WP3: Iterative amendment of the testing strategy

WP3 is a central work package of the ACuteTox project, which aims to reduce step by step the outliers from the *in vitro/in vivo* correlations, by examining the underlying reasons for deviations, such as the quality of *in vivo* and *in vitro* data, lack of information on ADME and/or target organ specificity. Consequently, WP3 interacts intensively with all the other WPs to identify a set of assays that will provide valuable alerts and corrector factors. The selected assays will be further introduced into a testing strategy.

The objectives in WP3 are the following:
1. Provide a database platform (Acutoxbase) to facilitate storage of SOPs, transfer of data from all partners (*in vivo* human and animal data, *in vitro* experimental data) and statistical analyses of larger data sets.
2. Identify outliers from the *in vivo*-*in vitro* comparisons of data obtained from WP1 and WP2.
3. Support with competence in multivariate modelling as performed in the MEIC (Multicenter Evaluation of In Vitro Cytotoxicity) project (Ekwall et al., 1998, 2000) and to carry out modelling of new data generated in WP1, WP2, and WP4-7.
4. Adapt *in vitro* methods to robotic platforms for high-throughput screening (HTS).

1. Acutoxbase
A pilot version of Acutoxbase was opened to all ACuteTox partners in March 2006.

The database consists of the following sections:
Chemicals section – contains the list of 97 ACuteTox reference chemicals with information about the provider (company, Cat number), a set of physico-chemical properties and a short description of the chemical (common use, reported target organ toxicities, some information on human biokinetics, etc.).
Animal *in vivo* section - contains oral acute toxicity data from animal experiments collected from literature on the 97 reference chemicals.
Human poisoning cases - gathers available data on human acute poisoning cases.
***In vitro* section** – is the largest part of the database. An accurately designed, standardized template enables to report practically any experiment performed *in vitro*, including detailed information about the protocol used, the conditions in which the particular experiment was carried on, and the raw data. The in vitro part contains also a section where the SOPs for all in vitro assays used in this project, and truthfully revised by experts in the field, are stored.
Kinetics - includes data from *in vitro* kinetics experiments and in silico modeling.
Reports – enables to generate summaries of all data in form of Excel files.

At the beginning of 2009 Acutoxbase contained a full set of data regarding the selected 97 reference chemicals, including molecular structure, physicochemical properties and summary descriptions. Moreover, 2204 data from acute oral toxicity studies *in vivo*, 2902 data sets from *in vivo* human blood poisoning reports, 10.300 files from in vitro experiments and 90 SOPs were introduced into the database. In summary, Acutoxbase has proven to be a very useful platform for data management and data exchange in such a large international project as ACuteTox (Kinsner-Ovaskainen A. et al 2009).

2. The robotic platform for HTS
One of the endeavours of the ACuteTox project is to provide an *in vitro* testing strategy composed of methods that are amendable to robotic HTS platforms and thus, could be easily

automised. In WP3, the 3T3/NR uptake and HepG2/MTT assays have successfully been adapted to two commercially available HTS robotic platforms.

3-4. Multivariate modelling and identification of outliers

In vitro-in vivo modelling of LC_{50} values for humans and LD_{50} values for rats have been performed using data from the 3T3/NRU assay. The model shows a number of outliers (17 in the comparison with human LC_{50} values and 16 in the comparison with rat LD50 values) (Table 1).

Furthermore, the *in vitro-in vivo* linear regression analysis has shown that the *in vitro* basal cytotoxicity assays correlated better with human peak lethal blood concentrations (LC_{50}) than with LD_{50} rat values. From this analysis it was also concluded that the *in vitro-in vivo* correlation based on mol/l and mol/kg was better than g/l and g/kg.

During 2007-08, 57 compounds (including outliers and non-outliers) were tested in WP4-7. The *in vitro – in vivo* modelling of LC_{50} blood concentration values for humans and LD_{50} values, including 75 *in vitro* assays, and data for the 57 chemicals were performed in 2008. Six of the 75 in vitro assays were basal cytotoxicity tests (see results **WP2**) and the remaining tests were organ target specific tests for neurotoxicity, nephrotoxicity, hepatocytoxicity etc (see results **WP4-7**). Partial least squares regression was used for the comparison. With the aim to find subsets of *in vitro* tests with good predictive capability, the influence of variable (assay) reduction was studied. The results showed that small batteries of a few *in vitro* tests give better correlations than batteries with many tests, both for models based on LD_{50} rat (R^2=0,59 and Q^2=0,57) and LC_{50} human (R^2=0,71 and Q^2=0,69). The assays contributing to the best models were mainly basal cytotoxicity tests and the target organ specific tests did only improve R^2 and Q^2 with about 0.02, compared models based on only basal cytotoxicity tests.
A principal component analysis based on four basal cytotoxicity tests and eight target organ specific tests showed that the information content is similar in all tests and could be summarized in two principal components that described 88% of the variance in the data.
The models contained many chemicals that deviated more than +-0,75log unit. For the best LD_{50} model, the most deviating chemicals with negative residuals (-0,75 to -2 log units) are Strychnine, Physostigmine, Warfarin, Sodium selenate, Parathion, Nicotine, Cycloheximide, and Epinephrine and with positive (0,75 to 1,5 log units) residuals are Amiodarone, Sodiumlauryl sulphate, 17a- Ethynylylestradiol, Cadmium chloride, Carbamazepine. For LC_{50} for humans the most deviating chemicals with negative residuals are Colchicine, Nicotine, Lindane, Atropine, Acetonitrile, Strychnine, Malathion, Cyclosporine, Parathion and the chemicals with positive residuals are Pentachlorphenol, Isopropyl alcohol, 2,4 dichlorphenoxyacid Dichlorvos, Diquat dibromide and Acetylsalicylic acid.

The result that small batteries of basal cytotoxicity tests predict acute human toxicity better than individual tests or models based on many test are similar to the results obtained in the MEIC study. Based on the results from the statistical analyses presented above, the ACuteTox Consortium draw the conclusion that further analysis of data is needed in order to select methods for the testing strategy. In 2009 a biostatistician was subcontracted to perform other statistical analysis using the raw data submitted by the partners to Acutetoxbase (see news from the project and results of WP9).

Table 1. Outliers (in bold) identified by comparing mean values of 3T3/NRU assay with LD_{50} and human LC_{50} values

	Reference Chemicals	Outliers (rat oral LD_{50})	Outliers (human LC_{50})	Comments
1	**acetaminophen**		**x**	
2	acetylsalicylic acid			
3	**atropine sulfate monohydrate**		**x**	
4	caffeine			
5	carbamazepine			
6	colchicine	No animal data		Variable in vitro data
7	cycloheximide		No human data	Variable in vitro data
8	diazepam			
9	**digoxin**	**x**	**x**	Variable in vitro data
10	isopropyl alcohol			
11	**malathion**		**x**	
12	mercury (II) chloride			
13	**pentachlorophenol**		**x**	Poor human data
14	**phenobarbital**	**x**		Poor human data
15	sodium lauryl sulfate			
16	sodium valproate			
17	**5-fluorouracil**		**x**	Variable in vitro data
18	benzene		No human data	
19	tert-butylhydroperoxide		No human data	
20	acrylaldehyde (acrolein)		No human data	
21	cadmium (II) chloride			Poor human data

22	phenanthrene		No human data	
23	pyrene		No human data	
24	1,2,3,4-tetrachlorobenzene		No human data	
25	pentachlorobenzene		No human data	
26	hexachlorobenzene		No human data	Variable in vitro data
27	benz(a)anthracene	No animal data	No human data	No in vivo data
28	amiodarone hydrochloride			Poor human data
29	(±)-verapamil hydrochloride			Poor human data
30	rifampicine			
31	tetracycline hydrochloride		No human data	
32	orphenadrine hydrochloride			
33	**nicotine**	**x**	**x**	Poor human data
34	**lindane**		**x**	Poor human data
bis 29	(±)-verapamil hydrochloride			
35	**D-amphetamine sulfate**	**x**	**No human data**	Restricted
36	**methadone hydrochloride**		**x**	Restricted
37	ethanol			
38	**parathion**	**x**	**No human data**	
39	dichlorvos		No human data	
40	**physostigmine**	**x**	**No human data**	
41	glufosinate-ammonium			Poor human

				data
42	***cis*-diammineplatinum (II) dichloride**		**x**	
bis 21	cadmium (II) chloride			
43	diethylene glycol		No human data	
44	**diquat dibromide**		**x**	
45	**ochratoxin A**	**x**	No human data	
bis 28	amiodarone hydrochloride			
bis 31	tetracycline hydrochloride		No human data	
bis 30	rifampicine		No human data	
46	**cyclosporine A**		**x**	
47	17α-ethynylestradiol		No human data	
48	sodium fluoride			
49	paraquat dichloride			
50	glycerol			
51	dimethylformamide			Poor human data
52	iron II sulfate			
53	amitriptyline hydrochloride			
54	ethylene glycol			
55	methanol			
56	phenol			Poor human data
57	**sodium chloride**		**x**	
58	xylene			
59	**potassium cyanide**	**x**		
60	lithium sulfate			

61	theophylline				
62	haloperidol				
63	propranolol hydrochloride				
64	arsenic trioxide				
65	thioridazine hydrochloride				
66	**thallium sulphate**	**x**			
67	**warfarin**	**x**			Poor human data
68	chloroform				
69	isoniazid				
70	dichloromethane				
71	hexachlorophene				
72	chloroquine diphosphate				
73	5,5-diphenylhydantoin				
74	chloramphenicol				
75	potassium chloride				
76	chloral hydrate				
77	**2,4-dichlorophenoxyacetic acid**		**x**		
78	meprobamate				
79	sodium pentobarbital				
80	**strychnine**	**x**	**x**		
81	glutethimide				
82	maprotiline				
83	disopyramide				
84	diphenhydramine				
85	chlormethiazole				
86	quinidine sulfate dihydrate				
87	procainamide hydrochloride				
88	**codeine**		**x**		Restricted
89	chlorpromazine hydrochloride				Poor human data

90	paraldehyde			Poor human data
91	**sodium selenate**	**x**		
92	acetonitrile			
93	sodium bicarbonate			
94	acrylamide			
95	**formaldehyde**	**x**	**No human data**	Not testable
96	**(-)-epinephrine**	**x**	**No human data**	
97	acetaldehyde			

References:

Ekwall, B. et al (1998) MEIC evaluation of acute systemic toxicity: Part VI. The prediction of human toxicity by rodent LD50 values and results from 61 *in-vitro* methods. *ATLA* 26: 617-658.

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