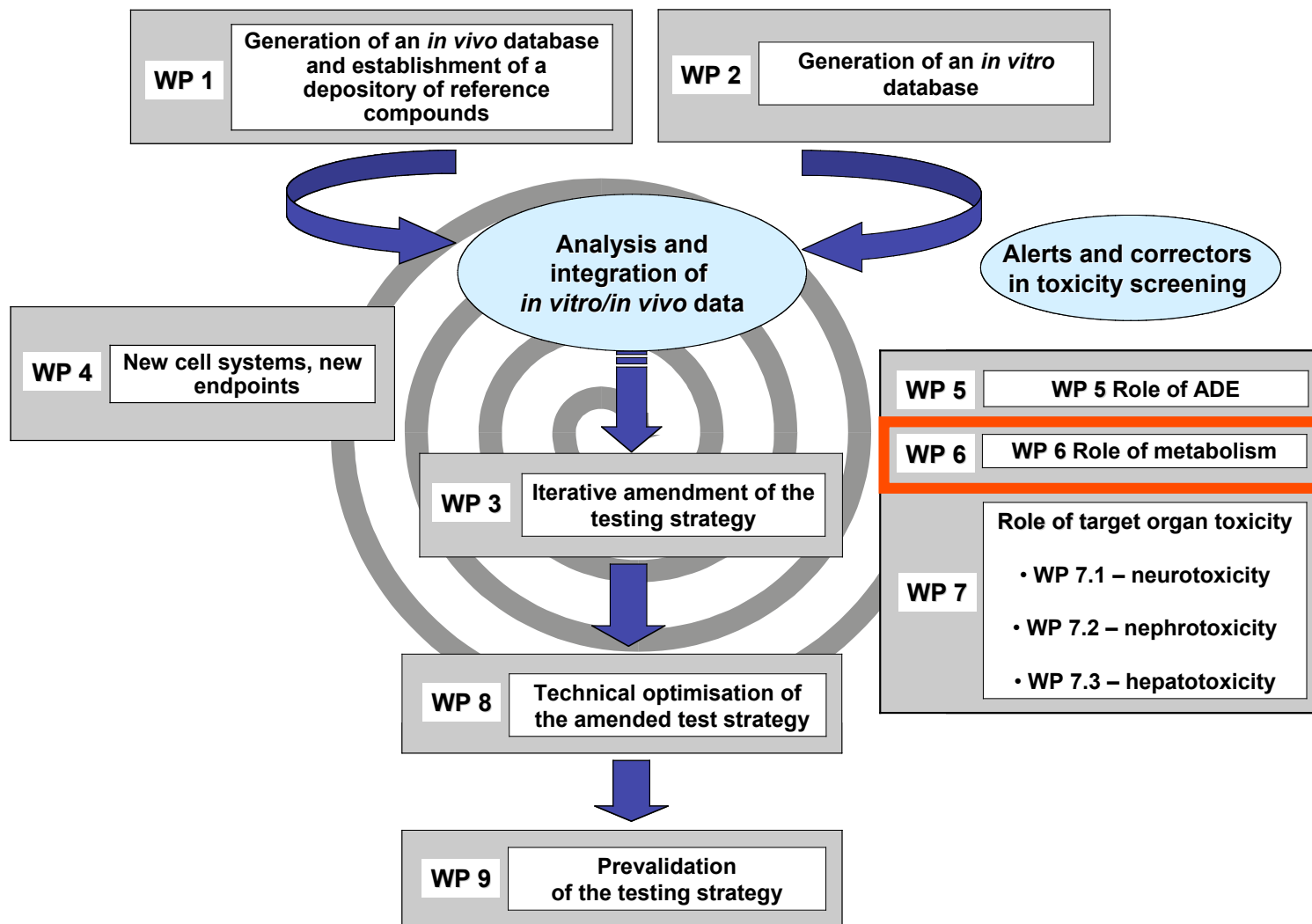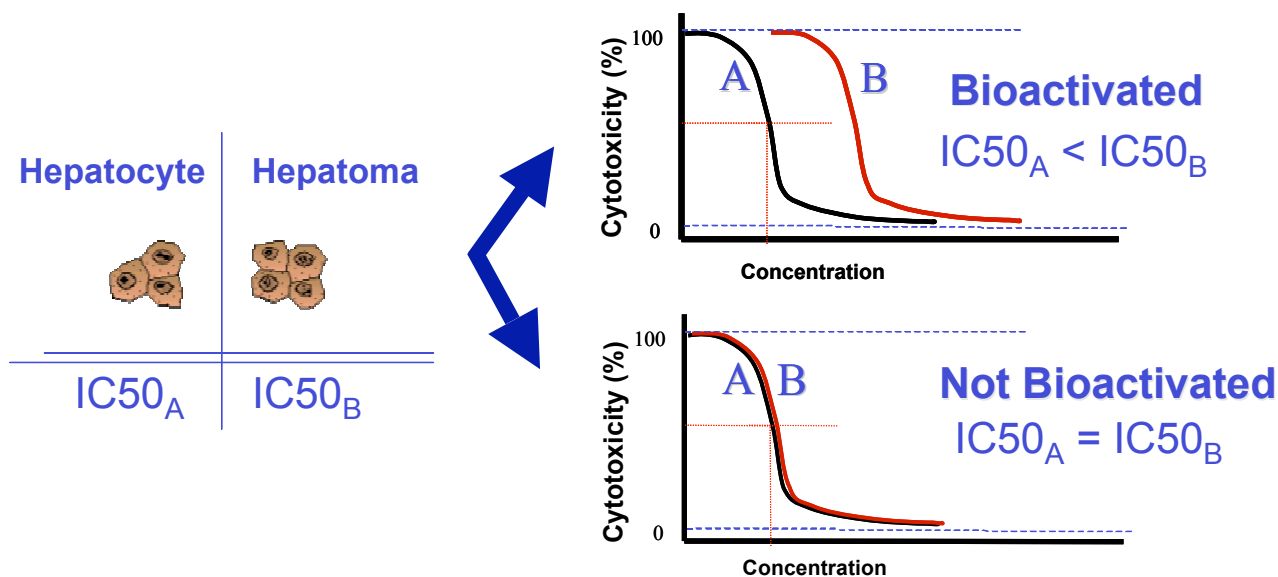
| Compound	Cas no	P15	P23	P31	Bayer	Mean	Comparison hepatocyes vs HepG2	Reported bioactivation
Atropine sulfate	5908-99-6	>1E-03	0,06	0,53	0,01	0,20	More toxic to hepatocytesthan to HepG2	YES
Mercury II	7487-94-7	0,04	0,18	0,75	0,18	0,29	More toxic to hepatocytesthan to HepG2	NO
Pentachlorophen	87-86-5	0,97	0,04	0,84	1,28	0,78	Slightly more toxic to hepatocytes than to HepG2	YES
Rifampicine	13292-46-1	0,85	0,56	1,18	0,67	0,82	Slightly more toxic to hepatocytes than to HepG2	NO
Tetracycline HCl	64-75-5	>1E-03	0,31	0,06	1,13	0,50	Slightly more toxic to hepatocytes than to HepG2	NO
Orphenadrine HCl	341-95-5	1,34	1,55	0,25	0,56	0,93	Similar toxicity to hepatocytes than to HepG2	NO
Diazepam	439-14-5	1,25	1,50	1,24	0,85	1,21	Similar toxicity to hepatocytes than to HepG2	NO
Malathion	121-75-5	1,46	1,46	>=1E-03	>1E-03	1,46	Similar toxicity to hepatocytes than to HepG2	YES
Amiodarone HCl	1951-25-3	1,35	1,02	1,10	1,54	1,25	Similar toxicity to hepatocytes than to HepG2	NO
SLS	151-2-3	1,63	0,42	1,69	1,45	1,30	Similar toxicity to hepatocytes than to HepG2	NO
Digoxin	20830-75-5	908,72	??	>=1000		>=1000	Less toxic to hepatocytes than to HepG2	NO
(±)-Verapamil HCl	152-11-4	8,85	2,75	0,31	1,70	3,40	Less toxic to hepatocytes than to HepG2	NO

WP 1 — Generation of an *in vivo* database and establishment of a depository of reference compounds

WP 2 — Generation of an *in vitro* database

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

Alerts and correctors in toxicity screening

WP 4 — New cell systems, new endpoints

WP 5 — WP 5 Role of ADE

WP 6 — WP 6 Role of metabolism

WP 3 — Iterative amendment of the testing strategy

WP 7 — Role of target organ toxicity
- WP 7.1 – neurotoxicity
- WP 7.2 – nephrotoxicity
- WP 7.3 – hepatotoxicity

WP 8 — Technical optimisation of the amended test strategy

WP 9 — Prevalidation of the testing strategy

WP7.1: Neurotoxicity

Human neuroblastoma SH-SY5Y cell line

- **Basal cytotoxicity**

- **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**
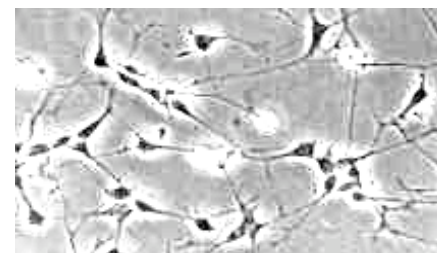
Serum-free aggregating rat brain cell cultures
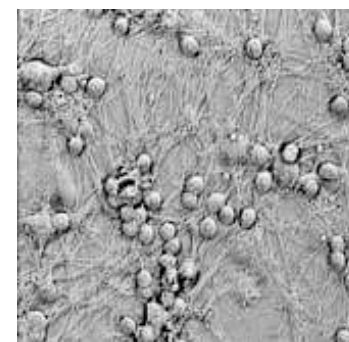
Primary cultures of cerebellar granule c

WP7.2: Nephrotoxicity

ions   **Cells: LLC-PK1**

BARRIER FUNCTION

- TER: sensitive indicator of nephrotoxicity
- TER: greater sensitivity for nephrotoxic chemicals
- Compounds requiring metabolism (diethylene glycol) did not show toxicity at concentrations used
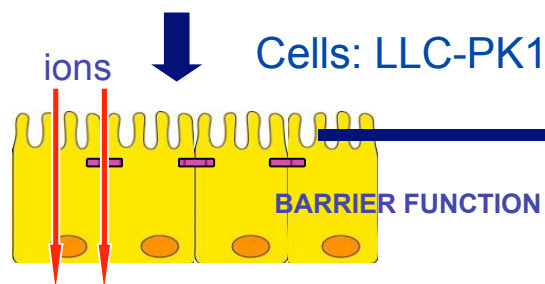
- Grown on permeable supports
- Current across epithelium
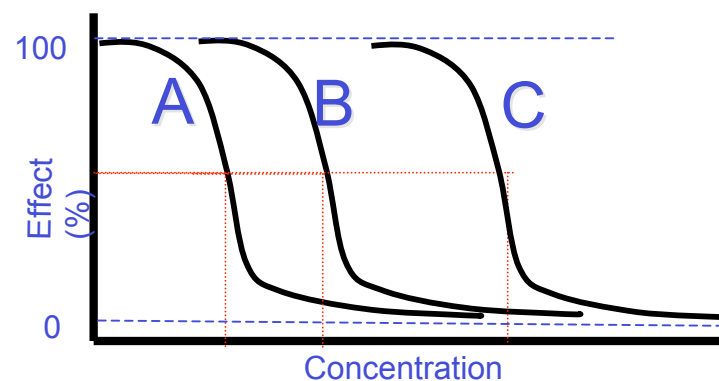- Rate of flux of ions
- Electrical resistance

REMS MACHINE

↓**TER**   -----------

↑**Permeability**

Loss of barrier function

WP7.3 Hepatotoxicity

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

$IC_{50}(A) < IC_{50}(B) \approx IC_{50}(C)$: "hepatotoxic" (bioactivable) → *alert*

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

$IC50(A) \approx IC50(B) \approx IC50(C)$: no hepatotoxic → *no alert*
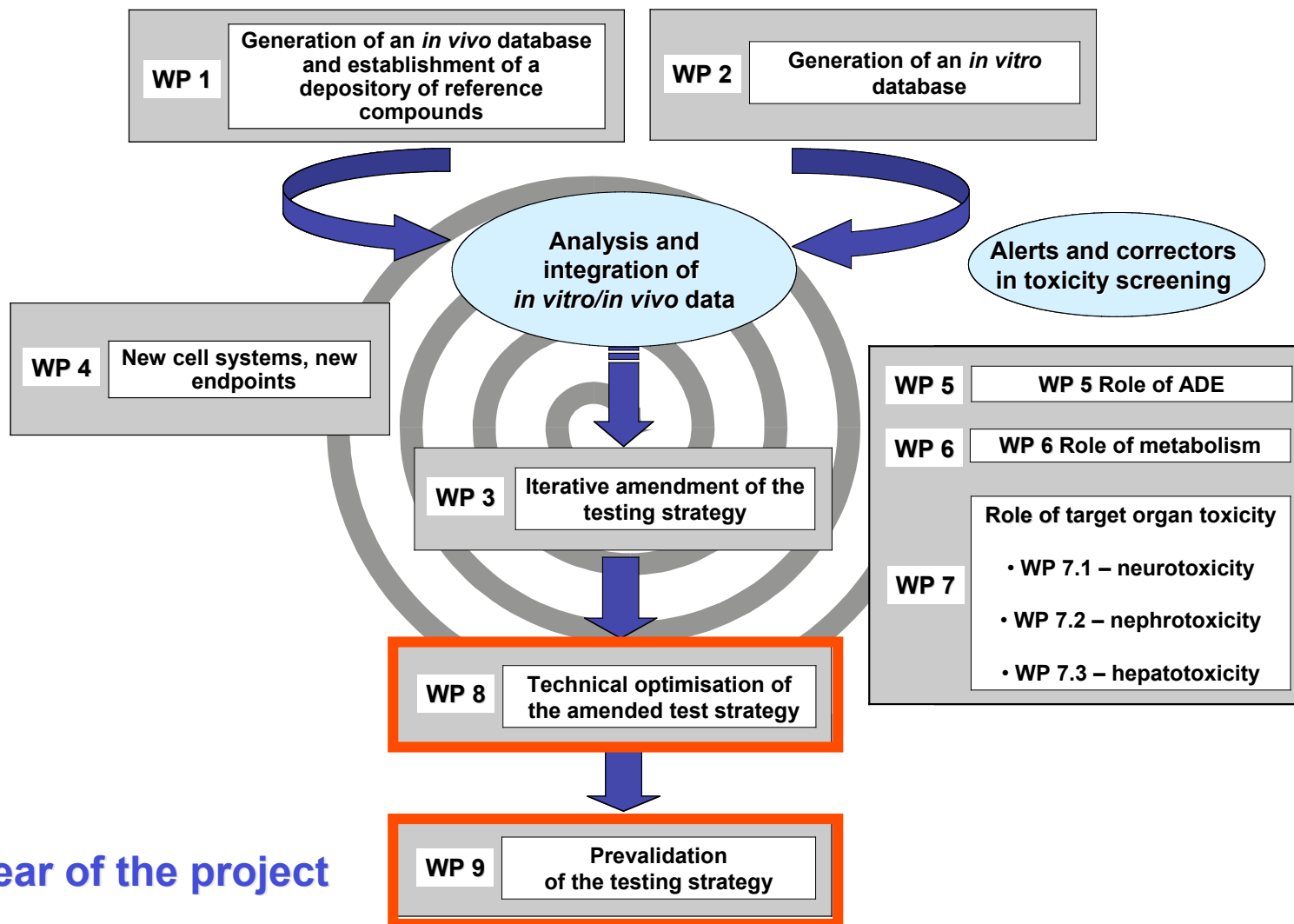
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Chair: T. Hartung (ECVAM)

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ECVAM follow-up validation study
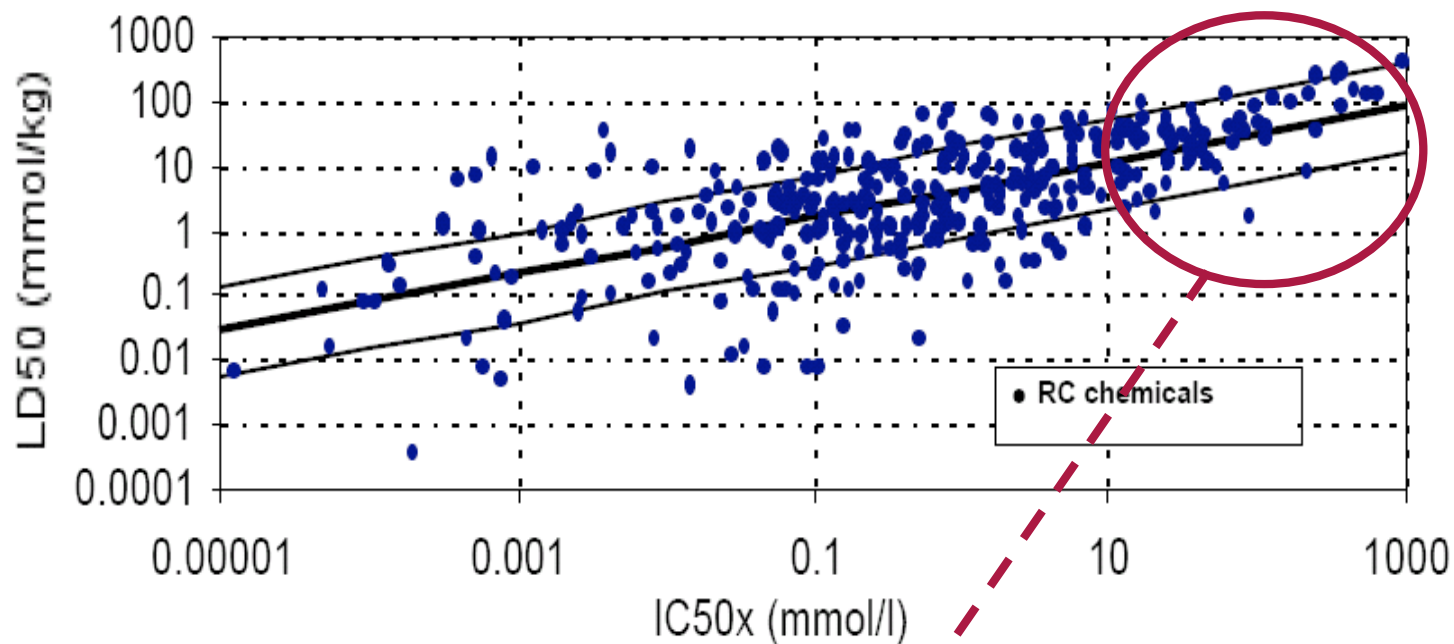
Chemicals (%)	LD50 (mg/Kg)
0	< 25
3	> 25 –200
21	> 200 –2000
76	> 2000

In this class are 76% of all new industrial chemicals

Ongoing evaluation of the 3T3 NRU assay for the prediction of non-toxic compounds