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ACuteTox
– Research Project For Alternative Testing

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The latest efforts in creating a test strategy for acute toxicity testing are heading for the prevalidation phase

ACuteTox is one of several large FP6 integrated projects, funded by the European Commission, that will make it possible for the European citizens to buy products containing better tested chemicals in some years. The particular aim of the ACuteTox project is to develop a simple and robust testing strategy for prediction of acute oral toxicity of chemicals without the use of laboratory animals.

There has been an intense activity within the project for the last sixth months to test an additional set of 41 reference chemicals in the 35 best performing methods that were selected at the mid-term meeting in July 2007. Recently this testing procedure has been completed and the data have been compiled in the database of the project, Acutoxbase. After analysing the data, the combination of tests that gives the best prediction will be selected in March-April 2008 and those tests will form the testing strategy. The prevalidation of the strategy will start in June 2008.

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Predictability of toxicity tests

The earlier MEIC (Multicenter Evaluation of *In Vitro* Cytotoxicity) programme showed that LD50 values from rat and mouse correlate to 65 % with the human lethal dose. That implies that the prediction of human acute effects is incorrect for approximately 35 % of the tested chemicals when using today's animal tests. The MEIC study also showed that the correlation increased to 70 % when test results (IC50 values) from basal cytotoxicity assays (i.e. data obtained from simple cell lines measuring growth or viability endpoints) were correlated with human lethal blood concentrations.

The ACuteTox project aims to improve the predictability further by combining a handful simple and robust tests measuring complementary parameters such as absorption, distribution and, metabolism and organ specificity. By introducing relevant assays we hopefully could increase the correlation to about 85-90 %.

One of the limitations of the existing *in vitro* models is that kinetics, i.e. absorption, distribution and elimination are not taken into consideration. This could be one of the reasons why certain compounds, when tested *in vitro*, do not correlate with the *in vivo* situation. Lack of correlation between *in vitro* basal cytotoxicity data and *in vivo* data could also be due to the fact that a compound is able to cause malfunction of specific organ cells at concentrations lower than those showing toxic effects to other cells. The organ malfunction results in the death of the organism without severe signs of cell death. The nervous system, the liver and the kidney are among the most frequent target organs for xenobiotic toxicity.

The ACuteTox testing strategy will therefore involve those parameters, mentioned above, that improve the prediction. Reference chemicals for which the *in vitro* basal cytotoxicity data does not correlate well with human and rodent toxicity data, outliers, have been identified. Subsequently, the effects of the outliers regarding absorption, distribution, metabolism and organ toxicity (liver, kidney and CNS) have been explored. This part, divided in several workpackages, is one of the major parts of the project. The objective of these research activities is to increase the knowledge on the mechanisms of toxicity of the compounds and to identify correctors/alerts assays in order to improve the *in vivo*/*in vitro* correlation.

Development of the ACuteTox strategy for testing of acute oral toxicity without the use of laboratory animals

The below scheme presents the work and strategy that the 35 partners of the ACuteTox project are carrying through to achieve the goal to present a strategy that has reached the level of maturity for subsequent formal validation and wide industrial and economic applications

Reference chemicals

Compilation of *in vivo* data WP1

Generation of *in vitro* data Basal Cytotoxicity Assays WP2

Compilation of in vivo and *in vitro* data in the database Acutoxbase for correlation by multivariate modeling WP3

Identification of outliers of the *in vivo-in vitro* correlation WP3

Improvement of the prediction of by use of new cell models from the hemapoetic system or new end-points. WP4

Exploring of outliers with the aim to identify alerts and correctors which could further improve the prediction of human acute toxicity

- Poor prediction because of different toxicokinetic behaviour which is related to their physicochemical and structural features **WP5**
- Poor prediction because of influence of metabolism **WP6**
- Poor prediction because of organ specific toxicity (the nervous system, the liver and the kidney) **WP7**

Development and optimisation of the testing strategy

Testing of 57 reference chemicals in approx 35 assays was finalised in the end of December 2007. **WP8**

Pre-validation of the testing strategy

The pre-validation of the strategy will start in May-June 2008. **WP9**

For more information about the results within each workpackage and the latest news from the project please visit our website www.acutetox.org.

Results from the ACuteTox project

Compilation of *in vivo* data (WP1)
- 2,200 LD50 values showed good reproducibility.
- 2,800 human poisoning cases
- Physicochemical properties for the reference chemicals including toxico kinetic and pharmacokinetic data

Generation of *in vitro* data (WP2)
97 reference chemicals tested in Basal Cytotoxicity Assays:
- Protein content (cell lines: Fa32, HepG2)
- ATP content (cell line: HL60)
- Neutral red uptake (cell lines: Fa32, 3T3, NHK)

The different cytotoxicity assays showed to give similar results. Since the Neutral red uptake assay with 3T3 cells has been validated, this assay was selected as the one used for identification of outliers.

Gradual improvement of predictive capabilities of *in vitro* tests (WP3)

Compilation of in vivo and in vitro data in the project database for correlation by multivariate modelling
The in vitro basal cytotoxicity assays (IC50 values) correlated better with human peak lethal blood concentrations (LC50) than with LD50 rat values.

Identification of outliers of the in vivo-in vitro correlation
16 outliers in the comparison with LD50 values.
17 outliers in the comparison with human LC50 values.
The identified outliers and some non-outliers, altogether 41 compounds, have now be tested (WP4 – 7)and evaluated in order to introduce further parameters which might improve the correlation.

Improvement of the prediction of by use of new cell and new end-points (WP4)

New cell systems:
- Cytokine secretion in human blood-derived cells
- CFU-GM assays (cytotoxic effects on cell differentiation)

Preliminary results with 21 reference

compounds showed a good correlation with the rat oral LD50 values but lower correlation with 3T3 cells for both assays.

New endpoints:
The selected flow cytometric assays showed good correlation when compared with *in vitro* cytotoxicity (3T3 cells), *in vivo* rodent toxicity, and *in vivo* human toxicity data. The method proposed by WP4 to continue testing up to December 2007 was Cytokine Secretion Assay evaluated by ELISA and Flow Cytomix.

Exploring of outliers with the aim to identify alerts and correctors which could further improve the prediction of human acute toxicity

Poor prediction because of different biokinetic behaviour which is related to the physicochemical and structural features of the compounds **(WP5)**
A set of rules or alerts has been developed to identify those chemicals for which one or more 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.

The work on neural network methods to estimate oral absorption and BBB passage has resulted in a tool that can be used as a first estimation of these parameters. The reference compounds (n=16) were classified with a 73 % and 72 % accuracy as compared to the *in vitro* models, respectively.

The BBB passage was measured *in vitro* using two different techniques:
a) transport of chemicals over a synthetic membrane (PAMPA) showed to be useful for interpreting physico-chemical properties for passive transport of a chemical over the BBB.
b) transport over a cellular system consisting of brain endothelial cells in co-culture with supporting cells showed relatively good correlation with *in vivo* data.

To determine oral absorption the Caco-2 cell system was used. Culturing the cell line in a two-compartment system makes it possible to measure the transport of chemicals over the cell layers. Results showed consistent outcomes with regard to toxicity.

Free concentration of a chemical in the culture medium was measured with solid phase microextraction (SPME) technique, and the results showed that it is possible to give a detailed description of the different parameters describing *in vitro* biokinetics.

Poor prediction because of influence of metabolism **(WP6)**
A simple model for metabolism-dependent toxicity is to compare effects on

metabolising cells (primary hepatocytes) with non-metabolising cells (HepG2) by the use of MTT assay. For four out of eleven compounds the assay indicated bioactivation and five out of eleven compounds indicated no bioactivation. New strategies to incorporate metabolic capabilities into cell lines was developed by transfecting HepG2 cells the major CYP genes involved in foreign compound metabolism.

Combined use of the softwares DEREK and METEOR can be used for in silico rank ordering and profiling of the possible toxicity of compounds and their major metabolites but not for predictions of acute toxicity or identification of outliers

Poor prediction because of organ specific toxicity **(WP7)**
Neurotoxicity – A sub-set of reference compounds 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 around 40 different endpoints. The results show that the broad collect of assays could in a very good way predict the toxicity of 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 end-points have been identified as good candidates and will be used for further testing: Cell membrane potential, Genomic biomarkers: NF-H, GFAP and Casp-3, GABAA receptor function and AChE activity.

Nephrotoxicity – To develop in vitro assays which reflect the role of the kidneys *in vivo* based on functional parameters the transepithelial electrical resistance (TEER) model was compared to the viability assay Alamar Blue. The overall results show that the TEER is a more sensitive indicator of toxicity than the Alamar blue assay, with greater sensitivity for nephrotoxic compounds compared to non-nephrotoxic chemicals.

Liver toxicity – To identify a set of markers characteristic of acute liver toxicity 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 a number of other biochemical functions have been examined in these cells, however, neither of them allowed a better discriminating effect than the MTT test.

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