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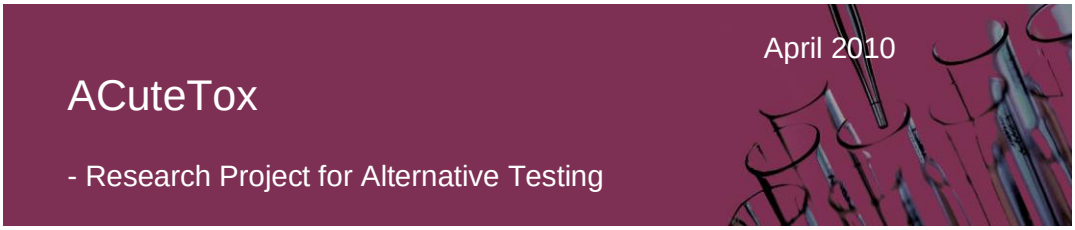

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The ACuteTox prevalidation phase has started

As a result of the below described statistical analysis, potential assays for the ACuteTox testing strategy have been selected (see Table 1) according to their reproducibility and reliability as well as to their potential to classify chemicals into the official acute oral toxicity classes (GHS[1] – global harmonisation system and CLP Regulation in EU). The selected tests are now challenged with a new set of 32 coded chemicals in a pre-validation study. During this last phase of the ACuteTox project the main focus will be on the assessment of the predictive capacity of three proposed tiered testing strategies and the identification of the combination that gives the best prediction.

What is the ACuteTox project?

ACuteTox is an integrated project funded by the EU 6FP, with the aim to develop and pre-validate a simple and robust testing strategy based on *in vitro* and computer models for the prediction of human acute oral systemic toxicity and classification of chemicals into the different EU and GHS[1] toxicity classes. Fast, cheaper and scientifically better tests for testing chemicals, cosmetics, and other products are urgently needed to replace the animal acute oral toxicity tests used today for regulatory purposes.

Table 1. The best performing in vitro assays, which were selected as candidates for the final tiered testing strategy, are further evaluated in the pre-validation phase.

Selected assay	Target (workpackage involved)
The neutral red uptake assay using the 3T3 fibroblast cell line (3T3/NRU)	General basal cytotoxicity (WP2)
The cytokine release assay using human whole blood (IL-1, IL-6, TNF-alpha)	Haemotoxicity (WP 4)
Cell differentiation in human cord blood-derived cells (CBC/CFU-GM)	Haemotoxicity (WP 4)
Gene expression (GFAP, HSP-32, MBP and NF-H) in primary rat brain aggregate cultures (see Figure 1)	Neurotoxicity (WP 7.1)
Uridine incorporation measuring the total mRNA synthesis in primary rat brain aggregate cultures	Neurotoxicity (WP 7.1)
Cytomic panel measuring oxidative stress (intracellular peroxidative activity, intracellular levels of superoxide anion, oxidized DNA base 8-oxoguanine) in HepG2, SH-SY5Y and A.704 cells	New endpoints (WP 4)
Cytomic panel for cytotoxicity screening (intracellular Ca^{2+} levels, mitochondrial membrane potential, plasma membrane potential) in HepG2, SH-SY5Y and A.704 cells	New endpoints (WP 4)
The MTT assay using primary rat hepatocytes	Metabolism (WP 6)
Kinetic parameters: volume of distribution, protein binding, clearance, and oral absorption (Caco-2 cells) for the estimation of the oral dose from the effective concentration observed *in vitro*	Biokinetics (WP5)
The estimation of compound passage through the blood-brain barrier using neuronal networks (for neurotoxicity assays) (see Figure 2)	Biokinetics (WP5)

EU contract no. LSHB-CT-2004-512051

Background

Starting in 2005, 24 European laboratories have tested 57 reference compounds in 75 *in vitro* assays, including assessment of basal cytotoxicity, organ- and system-specific toxicity, as well as analysis of biokinetic parameters. The generated data were used to assess the within-laboratory variability, the preliminary predictive capacity and in some cases also the between-laboratory variability of each *in vitro* assay. The outcome of this phase of the project is a large toolbox of *in vitro* methods, some of them evaluated to the level of prevalidation (Table 2 in Appendix).

The first approach to analyse the i*n vitro* data: multivariate PLS analyses

The *in vitro* IC_{50} values calculated in each laboratory were correlated with human (LC_{50} blood concentration values) and animal (oral LD_{50} values for rodents) data.

The results showed that the combination of 3 *in vitro* assays (3T3 cells/Neutral red uptake, Normal human keratinocytes/Neutral red uptake, Brain re-aggregation cultures/gene expression) slightly improve the correlation between *in vitro* IC_{50} and *in vivo* LD_{50} (R^2=0.59) and *in vitro* IC_{50} and human LC_{50} (R^2=0.71) compared to a single cytotoxicity assay (3T3 cells/Neutral red uptake).

However, a number of misclassified compounds were identified using this combination of assays.

When the *in vitro* data were correlated with animal LD_{50} data, physostigmine, warfarin, sodium selenate, parathion, nicotine and epinephrine were under-predicted, while amiodarone, 17-ethenylestradiol, sodium lauryl sulphate, cadmium chloride and carbamazepine were over-predicted.

When the *in vitro* data were correlated with the human LC_{50} data, colchicine, nicotine, lindane, atropine, acetonitrile, strychnine, malathion, cyclosporine, and parathion were under predicted. Pentachlorphenol, isopropyl alcohol, 2,4 dichlorphenoxyacid, dichlorvos, diquat dibromide and acetylsalicylic acid were over-predicted.

By including kinetic data (volume of distribution, protein binding, clearance, and oral absorption) in the in vitro models, the predication of rat LD_{50} values could be increased from R^2=0,59 to R^2=0,70.

However, the combination of three assays did not improve the prediction of the different EU and GHS[1] toxicity classes when compared to a single cytotoxicity assay (e.g. 3T3 cells/Neutral red uptake).

Based on the results from this multivariate analysis, it was concluded that further statistical analysis of data was needed in order to select the best performing methods for the testing strategy.

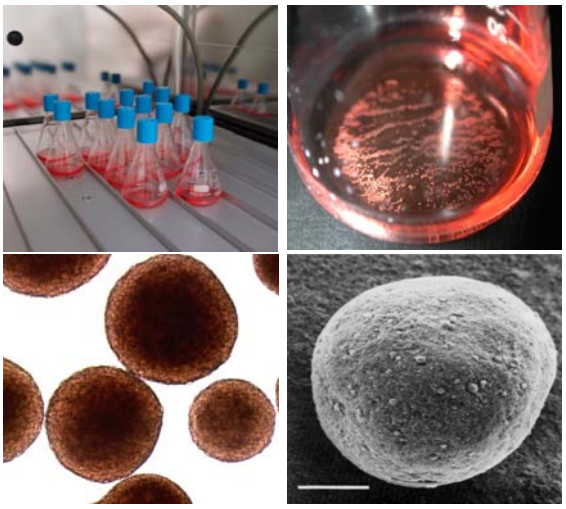

Figure 1.

Re-aggregated brain cell cultures. Top row: the cultures are kept in Erlenmayer flasks under constant rotation in an incubator. Bottom row: Microphotographs of rat brain aggregates (20 days *in vitro*)

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Additional statistical analyses aimed to select assays for the testing strategy

The selection of the *in vitro*/*in silico* methods for the prevalidation exercise from the total number of assays performed in the ACuteTox project was based on an *ad hoc* analysis of variability, repeatability and reproducibility of the single assays, as well as the assessment of preliminary predictive capacity using univariate and multivariate CART analyses (Table 2 in Appendix).

The first step of the analysis consisted in the re-calculation of EC_{20}, EC_{50} or LOEC values for every experiment using a standard approach. The assays were then compared with respect to their reproducibility and reliability by evaluating the variability of the assays at the level of raw response data and at the level of the estimated EC_{20}, EC_{50} or LOEC values.

Since the ultimate aim of the statistical analysis for ACuteTox is the classification of chemicals into the official acute oral toxicity categories (EU and GHS[1]), the Classification and Regression Trees (CART) was used as the classification algorithm of choice. CART is a non-parametric technique that produces either classification or regression trees, depending on whether the dependent variable is categorical or numeric, respectively. The application of the CART methodology meets with the approach suggested by the initial aim of ACuteTox, namely to 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. The CART analyses were performed using the untransformed EC_{50}, EC_{20} and LOEC values from the *in vitro* assays. In addition, for the neurotoxicity assays, a blood brain barrier (BBB) transformation was performed. Another approach was used in which the EC_{50}, EC_{20} and LOEC values from the *in vitro* assays were first transformed to rat LD_{50} values using a transformation algorithm suggested by WP5 (kinetic transformations), which took into account, the intestinal absorption, protein binding, clearance and volume of distribution. After derivation of a CART tree, misclassification rate was estimated from the data.

Twelve CART multivariate models have been investigated and three potential testing strategies have been identified. The best CART multivariate model found until now classified 65% (37 of the 57 reference chemicals) correctly into the 5 GHS acute oral toxicity classes. Toxicity was under-predicted for five compounds, by one hazard class for three compounds, by two classes for one compound and by three classes for one compound. Accordingly, toxicity was over-predicted for 15 of the compounds tested, by one hazard class for ten compounds, by two classes for three compounds and by three classes for two compounds. This misclassification rate is expected to be too optimistic because the correct classification rates are estimated from the training set. An unbiased estimate of the misclassification rate will be obtained from the application of the three selected CART trees to independent data, such as the data of the challenging exercise (prevalidation) that will involve testing of a new set of chemicals.

The database of the project

The “Acutoxbase” database is a “working” database that allows ACuteTox partners to submit and share in vitro and in vivo data online. The structure of the database is outlined in the publication *Kinsner-Ovaskainen et al, Toxicol In Vitro. 2009 23(3):476-85*.

The largest section of the database is of the part containing the *in vitro* data from all assays evaluated in the project. It also contains *in vivo* LD_{50} data on the 97 project chemicals (50% of which were drugs and 30% industrial chemicals, 20% others) as well as human data (human lethal blood concentrations). All *in vivo* data were collected from the literature.

1 The Globally Harmonized System (GHS) is an internationally standardized system for classifying chemicals. For acute oral toxicity the GHS categories are assigned based on the LD50 values: GHS 1 (LD50 < 5 mg/kg b.w., fatal if swallowed); GHS 2 (LD50 > 5 mg/kg < 50 mg/kg b.w., fatal if swallowed); GHS 3 (LD50 > 50 mg/kg < 300 mg/kg b.w., toxic if swallowed); GHS 4 (LD50 > 300 mg/kg < 2000 mg/kg b.w., harmful if swallowed); GHS 5 (LD50 > 2000 mg/kg b.w., may be harmful if swallowed; or LD50 > 5000 mg/kg, not classified).

It is envisaged that Acutoxbase will be made publicly available after the project is terminated. Parts of the data collected in the database have been already published:

- The human LC_{50} data are available in the publication by Sjöström et al. *Toxicol in Vitro 22 (2008) 1405–1411*.
- The summary of the 6 cytotoxicity assays (including all values) is presented in Clothier et al., *ATLA **36**, 503–519, 2008*
- The IC_{50}, IC_{70} and IC_{90} values for the chemicals tested in CFU-GM assay in Cerrato et al. *Toxicol Appl Pharmacol 238, 111-119*
- Effects on GABA-A receptor, GABA transport and Glutamate transport in primary cultured cortical neurons (neurotoxicity assays, EC_{50} values) are summarized in Galofre et al. *Neurotoxicol Teratol. 2010, 32(1):52-61*.
- Evaluation of transcriptional activity of caspase-3 gene as a marker of acute neurotoxicity in rat cerebellar granular cells (IC_{50} values published in Folch et al., *Toxicol In Vitro. 2009*).
- Neurofunctional endpoints assessed in human neuroblastoma SH-SY5Y cells for estimation of acute systemic toxicity are presented in Gustafsson et al. *Toxicol Appl Pharmacol*. 2010–in press
- doi:10.1016/j.taap.2010.02.018
- The publication on the analysis of the *in vivo* rat and mouse data and the summary tables of the LD_{50} values is in preparation.

Next Newsletter will be published in June 2010

More information about the project and publications can be found at www.acutetox.org

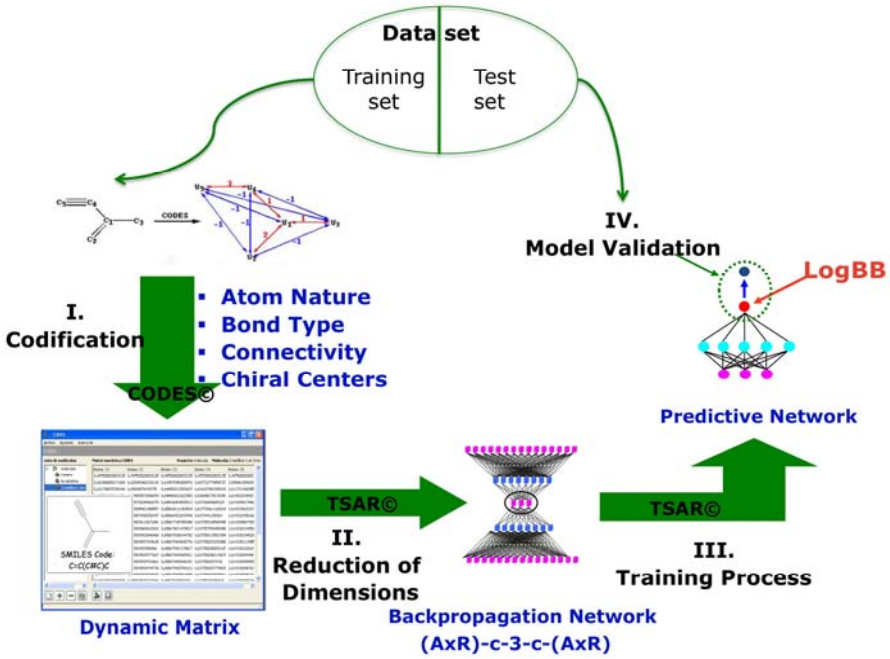

Figure 2.

Strategy used to develop a neural network model to predict the passage of the blood-brain barrier. The key steps of this methodology are: I) codification using CODES program, II) reduction of dimensions of the dynamic matrix, III) training of the neural model and IV) model validation

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Table 2 *In vitro* assays evaluated in the different Work Packages (WPs) of the ACuteTox project. The Table presents the status of the assays, including an indication whether within-laboratory reproducibility, between-laboratory reproducibility and preliminary predictive capacity were evaluated.

***In vitro* assay**	**Test system**	**Within-lab reproducibility**	**Between-lab reproducibility**	**Preliminary predictive capacity**	**Candidates for tiered testing strategy**
Cytotoxicity					
ATP content	HL-60 human cell line	X			no
Protein content (CBQCA assay)	HepG2 human hepatoma cell line	X			no
Protein content (CBQCA assay)	Fa32 rat hepatoma cell line	X			no
Neutral red uptake	Balb/3T3 mouse fibroblasts	X	X	X	**YES**
Neutral red uptake	Normal human keratinocytes	X	X	X	no
Neutral red uptake	Fa32 rat hepatoma cell line	X			no
New cell systems, new endpoints					
Multiplexed flow cytometry analysis of cytokine release (IL-12p70, IFN-γ, IL-2, IL-10, IL-8, IL-6, IL-4, IL-5, IL-1β, TNF-α, TNF-β)	Human peripheral blood mononuclear cells (PBMC)	X		X	**YES**
ELISA analysis of cytokines IL-5, IFN-γ and TNF-α release	Human peripheral blood mononuclear cells (PBMC)	X		X	no
Cytokine release (IL-1, IL-6, TNF-α)	Human whole blood			X	**YES**
Colony forming unit-granulocyte/macrophage (CFU-GM)	Human cord blood cells	X	X	X	**YES**
Cytomic Panel for Cytotoxicity Screening including:	Performed in three cell lines: - A.704 kidney	X		X	**YES**

- intracellular Ca^{2+} (Fluo-4 probe) - mitochondrial membrane potential (rhodamine123) - plasma membrane potential (DIBAC probe) - intracellular lipid content (BODIPY probe)	adeno-carcinoma - HepG2 human hepatoma cell line - SH-SY5Y human neuroblastoma cell line				
Cytomic Panel for Oxidative Stress Screening including: - intracellular peroxides - mitochondrial generation of superoxide - intracellular levels of the oxidized DNA base 8-Oxo-Guanine	Performed in three cell lines: - A.704 kidney adeno-carcinoma - HepG2 human hepatoma cell line - SH-SY5Y human neuroblastoma cell line	X		X	**YES**
Biokinetics					
PAMPA assay	Not available				no
Intestinal absorption (permeability assay)	Caco-2 intestinal cell line*	X	X	X	**YES, a)**
Intestinal absorption (toxicity assay - ^{14}C-Mannitol)	Caco-2 intestinal cell line*	X	X	X	no
Intestinal absorption (toxicity assay – Lucifer yellow)	Caco-2 intestinal cell line*	X	X	X	no
Blood-brain barrier passage (permeability assay)	Blood-brain barrier model **	X			no
Blood-brain barrier (toxicity assay – Lucifer yellow)	Blood-brain barrier model **	X			no
Blood-brain barrier (toxicity assay - ^{14}C-sucrose)	Blood-brain barrier model **	X			no
Aqueous solubility	Not available	See note a)			**YES**
Plasma protein	Pooled human	See note a)			**YES**

binding	plasma***				
Chromatographic Hydrophobicity Index (CHI)	Not available				
Metabolic stability	Rat liver micro-somes****	See note a)			**YES**
Metabolic stability	Human liver micro-somes****	See note a)			**YES**
Metabolic stability	Primary rat hepatocytes	See note a)			**YES**
Metabolic stability	Cryopreserved human hepatocytes	See note a)			**YES**
Neurotoxicity					
Alamar Blue assay	Primary rat cerebellum granule cells (CGCs)	See note b)			no
Mitochondrial membrane potential	Primary rat cerebellum granule cells (CGCs)	See note b)			no
AChE inhibition	Pure enzyme	See note b)			no
AChE inhibition	SH-SY5Y human neuroblastoma cell line	X			no
LDH leakage	SH-SY5Y human neuroblastoma cell line	See note b)			no
LDH leakage	Rat brain slices	See note b)			no
Ca^{2+} overload	SH-SY5Y human neuroblastoma cell line	See note b)			no
GABA-A receptor function	Primary mouse cortical neurons	X		X	no
GABA uptake	Primary mouse cortical neurons	See note b)			no
Cell membrane potential (CMP)	Primary mouse cortical neurons	See note b)			no

Glutamate uptake	Primary mouse cerebellum granule cells (CGCs)	See note b)			no
Cell membrane potential (CMP)	SH-SY5Y human neuroblastoma cell line	X		X	**no**
Noradrenalin uptake	SH-SY5Y human neuroblastoma cell line	See note b)			**no**
Voltage operated Ca^{2+} channel function	SH-SY5Y human neuroblastoma cell line	See note b)			**no**
Acetylcholine receptor function	SH-SY5Y human neuroblastoma cell line	See note b)			**no**
Gene expression (GFAP, MBP, NF-H, NF-M, PPAR-gamma, HSP-32, iNOS)	Rat re-aggregated brain cells culture	X		X	**YES**
Enzyme inhibition/activation (ChAT, GS, AChE, LDH, 2,3-CNP)	Rat re-aggregated brain cells culture	See note b)			no
Methionine uptake (protein synthesis)	Rat re-aggregated brain cells culture	X			no
Uridine uptake (RNA synthesis)	Rat re-aggregated brain cells culture	X		X	**YES**
2-deoxyglucose uptake	Rat re-aggregated brain cells culture	X		X	no
Caspase-3 mRNA expression	Primary rat cerebellum granule cells (CGCs)	X			no
MTT assay	Primary rat cerebellum granule cells (CGCs)	See note b)			no
Glutamate induced cytosolic Ca^{2+} increase	Primary rat cerebellum granule cells	See note b)			no

	(CGCs)				
ROS production	Primary rat cerebellum granule cells (CGCs)	See note b)			no
LDH release	Primary rat cerebellum granule cells (CGCs)	See note b)			no
Microarray quantitative mRNA expression analyses of 31 genes	Primary rat cerebellum granule cells (CGCs)	See note b)			no
Nephrotoxicity					
Alamar Blue	LLCPK-1 renal epithelial *cell* line	X		X	no
Transepithelial electrical resistance (TEER)	LLCPK-1 renal epithelial *cell* line	X		X	no
Hepatotoxicity, metabolism and bioactivation					
MTT assay	Rat hepatocytes	X	X	X	**YES**
MTT assay	Balb/3T3 mouse fibroblasts	X	X	X	no
MTT assay	HepG2 human hepatoma cell line	X	X	X	no

[a)] WP5 - These assays provided input data for the PBBK modelling (biokinetics)

[b)] WP7.1 – only 20 chemicals have been tested.

** Three different protocols for culturing Caco-2 cells and for the assessment of toxicity and prediction of intestinal absorption in Caco-2 cells have been evaluated in WP5. The cell models were: Caco-2/TC-7 clone, CacoReady™ (Advancell, Spain), Caco-2 from ATCC.*

*** Two different models of Blood-brain barrier (BBB), as well as three different protocols for the assessment of toxicity and prediction of BBB passage have been evaluated in WP5*

**** Two different protocol for plasma protein binding were used in WP5*

***** Two different protocol for rat and human microsomal stability were used in WP5*