## Publishable executive summary AcuteTox, 2006

The ACuteTox project is an EU-funded project that started in January 2005 with the overall aim to develop and pre-validate a simple and robust *in vitro* testing strategy for prediction of human acute systemic toxicity, which could replace the animal acute toxicity tests used today for regulatory purposes. The extensive amount of work performed since the 70s has led to a large number of existing *in vitro* models for acute toxicity testing. Many studies have shown good correlation between *in vitro* basal cytotoxicity data and rodent LD50 values. In addition, the MEIC (Multicenter Evaluation of *In Vitro* Cytotoxicity) programme showed a good correlation (around 70%) between *in vitro* basal cytotoxicity data and human lethal blood concentrations. This means, however, that when using the existing tests, a certain number of misclassifications will occur. ACuteTox aims to identify factors that can optimise the *in vitro-in vivo* correlation for acute systemic toxicity.

The project is divided in to 9 scientific workpackages:

- WP1: The generation of a high quality *in vivo* database
- WP2: The generation of a high quality *in vitro* database
- WP3: Iterative amendment of the testing strategy
- WP4: New cell systems and new endpoints
- WP5: Alerts and correctors in toxicity screening (I): Role of ADE
- WP6: Alerts and correctors in toxicity screening (II): Role of metabolism
- WP7: Alerts and correctors in toxicity screening (III): Role of target organ toxicity
- WP8: Technical optimisation of the amended test strategy
- WP9: Pre-validation of the test strategy

Known outliers of *in vitro-in vivo* correlations will be evaluated in order to introduce further parameters which might improve the correlation, such as absorption, distribution and excretion, metabolism and organ specificity. Reference chemicals selected mainly from previous studies such as the MEIC and ECVAM/ICCVAM validation studies are tested in different *in vitro* and *in silico* assays. This allows integration of alerts and in a prediction algorithm, which together with robust implementation of medium throughput approaches, would enable the establishment of a new testing strategy with a better prediction of toxic classification. The project will last for 5 years and during the last 2 years the testing strategy will be pre-validated.

The work in **WP1** and **WP2** have been finalised. In **WP1** animal and human data for the 97 ACuteTox reference compounds were compiled. The database contains LD50 values from 2206 animal studies as well as human data from 2902 cases reports, including acute sub-lethal and lethal blood concentration data. Furthermore, descriptive summaries containing physico-chemical data, LD50 values, human toxicity data, pharmacokinetics-/ toxicokinetics data, metabolism, toxicological mechanisms, target organs for all 97 reference chemicals have been compiled.

Testing of the ACuteTox reference chemicals in 6 basal cytotoxicity tests (**WP2**) have been completed. Data for most of the 97 reference compounds are available for the Fa32/NR uptake, Fa32/protein content, 3T3/NR uptake and NHK/NR uptake systems and for the HepG2/protein content and HL60/ATP content systems data are available for half of the compounds. Data from **WP1** and **WP2,** as well as from **WP4-WP7,** are stored in AcuBase, a database developed in **WP3** to facilitate SOPs storage, data transfer from all partners and statistical analysis of larger data sets. The data from **WP1** and **WP2** have been compared in some preliminary multivariate analyses with the aim to identify additional outliers that will be tested by the Partners before the final selection of methods that will enter the pre-validation phase. A number of outliers were identified, but since data are preliminary they will not be

presented in this report. The results of the definitive analyses are expected in the end of February 2007. One of the aims of the project is to adapt the methods of the testing strategy to high-throughput screening. In **WP3** a number of test assays, such as the 3T3/NR uptake and HepG2/MTT assays have successfully been adapted to two commercially available high-throughput (HTS) robotic platforms.

In **WP4-WP7** the testing of the first 2 sets of reference chemicals (no. 1-46) have continued and the results are reported to AcuBase. This testing will continue until month 30 when the data sets will be integrated in the *in vitro-in vivo* comparisons with the aim to select the methods that could improve the correlation and thus be candidates for inclusion in the testing strategy.

The aim of **WP4** is to provide an alternative way to improve the prediction of acute toxicity by incorporating more specific end-point parameters, and/or cell models from the haematopoietic system in the testing strategy. Effects on the *in vitro* production of cytokines in whole human blood cultures as well as effects on the CFU-GM and progenitors of megakaryocytes have been measured for 20 chemicals and the results are now being analysed. The miniaturizing of the Cytomic Panels for Cytotoxicity and Oxidative Stress Screening as well as testing of 20 reference chemicals have been finalized and the results show good correlation with human blood LC50 values. A new system for High-Content Cell-based Assays InCell Analyzer 1000 has been installed and testing has started.

The most crucial parts of the kinetic behaviour have been studied in **WP5**. For this purpose, the determination of kinetic parameters is being performed either by experimental, *in vitro* test or computer-based kinetic modelling. Neural network methodologies that are useful to estimate oral absorption and blood brain barrier passage have been developed. Results from three variants of the Caco-2 model for prediction of oral absorption, used in three different laboratories, have been compared. So far, the results show good agreement between the different variants. Toxicity studies and permeability studies using *in vitro* BBB models have been performed for 22 and 19 compounds respectively, showing relatively good correlation with *in vivo* data. Another aim of **WP5** is to investigate the partitioning behaviour of a number of polycyclic aromatic hydrocarbons (PAHs) to different components of a typical *in vitro* assay by using solid phase microextraction. This technique was proven to accurately measure the free concentration of compounds such as PAHs and also easy to use. Finally, *in vitro* plasma protein binding of reference compounds has been performed. The data obtained from **WP5** are now the basis of further biokinetic modelling.

In order to evaluate if toxicity is dependent on metabolism, the effects of 21 reference compounds have been compared between a metabolic competent model (primary hepatocytes) and a non-metabolising cell type (HepG2) by use of MTT (**WP6**). By comparing the concentration-toxicity curves of each compound in both models it is possible to ascertain whether the molecule elicits toxic effects preferentially on hepatocytes suggesting that a bioactivation of the xenobiotic is required. A recombinant-defective adenoviral vectors encoding major CYP genes involved in foreign compound metabolism have been developed as a way to overcome the problem with non-metabolising cell systems. For Tamoxifen, Cyclosporin A, and to some extend also Tetracyclin, which toxicity is dependent on 3A4-dependent biotransformation, toxicity was observed only in HepG2 cells that was infected with adenovirus encoding for CYP450 3A4. Another aim of **WP6** was to investigate how METEOR and DEREK software performs in prediction of metabolic fate of compounds with known biotransformation. Fourteen ACuteTox reference compounds were tested, showing that METEOR seems as an interesting alternative for *in silico* prediction of metabolism. Furthermore, it was concluded that even if the DEREK program does not predict acute toxicity *per se*, important information of the toxic profile of the tested substances can be gained.

In the neurotoxicity **WP 7.1** 26 chemicals (half of them neurotoxic) have been studied in native or differentiated human neuroblastoma SH-SY5Y cells, primary cultures of mouse or rat neurons, and mature re-aggregated rat brain cells by using more than 20 different endpoints (such as mitochondrial membrane potential, $GABA_A$ receptor function, [$^3$H]GABA uptake, GAD activity, [3H]aspartate uptake, glutamate release, AChE activity, ChAT activity NMDA-glutamate receptor, acetylcholine esterase activity, uptake of [$^3$H]noradrenalin, depolarization- and carbachol-evoked changes in CMP, intracellular free $Ca^{2+}$ concentrations, gene expression, ROS, 2-deoxyglucose uptake, [$^3$H]uridine incorporation, [$^{35}$S]methionine incorporation, GS activity, CNP activity). The results show that the broad collect of assays could in a very good way predict the neurotoxic compounds. However, the challenge is to find more general assays that could pick up several different neurotoxic mechanisms of action.
For the measurement of nephrotoxicity in **WP7.2,** transepithelial resistance (TEER) was chosen as the functional assay and the LLC-PK1 cell line as the test system. The REMS automated device was selected for measurement of TEER. Twenty one reference chemicals (of which 5 is nephrotoxic) were tested and the overall results show that the TEER model using renal epithelial cells is a promising model for detection of nephrotoxicity.

The main goal of **WP7.3** is to identify a set of markers characteristic of acute liver toxicity that could be of use in high throughput screening. Metabolic competent cells (rat hepatocytes), non-competent hepatic cells (HepG2) and non hepatic cells (3T3 fibroblasts) were exposed to the 21 selected compounds using the MTT assay. Also, the following biochemical functions were examined in the cells: cellular ATP levels, formation of reactive oxygen species (ROS) as an index of oxidative stress, cellular protein content and mitochondrial membrane potential. The results were compared with the MTT assays and suggest that neither ATP levels, ROS formation or protein content, when measured at early times (5 hours of incubation) or late times (24 hours of incubation) allowed a better discriminating effect than the one obtained by the MTT test. Another objective of **WP7.3** is to develop an *in vitro* strategy to assess the effects of chemicals on the impairment of hepatocyte bile acids and bilirubin transport. This is done by generating a cell bank expressing the various hepatocellular transport systems and establishing assays as well as to characterize the interaction of chemicals *in vitro* with individual transporters. As a result of these efforts, a protocol outlining a test system to identify (alert) for substances which are potentially hepatotoxic because of their capability to impair hepatic transport is expected.

Within **WP4-WP7**, testing will continue until month 30 when the evaluation of data will be performed and the best performing tests which could improve the predicting of acute toxicity will be selected. However, the aim is not just to improve the prediction, but also to signal, which compounds require further testing because their acute toxicity can not be properly predicted. A second draft of the testing strategy has been formulated by **WP8**. The strategy, which consists of a step wise procedure where each step (toolbox) refers to the application of information and methodologies will be further developed during 2007. It is expected that a formal validation of the definite testing strategy will lead to regulatory acceptance and its incorporation into the set of standardized test guidelines for chemicals hazard assessment. The proposed testing strategy has the potential to replace EU methods B.1bis and B.1tris in Annex V of Dir 67/548 EEC and consequently the corresponding OECD Test Guidelines 420 and 423, as well as 425. To facilitate the prevalidation process during the two last years of the project, the work in **WP9** has already started. Common templates for SOPs have been prepared and are used by all Partner (72 SOPs are available). Furthermore, a list of criteria for the selection of methods that will be included in the testing strategy have been prepared as well as a common template, which will be used by WP leaders to report the results of the promising models that will enter evaluation before the prevalidation exercise starts.

The second version of the Plan for using and disseminating knowledge has been produced by the ACuteTox consortium. A general ethical problem consists in the fact, that methods of central interest for human toxicity testing should be protected on the one hand, but also be freely available to the public, in particular abroad in developing countries. At the end of the ACuteTox project, a final version of the plan will be prepared by the Consortium and presented to the Commission. In this plan, the Consortium shall list all the technologies produced in the project and state how and by whom each of these are being exploited and will be exploited after the project. The contractors of the ACuteTox consortium have been very active in disseminating the knowledge both to the scientific community and to the general public in the year 2006. As an example, more than 20 project related articles and posters were published. A list of all publications produced by the Consortium is available the web site www.acutetox.org.

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