Publishable executive summary AcuteTox, 2005

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 great 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
- WP 8: Technical optimisation of the amended test strategy
- WP9: Pre-validation of the test strategy

The existing outliers of the *in vitro*-*in vivo* correlation 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 the ECVAM/ICCVAM validation studies will be tested in the different *in vitro* and *in silico* assays. This would allow 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 ACuteTox project proceeds more or less as planned in the Technical annex of the EC contract; only some minor delays are foreseen in some of the nine WPs. Since the start of the project three sets of all together 97 chemicals have been selected for testing. Thirty-one percent of these are industrial chemicals, 52% drugs 12% pesticides and 5% others. A substantial number of *in vivo* toxicity data (human and animal) have in WP1 been derived for the 97 reference chemicals. These data will be used during year 2 for in *vitro*-*in vivo* comparisons. In WP1 also “short descriptions” containing physico-chemical data, LD50 values, human toxicity data, pharmacokinetics /toxicokinetics data, metabolism, toxicological mechanisms, target organs etc. have been compiled for approximately 50% of the chemicals. These data will be used by all partners for evaluation of the performance of test methods etc.

In WP2 basal cytotoxicity data are generated from 6 basal cytotoxicity tests (HL-60/ATP assay, 3T3/NRUptake, NHK/NRUptake, Hep-G2/protein content and Fa32/protein content and NRUptake). More than 50% of all data that should be produced by WP2 are available and the testing will be finalised month 18. The data produced will be used for *in vitro*-*in vivo* comparisons (in WP3) during year 2 with the aim to identify additional outliers.

The development of a database (AcuBase) for efficient management of data in WP3 is a central and important part of the project. In this database all *in vivo* and *in vitro* data of the project as well as standard Operating Procedures (SOPs) for all methods used should be compiled. A first draft version of AcuBase, has been produced and will be available to Partners in March 2006 for uploading of results. Draft versions of templates for SOPs and raw data collection to be used by partners for uploading information to AcuBase have also been produced. Other tasks of WP3 are to provide a robotic platform for high-throughput testing and to optimize the transfer from manual protocols to automated protocols for methods that will be part of the testing strategy. The preparation of the versatile laboratory facilities for the robotic platform has started. An automated version of the 3T3 NRUptake test has been designed and first training runs with good results have been performed.

To identify factors such as absorption, distribution, excretion, metabolism and organ specificity that will improve the correlation between *in vitro* and *in vivo* toxicity (WP4-7) and to define an algorithm that counts for this (WP3) is essential parts of the project. In WP4-7, SOP's for many of the assays that will be used during the first 18 months have been produced and results from testing of the first 2 sets of reference chemicals (no. 1-46) have been generated.

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 CFU-GM progenitors have been measured for a certain number of chemicals. The miniaturizing of the Cytomic and the Oxidative Stress Cytomic assays has been successful and testing of chemicals in these systems has started. Furthermore, 20 chemicals have been tested for delayed toxicity in a rat cell line (Fa32 cells). This study confirmed that the human acute toxicity is better predicted by the delayed than by the acute cytotoxicity. However, since the human acute toxicity only was slightly better predicted by the delayed cytotoxicity assay it was decided not to further investigate delayed toxicity.

The aim of WP5 is to study the most crucial parts of the kinetic behaviour in order to identify which type of kinetic information is needed in order to improve the prediction. The following systems are used 1. Neural networks to predict transport on basis of structural characteristics, 2. Systems to measure barrier transport, a. oral absorption and b. blood-brain barrier (BBB), 3. Systems to evaluate free concentration, a. protein binding, b. Solid-phase microextraction measurement of actual free concentration and 4. Kinetic modelling. The construction of biokinetic models and an evaluation of the importance of physico-chemical parameters for compounds being outliers have started. Furthermore, for the first set of reference chemicals determination of free concentration and measurement of BBB passage have been performed.

Aims of WP6 were to 1. determine whether toxicity is associated with the metabolism of a tested compound, by using simple methods 2. develop new strategies to incorporate metabolic competence into cell lines and 3. to implement computer-based prediction of metabolism and to integrate the data into toxicity screening. In order to evaluate if toxicity is dependent on metabolism, the effects of compounds are investigated in a metabolic competent model (primary hepatocytes) and in a non-metabolising cell type (HepG2). 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. Several chemicals have been tested in the two cell types with the use of MTT assay. Also the work on new strategies to incorporate metabolic capabilities into cell lines has started. Amplification, titulation, and testing activity of adenoviruses for CYP2E1 and 3A4 and for CYP1A2 and 2A6 have been performed. Preliminary experiments have been conducted to define the conditions for the adenoviral infection of

HepG2 cells. Identification of metabolites by use of the METEOR software has been performed for a smaller number of compounds. Furthermore, results from the first 16 reference chemicals on metabolic stability *in vitro* have been presented.

In WP 7.1 (neurotoxicity), 21 chemicals (10 neurotoxic and 10 non-neurotoxic) have been tested in a test battery comprising of 15 different endpoints studied in native or differentiated human neuroblastoma SH-SY5Y cells, primary cultures of mouse or rat neurons, and mature re-aggregated rat brain cells. For the measurement of nephrotoxicity in WP7.2 transepithelial resistance (TER) 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 TER. Optimisation of the test system has been undertaken and testing of the reference chemicals have started. In WP7.3 (hepatotoxicity) the main goal is to generate an integrated cell system and to select end-point parameters capable of identifying substances with a preferential toxic action on hepatocytes that could be amenable for high throughput screening. The development of new indicators for acute hepatotoxicity has started. Two fluorescent assays addressed to the measurement of reactive oxygen species formation (ROS) and mitochondrial membrane potential in 3T3 and HepG2 cell lines have been set up. 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, by generating a cell bank expressing the various hepatocellular transport systems (sinusoidal uptake systems, canalicular export systems and export pumps) and establishing assays to characterize the interaction of chemicals *in vitro* with individual transporters. A first successful round of stable transfection of CHO cells with rat Oatp1a4, and preliminary characterization with the aid of a fluorescent bile acid have been performed.

The testing within WP4-7 will continue until month 30 when the evaluation of all data will be performed and the best performing tests which could improve the prediction of acute toxicity will be selected for inclusion in the testing strategy. However, the aim is not only to improve the prediction, but also to signal, which compounds require further testing because their acute toxicity can not be properly predicted. A pre-validated testing strategy and a pre-validated associated prediction model for acute systemic toxicity will be provided in the end of the project. The first step in designing the testing strategy has been taken by the leader of WP8. 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.

The implementation of REACH will result in the need for further assessment of up to 30.000 existing chemicals. It is estimated that the testing of these chemicals should result in the use of around 4 million animals. The costs for safety testing of chemicals are high and will tremendously increase when REACH is implemented within the EU. The optimisation of the *in vitro* testing strategy within the ACuteTox project will contribute to the establishment of more convenient, less expensive and more scientific-based safety testing.

A first 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 year 2005. As an example, more than 15 project related articles, abstracts and book chapters were published. A list of all publications produced by the Consortium is available the web site www.acutetox.org.

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