Executive summary

Introduction

The ACuteTox project is a 5-years 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 *in vitro* 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 are evaluated in order to introduce further parameters which might improve the correlation, such as absorption, distribution and elimination, 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.

Results

The work in **WP1** and **WP2** has been finalised. In **WP1** animal and human data for the 97 ACuteTox reference compounds were compiled in a database. The database contains LD50 values from 2206 animal studies (**P29**) as well as human data from 2902 cases reports (**P36**), 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.

A statistical evaluation of the animal data collected in **WP1** (**P4, P24**) indicated good reproducibility of LD50 values for the vast majority chemicals. Furthermore, rat and mouse mean LD50 were highly correlated with two exceptions: warfarin and cycloheximide were much more toxic in rat. Regression of human acute lethal doses with rat oral LD50 data for 30 reference chemicals resulted in a coherent correlation with slope 0.955, intercept -0.615, and coefficient of determination 0.571, which was similar to results obtained in the MEIC study. For 62 reference chemicals, EU/GHS toxicity classifications/categories were allocated, corresponding to respective maximum and minimum LD50 values. Fifty seven (92%) and 53 (85%) of the chemicals (EU and GHS, respectively) display individual ranges of LD50

limited to two adjacent classification categories. For 4 (6%) and 7 (11%) of the chemicals (EU and GHS, respectively) LD50 values span three different classifications. For 1 (1.6%) and 2 (3%) of the chemicals (EU and GHS, respectively) cited LD50 values allow scope for more than three different classifications.

From **WP2** (**P8, P14, P15, P23, IIVS**) data for most of the 97 reference compounds are available from the Fa32/NRU, Fa32/protein content, 3T3/NRU and NHK/NRU assays. For the HepG2/protein content and HL60/ATP assays data are available for respectively, 61 and 47 compounds. All data from **WP1** and **WP2**, as well as from **WP4-WP7**, that has passed the acceptance criteria, are stored in Acutoxbase, a database developed in **WP3** (**P19**) to facilitate SOPs storage, data storage and transfer between partners and statistical analysis of larger data sets. All 6 basal cytotoxicity assays give similar results, which confirm the results from the MEIC study (1). The toxicity ranking of the compounds is the same when the cell types are compared, with the exception of colchicine, cyclohexamide, hexachlorobenzene, digoxin and 5-fluorouracil. The Hep-G2, HL-60 and NHK cells showed a somewhat higher viability in the IC_{50} data than the 3T3 and Fa32 cells. Since the 3T3 assay has been validated, this assay was selected as the one used for the identification of outliers in **WP3**.

In *vitro – in vivo* modeling of LC_{50} values for humans and LD_{50} values for rat have been performed (**WP3**) using different combinations of the 6 basal cytotoxicity tests (**P26**). The models based on the 3T3 assay and LD50 or human LC values, show a number of outliers (16 and 17, respectively) detected by normal probability plots. Furthermore, the *in vitro-in vivo* correlation based on mol/l and mol/kg was superior to g/l and g/kg. The results also showed that the presently available data are not sufficient to obtain a general model for all chemicals and LD_{50} for rat and LC-values for humans (2) with good predictive capabilities. However, the models are better for the LC_{50} values in humans compared to LD_{50} values in rat. The identified outliers as well as non-outliers, altogether 41 compounds, have in **WP4-7** during the last part of 2007 been tested in selected assays.

One of the aims of the project is to adapt the methods of the testing strategy to high-throughput screening. In **WP3** the 3T3/NR uptake assay has successfully been adapted to commercially available high-throughput (HTS) robotic platforms and 24 chemicals have been tested (**P4**). Automation of a new cell system assay based on the use of primary cells Neo-hepatocytes has started and preliminary data on cytokine release by these cells have been obtained in collaboration with **WP4** (**P4, P9**).

In July 2007 the mid-term meeting of the project was held in Stockholm. At this meeting a first selection of the best performing methods was done. From **WP4-7** approximately 50 of the best performing assays have been selected for further testing of the 41 reference compounds. This testing was finalized in December 2007. After analyzing the data in the beginning of 2008, the best performing assays will be selected in March 2008 to be included in the testing strategy. The pre-validation of the strategy is planned to start in June 2008.

In **WP4-WP7** the testing of 57 reference compounds have been finalised and the results have been reported to Acutoxbase. 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 (3, 4). With the first set of 20 chemicals the best performing methods were the Cytokine secretion (IFN-γ, IL-5, and TNF-α) in human blood-derived cells (**P21**), and CFU-GM assay performed in human cord-blood-derived cells (**P11**) showing a very good correlation with the rat oral LD50 values (R^2 around

0.85). Correlation between the standard ELISA immunoassay and novel miniaturized multiplexed flow cytometric assays for the analysis 11 (IL-12p70, IFN-γ, IL-2, IL-10, IL-8, IL-6, IL-4, IL-5, IL-1β, TNF-α, TNF-β) cytokine levels using the next set of 41 compounds have been carried out. Furthermore, Cytomic assays, including: Multiparametric cytomic assay of Calcium/Mitochondrial membrane potential/Superoxide in HepG2, SH-SY5Y and A704 cell lines and High-Content Bioimage Analysis of 8-oxoG in A704 and SH-SY5Y cell lines were selected for the additional testing (**P9**). The data from the additional testing are now being analysed.

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 models for oral absorption and BBB passage (**P13**) classify the compounds (n=16) with a 73% and 72% accuracy as compared to the in vitro models, respectively (5) . The analyses have been also been extended with more compounds. However, the results have not yet been evaluated. Toxicity and permeability studies of 26 chemicals using the *in vitro* BBB models **(P5, P15, P35)** show relatively good correlation with *in vivo* data. The analysis of the results of 96 compounds is presently being made. Furthermore, based on this original BBB *in vitro* model, **P34** has developed a new BBB *in vitro* model to fit with the needs when screening large numbers of compounds (6). Reliable values for permeability coefficients (P_{app}-values) in different Caco-2 systems **(P5, P15, P27, P34)** are available for more than 50 compounds. Although the absolute values were different, the rank order of the P_{app} values was strikingly comparable. A set of rules or alerts has been developed to identify those chemicals for which one or more of these processes may lead to a reduction of the actual or bioavailable concentration in the *in vitro* cytotoxicity assay. These alerts are based on physical and chemical properties, including protein binding affinity and the octanol-water and air-water partition coefficient. Based on these rules, problematic chemicals from the list of 97 chemicals have been identified. The data obtained from **WP5** are now the basis of further biokinetic modelling (**P6**). This modelling enables an estimate of the relationship between the oral dose of a compound and the plasma concentrations resulting from that oral dose. The most important parameters; oral absorption, intrinsic clearance, and free fraction (7), have been incorporated in the model.

In order to evaluate if toxicity is dependent on metabolism (**WP6**), the effects of 57 reference compounds have been compared between a metabolic competent model (primary hepatocytes) and a non-metabolising cell type (HepG2) by use of MTT **(Partners 3, 15, 23 and 31)**. 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. Altogether 57 compounds have been tested in the two different models. To examine the robustness of this strategy, intra-assay, inter-assay as well as intra-laboratory variability was investigated for each cell system. A low variability (%CV<10%), both intra-plate and intra-assay, was obtained in all laboratories, however, the intra laboratory variability needs to be reduced. The higher intra-laboratory variation is probably due to several reasons, for example the physicochemical properties of the compounds assayed, many showing hydrophobicity and poor water solubility. The Adeno-CYP HepG2 model developed earlier (8, 9) was adapted to 96-well plates. Up to now, tamoxifen, tetracycline, cyclosporine A, amiodarone, atropine sulphate, verapamil-HCl and SLS **(P3 and P15)** have been tested in the new ready-to-use models (adenoCYP3A4- and 2E1-HepG2). Data on protein binding and metabolic stability (using rat and human liver microsomes and/or human and rat hepatocytes) have been generated for a sub-set of the reference compounds (**P7**) and are used in **WP5**.

In the neurotoxicity **WP 7.1** native or differentiated human neuroblastoma SH-SY5Y cells, primary cultures of mouse or rat neurons, and mature re-aggregated rat brain cells have been used to test the 1st set of 26 reference chemicals in around 40 different endpoints (10, 11, 12). The results show that the broad collect of assays could in a very good way predict the neurotoxic compounds. However, no general test is available that can identify all neurotoxic outliers. The challenge is to find a limited number of more general assays that could pick up several different neurotoxic mechanisms of action. The following assays were identified as good candidates and have been used for the testing of the 2nd set of 41 compounds: SHSY5Y-AChE-60 min (**P5**), SHSY5Y-CMP-3 min (**P16**), SHSY5Y-CMP-3 min (**P13**), CGC-Casp3-16hr (**P37**), and A global evaluation of: AGGR-GFAP gene-48hr; AGGR-MBP gene-48hr, AGGR-NF-H gene-48hr; AGGR-HSP32 gene-48hr; AGGR-2DG-48hr; AGGR-URD inc.-48hr (**P20**). Data from theses tests are now being analysed.

For the measurement of nephrotoxicity in **WP7.2,** trans-epithelial 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. Fifty five reference chemicals (including some nephrotoxic) were tested (**P18 and P25**) and the overall results show that the TEER is a sensitive predictor of toxicity. TEER showed greater sensitivity for nephrotoxic chemicals compared to non-nephrotoxic chemicals. However, compounds requiring metabolism, such as diethylene glycol did not show toxicity at the highest concentration tested.

The main goal of **WP7.3** has been 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 57 selected compounds (13, 14) using the MTT assay (**P3, 12, 15, and 31**). The data will be analysed as soon as the new software Acusoft is ready in the beginning of 2008.By loading the plasma membranes with cholesterol, it was possible to increase the reproducibility of the ATP hydrolysis of Bsep assay (**P28**). However, the signal-to-noise ratio is not satisfactory and the conclusion is that this ATPase assay will not be considered for the prevalidation phase of ACuteTox. Two fluorescent bile acid derivatives (namely, 3α and 7α NBD substituted) have been used with a subset of cholestasic and non-cholestasic compounds (**P28**). The work performed in 2007 has provided a proof of concept of this assay and the results are now being analysed.

The ACuteTox testing strategy has been further developed **(P2, P4, P6, P16, P25 and P26)** and a draft scheme will be presented at the Consortium meeting in February 2008.

A list of all publications produced by the Consortium is available the web site www.acutetox.org.

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