WP3: Database, statistical analysis, assay automation (P4, P10, P17, P19, and P26)

Establishment of AcutoxBase (P4, P19)

AcutoxBase is a unique database that combines *in vivo*, *in vitro* and *in silico* data on acute toxicity and biokinetics of 97 selected reference chemicals. It functions as a central element of the project, with regard to reporting and management of the data and allows easy, quick and reliable exchange of the generated datasets, enables proper documentation and traceability of all the experimental procedures, protocols, raw data and final results. The database is provided as an Internet application, thus ensuring easy access for all the ACuteTox partners all over Europe. The data can be entered and retrieved on-line by all project participants equipped with any modern web browser.

To allow a better control of the data and to ensure that every person conducting a test, properly records all the necessary information, a unified template for reporting of *in vitro* experiments has been carefully designed, that allows to introduce data from experiments based on different cell types and performed in various types of culture-ware (e.g. 96-well plates, Erlenmeyer flasks, etc.), with different exposure times, number of concentrations tested and diverse outputs. Also, it has been implied that for every single experiment the raw data must be attached. This feature has proven to be very useful in the second phase of the project (selection of assays for prevalidation) and gave the possibility re-evaluate the raw data during the last six months of the project and to make additional calculations and statistical analyses by the subcontractor (DKFZ).

AcutoxBase compiles also documented animal *in vivo* acute toxicity (LD_{50}) studies for the 97 reference chemicals selected for the optimisation phase of the ACuteTox project.
Another source of *in vivo* data collected in AcutoxBase is the human acute poisoning cases (blood concentration measurements, including victim observations) available from clinical/forensic medical reports. For more information on the compilation of *in vivo* data see work performed and results for WP1.

AcutoxBase serves also as a large compendium of SOPs from a large number of *in vitro* assays in several toxicological areas (basal cytotoxicity, biokinetics, neurotoxicity, nephrotoxicity, hepatotoxicity, haematotoxicity, immunotoxicity, cytomics). This collection of SOPs might serve in future as a valuable source of protocols for any user interested in the technical details of the assays, and will allow transferring the documented methods to other laboratories.

At the end of the project, AcutoxBase contains a full set of data regarding the selected 97 reference chemicals, including molecular structure, physicochemical properties and summary descriptions on use, toxicity and *in vivo* biokinetics. Moreover, about 2.200 data from acute oral toxicity studies *in vivo*, 2.900 data sets from human blood poisoning reports, 10.300 files from *in vitro* experiments and 118 different SOPs for *in vitro* assays were introduced into the database. These data (both the summary, as well as the raw data) can be easily retrieved as 'Excel' files and used for any type of analysis (e.g. statistical calculations, comparisons, etc.), both within the frame of this project, as well as for future follow-up activities.

Details on the structure and functionality of AcutoxBase can be found in the publication by Kinsner-Ovaskainen *et al*, 2009.

Automation of assays to high-throughput (HTS) robotic platforms (P4, P10, P17, P9)

Over the last two decades robotic systems have been increasingly used for drug discovery and development, as they allow reducing cost and time, and thus shortening the pipeline of drug development. Assay automation is now being introduced also in the field of toxicology, since it generate large datasets to develop strategies for toxicity testing. Examples of large programs based on high-throughput toxicity testing have already started in the US (e.g. ToxCast, http://epa.gov/ncct/toxcast).
In the first three years of the ACuteTox project, a few assays, with already well defined protocols, have been adopted to the robotic platform established at P4:

a) the 3T3 Neutral Red Uptake,
b) the MTT assay in HepG2 cells and rat hepatocytes
c) the LDH release assay in NeoHep.

A: Automation of 3T3/NRU protocol

The original, manual protocol from the NICEATM-ECVAM validation study of the Neutral Red Uptake assay in 3T3 cells have been transferred to the robotic platform (Hamilton, CH) available at the labs of P4. The transfer was done in collaboration with P10, experienced in assay automation, and P8, who provided practical advice, as it was one of the participant laboratories in the NICEATM/ECVAM validation study.

The successful transfer was confirmed by re-testing 24 chemicals from the above-mentioned validation study on the robot. The results showed that reproducibility of the assay was good or even improved when using the automated protocol, with less intra-plate and intra-assay variability. This pilot study has indicated the importance for the process of automation of very well prepared and detailed SOPs, for which the between-laboratory reproducibility and transferability should preferably be previously assessed. The automated 3T3/NRU assay has been used in the second phase of the project (WP9) to generate cytotoxicity data on 32 prevalidation compounds.

B: Automation of MTT hepatocytes protocol

Another assay successfully automated was the MTT test performed in HepG2 cells and rat hepatocytes. The manual protocol was developed by P3 in WP6, and then transferred to a robotic platform at P17. After a successful automation of the assay, P17 tested all the 97 ACuteTox reference chemicals in both cell types. Also in this case the statistical analysis has shown that the reproducibility of the automated assay is good.

c) Automation of LDH assay in NeoHep

A new cell system assay adapted to the robotic platform was LDH release in Neohepatocytes. These cells are derived from a procedure of de-differentiation and subsequent differentiation of human monocytes into hepatocyte-like cells. Initial results have been obtained on the LDH release, and in collaboration with P9, on cytokine production (IL-12p70, IFN-γ, IL-2, IL-10, IL- 8, IL-6, IL-4, IL-5, IL-1β, TNF-α, TNF-β) by Neo-hepatocyte cells exposed to test compounds using the multiplexed flow cytometry analysis. Both assays in combination determine the secretion of cytokines by Neo-hepatocyte cells as a response to the challenge with sub-lethal concentrations of test compounds, thus allowing distinguishing between specific immunotoxicity and general cytotoxicity.

Calculation of human LC_{50} values from human acute sub-lethal and lethal blood concentration data (P26)

An approach to estimate human lethal concentration (LC_{50}) values derived from time related human sub-lethal (LC_0) and lethal (LC_{100}) data determined from human acute poisoning cases was developed. Using this approach the LC_{50} values were calculated for 78 out of the 97 chemicals (i.e. for those chemicals which have quality human data collected in AcutoxBase).

The calculated human LC_{50} values were then used as inputs for *in vivo – in vitro* modelling, which was based on a statistical correlation between *in vitro* IC_{50} 3T3/NRU cytotoxicity values and human LC_{50}. Linear regression analysis between IC_{50} and LC_{50} gave an explained variance R^2=0.56 for the 67 reference chemicals, for which both sets of data were available. This R^2 value shows that additional organ-specific and biokinetic tests are needed in order to improve the predictability.

The detailed approach for estimation of human blood LC_{50} values and the results were published in Sjöström *et al*., 2008.

Identification of outliers from the in vivo-in vitro correlations of the data obtained from WP1 and WP2 (P26)

In vitro – in vivo modelling of LC_{50} values for humans and LD_{50} values for rat (collected and analysed in WP1) have been performed using different combinations of *in vitro* cytotoxicity tests (performed in WP2). Ultimately, the models based on the IC_{50} values from the 3T3/NRU cytotoxicity assay and rat LD_{50} or human LC_{50} values have been used to identify outliers (Table 2), detected by normal probability plots. The outliers identified were defined as 0.75 log deviation of IC_{50} from LC_{50} or LD_{50}

Detailed results from these analyses can be found in the publications of Sjöström *et al*, 2008, and Clothier *et al*, 2008.

The identified outliers (Table 2) as well as well-balanced a number of non-outliers, altogether 56 reference chemicals, were tested by WP4-7 in selected tests (for details see specific WPs descriptions).

Table 2. Outliers identified by comparing mean IC_{50} values of 3T3/NRU Cytotoxicity assay with animal LD_{50} and human LC_{50} values.

Chemical name	**Outlier in linear regression between IC_{50} and rat LD_{50}**	**Outlier in linear regression between IC_{50} and human LC_{50}**
(-) epinephrine	X	No human data
2,4-dichlorophenoxyacetic acid		X
5-fluorouracil		X
Acetaminophen		X
Atropine sulfate monohydrate		X

Chemical name	Outlier in linear regression between IC_{50} and rat LD_{50}	Outlier in linear regression between IC_{50} and human LC_{50}
Cis-diammineplatinum (II) dichloride		X
Codeine		X
Cyclosporine A		X
D-amphetamine sulfate	X	No human data
Digoxin	X	X
Diqaut dibromide		X
Formaldehyde	X	X
Lindane		X
Malathion		X
Methadone hydrochloride		X
Nicotine	X	X
Ochratoxin A	X	No human data
Parathion	X	No human data
Pentachlorophenol		X
Phenobarbital	X	
Physostigmine	X	No human data
Potassium cyanide	X	
Sodium chloride		X
Sodium selenate	X	
Strychnine	X	X
Thallium sulfate	X	
Warfarin	X	

Statistical analysis of the in vitro data obtained from testing 57 reference chemicals in 75 endpoints (WP1 and WP3-7) by use of PLS multivariate analysis (P26)

In vitro-in vivo models based on rat LD_{50} (mol/kg) for 56 chemicals and LC_{50} human blood concentrations (M) for 46 chemicals using IC_{50} values (M) obtained in basal cytotoxicity (WP2) and specific target organ toxicity tests (WP4-7) were developed using Partial Least Squares (PLS) regression. The data was extracted from AcutoxBase and the models were based on log 10 transformed data and on subsets with at the most 35% missing values. With the aim to find subsets of *in vitro* tests with good predictive capability, the influence of variable reduction was studied.

The *in vitro-in vivo* modelling with PLS showed that the predictive capabilities of the *in vitro* tests, expressed as explained variance (R^2) and predictive variance from cross-validation (Q^2), were for models based on LD_{50} rat - R^2=0,59 and Q^2=0,57, and for human LC_{50} - R^2=0,71 and Q^2=0,69. However, the variables contributing to the best models were mainly

basal cytotoxicity tests and the target organ specific tests only improved R^2 and Q^2 with about 0.02, compared to models based on basal cytotoxicity tests alone.

Summing up, the results from the PLS analyses did not allow to select among the investigated *in vitro* tests, those that would substantially improve the correlation and predictive capability compared to the basal cytotoxicity tests. Moreover, being a regression analysis, the PLS could not be used to identify possible strategies that would allow to classify chemicals into the official acute oral toxicity categories (EU CLP and GHS) (which is the ultimate goal of acute toxicity testing for regulatory purposes).

It was therefore decided that an independent statistical analyses should also be done, with the aim to identify methods to be included in the testing strategy. The additional analysis, performed under WP9, was targeted to select assays not only according to their reproducibility and reliability, but most importantly, according to their potential to classify chemicals into the GHS classes that was determined by application of classification algorithms.