



ACuteTox

- Research Project for Alternative Testing

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ACuteTox: Prevalidation, Highlights and Main Achievements of the Project

Currently, acute oral toxicity is assessed in rats in accordance with the OECD Test Guidelines 420 (Fixed Dose Procedure, FDP), 423 (Acute Toxic Class Method, ATC), 425 (Up and Down Procedure, UDP).

The ACuteTox project undertook, for the first time, a challenging goal to develop and optimise an integrated testing strategy to replace the animal testing used today for predicting human acute oral systemic toxicity, which is based exclusively on *in vitro* and *in silico* methods. This project represents also the first attempt to prevalidate a testing strategy based exclusively on non-animal methods.

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 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. The project was finalized in June 2010.

In this newsletter we focused on the outcome of the prevalidation study performed during the 6-month extension period of the ACuteTox project. This second phase of the project centered mainly on the assessment of the predictive capacity of few tiered testing strategies that were proposed based on results generated in the first phase, and on the identification of the combination of test methods that gave the best prediction in terms of classification of chemicals in the official acute oral toxicity categories. Furthermore, we sum up the overall conclusions from the ACuteTox project.



Lessons learned from the Prevalidation exercise

Test methods considered as promising building blocks for the testing strategy were identified in the first phase of the project on the basis of the so-call training set of compounds (57 compounds used in the optimisation phase of the project) (see Table 1).

During the Prevalidation phase, these selected test methods were challenged under blind conditions with a new set of 32 test compounds. This work was undertaken in Work Package 9 (WP9).

Table 1. The best performing *in vitro* assays, which were selected during the optimisation phase of ACuteTox as candidates for the final tiered testing strategy, and further evaluated in the prevalidation phase.

Selected assay	Target (work package 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)	Haematotoxicity (WP 4)
Cell differentiation in human cord blood-derived cells (CBC/CFU-GM)	Haematotoxicity (WP 4)
Gene expression (GFAP, HSP-32, MBP and NF-H) in primary rat brain aggregate cultures	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 <i>in vitro</i>	Biokinetics (WP5)
The estimation of compound passage through the blood-brain barrier using neuronal networks (for neurotoxicity assays)	Biokinetics (WP5)



Specific objectives of WP9

- To guarantee that the protocols of the selected test methods were sufficiently standardised and optimised (module 1 of ECVAM's modular approach).
- To assess the reproducibility of each test method (building block) composing the final tiered testing strategy, using a defined set of chemicals (module 2 of ECVAM's modular approach).
- To perform a preliminary assessment of the relevance of the final tiered testing strategy (module 5 ECVAM's modular approach).

Participant laboratories

Due to time constraints the assessment of transferability of the methods to a second independent laboratory was not feasible, as it requires extensive training of the naïve laboratory. Therefore, laboratories which were previously involved in the development and optimisation of the selected test methods, took part in the prevalidation exercise.

The nine participant laboratories tested the coded chemicals in a pre-defined order, in three phases (10-11 chemicals per phase), according to the study plan approved in January 2010.

Raw data were sent to DKFZ (biostatisticians) for calculation of concentration-response curves and calculation of the characteristic values (see below).

The laboratories reported solubility problems or technical difficulties with some of the tested chemicals.

In silico data (LogBB and HIA) were not estimated for 6 test chemicals due to few atoms or problems with CODES program. *In vitro* kinetic data were not generated for 9 compounds since no analytical methods were available in any of the laboratories participating in the prevalidation.

Table 2. Main activities carried out during the Prevalidation and time lines.

Task number		January	February	March	April	May	June	
WP9	Prevalidation of the tiered testing strategy							
9.1	Acquisition, depository and distribution of reference chemicals							
9.2	Revision of SOPs							
9.3	Testing – phase 1							
	Testing – phase 2							
	Testing – phase 3							
9.4	Finalisation of templates for raw data entry							
	Reporting of raw data							
9.5	Statistical analysis/provision tiered testing strategy							



Data analysis

The evaluation of raw data was performed in the same manner as for the optimisation phase of the project, including the statistical evaluation of the concentration-response curves and the computation of a characteristic value (EC_{20} , EC_{50} or LOEC) for every experiment performed.

An example of variability analysis performed with data generated with the cytomic panels measuring oxidative stress and for cytotoxicity screening in HepG2 cells is shown in Figure 1.

Since the ultimate aim is the classification of chemicals into the official acute oral toxicity categories (EU CLP and GHS), the Classification and Regression Trees (CART), used in the first phase of the project, and the Random Forest model were used for the classification task during the prevalidation.

Two classification approaches were evaluated:

- 1) a single step procedure only with Random Forest classification algorithm and
- 2) a two step tiered testing strategy

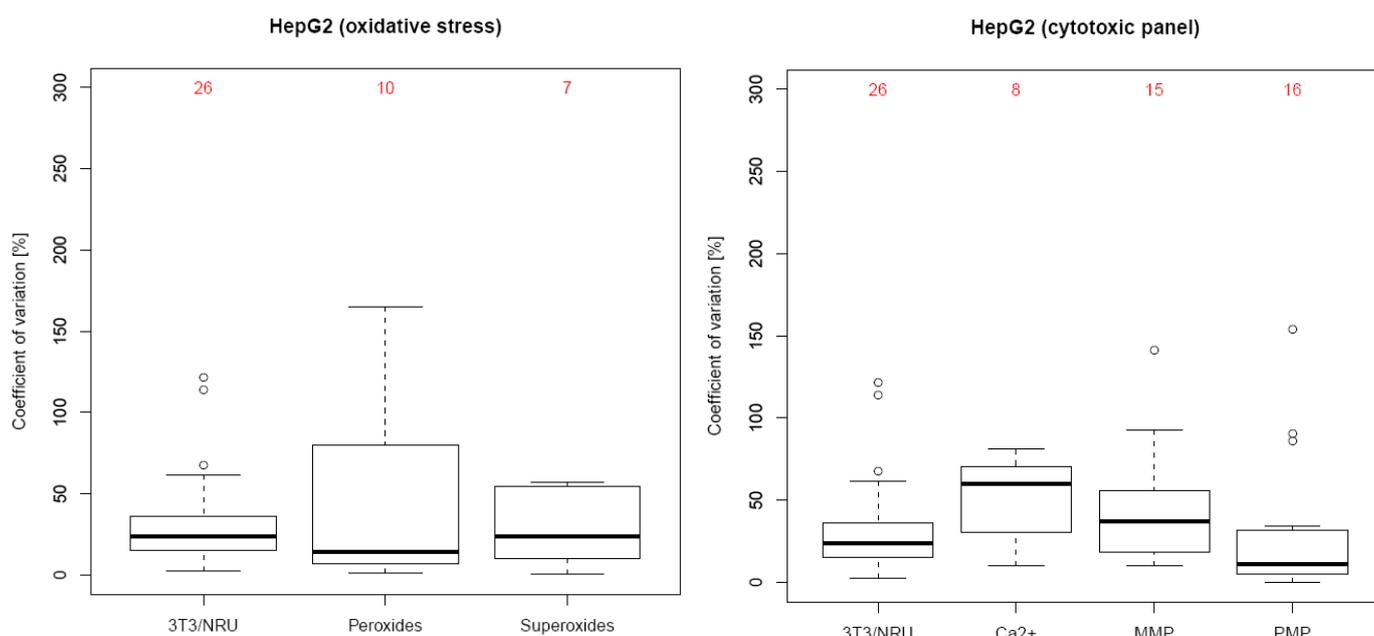


Figure 1: Boxplots for coefficient of variation calculated from EC_{xx} estimates in the cytomic panel assays with HepG2 cells and IC_{50} estimates in the 3T3/NRU cytotoxicity assay.

Results

Based on the classification analysis carried out, 10 *in vitro* testing strategies were formulated and evaluated in terms of predictivity, five using the full set of compounds and another five ignoring in the analysis compounds with a logP value larger than 5.

The strategy that uses the Random Forest model including 9 endpoints (CFU-GM/human cord blood cells assay; 3T3/NRU assay; Primary rat brain aggregates including the following 3 endpoints: lowest gene expression, HSP-32 expression, NF-H expression; SH-SY5Y cell line/Lowest EC value obtained in cytotoxic panel; HepG2 cell line/MMP; rat hepatocytes/MTT assay; whole blood/IL-1 release) in a single step procedure, gave the best correct classification rate (69.26%) and resulted in only 2 compounds with underestimated toxicity (Brucine, Paraquat), as compared to the official acute toxicity classification.

The two biokinetic transformations evaluated in the ACuteTox project, “estimation of oral dose” or “incorporation of blood-brain barrier passage plus estimation of oral dose” were not incorporated in the ten testing strategies evaluated. The reason is that, for these transformations, not enough data were generated for useful application of the developed testing strategies to the test set (32 chemicals).

The results of the classification analysis showed that the incorporation of additional endpoints did not improve significantly the outcome of the 3T3/NRU cytotoxicity assay alone, and only compounds with oral LD₅₀ > 2000 mg/kg (e.g. unclassified chemicals according to EU CLP classification system) are best classified.

However, the outcome of this analysis reinforced previous results obtained with the 3T3/NRU cytotoxicity assays and supports the use of this validated cytotoxicity assay to identify unclassified chemicals (LD₅₀ > 2000 mg/kg) according to the EU CLP classification system, as a first step in a tiered testing strategy.

Main Achievements of the ACuteTox project and Impact

This project provides an excellent case study for ECVAM on the prevalidation of a testing strategy based exclusively on non-animal methods and helps progressing the discussions on validation of testing strategies which are currently ongoing at several levels (ECVAM, European Partnership for Alternative Approaches to Animal Testing (EPAA), COLIPA, ECHA and others). Indeed, this case study has been already presented at the recent EPAA/ECVAM workshop on Validation of Integrated Testing Strategies (Ispra, Italy, 12-13 October 2009).

In the first phase of the project, a very large number of *in vitro* test methods (approximately 75 endpoints) have been evaluated in terms of their within-laboratory variability (and in some cases also the between-laboratory variability), preliminary predictive capacity and the potential to identify alerts for organ-specific toxicity. The outcome of this phase of the project is a large toolbox of *in vitro* methods with associated optimised protocols, some of them evaluated to the level of prevalidation.

Several *in vitro* assays have proved to be useful to identify alerts for tissue specific toxicities, such as



Recommendations

neurotoxicity (Gustafsson et al., 2010) and nephrotoxicity. However, the results of the classification analysis showed that complementing the 3T3/NRU assay with those *in vitro* assays is not improving significantly the classification of compounds in acute oral toxicity categories 1 to 4. This outcome is largely linked to the fact that the current classification systems are based on arbitrarily assigned cut-offs for the rat LD₅₀ values, and this animal test does not include more detailed scientific (mechanistic) information on the compounds.

Most of the results obtained in the course of the ACuteTox project have resulted in peer review publications, which proves the scientific quality of the data generated. In addition, a Special Issue on ACuteTox is under preparation and will be published by Toxicology in Vitro.

Some of the knowledge derived from the project has been already applied in the daily activities of companies which are ACuteTox partners (e.g. pharmaceutical sector). This goes beyond the main objective of the ACuteTox project (prediction of acute oral toxicity) and helps improving European competitiveness. Examples include, for instance, the case of Noscira S.A. (Spain), which increased the confidence in the results obtained by using some of the neurotoxicity models that were optimised during the project.

In the same direction, the project allowed to establish interesting specific collaborations with individual partners covering e.g. aspects of hepatotoxicity and metabolism, not only at the experimental level, but also in the preparation of dossiers and documents being presented to both the European Medicinal Agency and the US-FDA, which in the end is a definitive success of the project.

The results obtained from the classification analysis performed in the ACuteTox project lead us to question the scientific motivation for the current classification systems for acute oral toxicity and to suggest the revision of the GHS/EU CLP systems.

The outcome of the analysis of consistency in classification (GHS and EU CLP acute oral categories) corresponding to reported ranges of LD₅₀ respective of individual 97 substances included in the ACuteTox project give an indication of potential consequence for ambiguity in corresponding classification (Hoffmann et al., 2010). The analysis showed that (with at least 90% probability) ~50% of the substances would be unequivocally classified by a single category, ~40% would ambiguously occur within the limits of two adjacent classification categories, and ~10% of the substances have LD₅₀ ranges of sufficient scope to span three or more different classifications. This analysis reinforced previous findings by Rudén and Hansson (2003) and leads to the same recommendation of revision of the GHS/CLP classification system.

The estimation of the oral dose by including kinetic parameters needs to be further evaluated, in particular the availability of well established in house validated analytical methods for non-drug like compounds is at present a limiting factor, and requires further investment in future strategies.

If more data on kinetic parameters were available, a better evaluation of the impact of the different kinetic factors, i.e absorption, distribution (e.g. lipophilicity, protein binding) could be made.



Relevant publications

Anon 2008. Regulation (EC) No. 1336/2008 of the European Parliament and of the Council of 16 December 2008 amending Regulation (EC) No. 648/2004 in order to adapt it to Regulation (EC) No. 1272/2008 on classification, labelling and packaging of substances and mixtures. Official Journal of the European Union, L354 (vol. 60). Office for Official Publications of the European Communities, Luxembourg.

Gustafsson H., Runesson J., Lundqvist J., Lindegren H., Axelsson V., Forsby A. Neurofunctional endpoints assessed in human neuroblastoma SH-SY5Y cells for estimation of acute systemic toxicity. *Toxicology and Applied Pharmacology* (2010), 245: 191–202.

Hoffmann S., Kinsner-Ovaskainen A., Prieto P., Mangelsdorf I., Bieler C., Cole T. Acute oral toxicity: Variability, reliability, relevance and interspecies comparison of rodent LD(50) data from literature surveyed for the ACuteTox project. *Regul Toxicol Pharmacol.* (2010), doi:10.1016/j.yrtph.2010.08.004

Rudén, C., Hansson, S.O. How accurate are the European Union's classifications of chemical substances. *Toxicol. Lett.* (2003), 144: 159–172.

Participating partners in WP9

P4, Pilar Prieto and Agnieszka Kinsner
JRC/ECVAM, Italy

P8, Richard Clothier
University of Nottingham, UK

P9, Enrique O'Connor, Guadalupe Herrera
University of Valencia, Spain
P11, Laura Cerrato, Beatriz Albella
CIEMAT, Spain

P13, Nuria Campillo
CSIC, Spain

P15, Maria Jesus Zrubano, Lourdes Gombau,
Guillermo Quintas
ADVANCELL, Spain

P16, Anna Forsby
Stockholm University, Sweden

P18, Gabriella Hawksworth
University of Aberdeen, UK

P20, Paul Honegger, Marie-Gabrielle Zurich
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Université de Lausanne, Switzerland

P27, Laura Turco
Istituto Superiore di Sanità, Italy

P34, Per Artusson, Richard Svensson
Uppsala University, Sweden

P35, Romeo Cecchelli, Christophe Landry
Université D'Artois, France

Biostatisticians, Annette Kopp-Schneider,
Sven Stanzel
DKFZ, Germany



The AcuteTox partner Dr. Richard Clothier, receives the Bjorn Ekwall Memorial Award 2010

The AcuteTox partner Dr. Richard Clothier, receives the Bjorn Ekwall Memorial Award 2010, in recognition of his outstanding contributions in the field of in vitro toxicology, and in particular with respect to the development, implementation and validation of alternative toxicity test methods, and for his substantial contribution to the Fund for the Replacement of Animals in Medical Experiments (FRAME) Research Programme.

Richard was a co-founder (in 1983) of the FRAME Alternatives Laboratory (FAL) at the University of Nottingham Medical School, and was the director of the FAL from 1997 to 2005. He has played a substantial role in the field of in vitro toxicology, by contributing to the development and application of alternative assays for replacing animal toxicity testing, e.g. the Neutral Red Uptake (NRU) and Neutral Red Release (NRR) tests for basal cytotoxicity, the Fluorescent Leakage assay, the Alamar Blue (Resazurin) metabolism test for toxicity, and many others.

As an expert in national and international trials, he has contributed significantly to the further advancement of the acceptance of alternative assays for toxicity through his commitment to a number of studies, including a FRAME-led study in 1983 - 1986, the EC/HO and COLIPA international validation study on alternatives to the Draize eye irritation test, the EU/COLIPA international validation study on the in vitro 3T3 NRU test for phototoxicity, and the ECVAM/ICCVAM assessment of the NRU assay for measuring basal toxicity.

The award will be handed over at the 2010 FRAME Annual Lecture at The Kennel Club in London the 4th of November.

More information about the project and publications can be found at

www.acutetox.eu



Dr. Richard Clothier

