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ACuteTox

Optimisation and pre-validation of an *in vitro* test strategy for predicting human acute toxicity

Instrument: Integrated Project

Thematic Priority

Periodic activity report

Period coved: from1st January 2008 to 31st of December 2008
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Final version

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

During the reporting period the ACuteTox partners (**WP4-7**) have finalized the testing of 57 reference chemicals in approximately 70 assays and uploaded, checked and corrected the data in Acutoxbase (**WP3**), the database of ACuteTox (1). At the end of 2008 Acutoxbase (**P19 and 4**), contained a full set of data regarding the 97 ACuteTox reference chemicals, including molecular structure, physicochemical properties and summary descriptions on use, toxicity and *in vivo* biokinetics. Moreover, about 2.200 data sets from acute oral toxicity studies *in vivo*, 2.900 data sets from human blood poisoning reports, 10.300 files from *in vitro* experiments and 90 different SOPs for *in vitro* assays were introduced into the database. Acutoxbase has proven to be a useful platform for data management and data exchange. Indeed, the statistical analyses performed (**P26**) so far are based on the records from Acutoxbase. The database served as source of human and *in vitro* cytotoxicity data for the estimation of human blood LC50 values (2) and for the *in vitro – in vivo* modelling.

The *in vitro – in vivo* modelling of LC_{50} blood concentration values for humans and LD_{50} values have been a central activity of the project during 2008, including 75 *in vitro* variables (tests), and data for 57 chemicals (**WP3**). Six of these variables are basal cytotoxicity tests

(**WP2**) and the remaining variables are from organ target specific tests for neurotoxicity, nephrotoxicity, hepatocytoxicity etc (**WP4-7**). Partial least squares regression was used for the comparison (**P26**). With the aim to find subsets of in vitro tests with good predictive capability, the influence of variable reduction was studied. From the models with the best predictive capabilities, outliers were identified.

The results showed that small batteries of a few in vitro tests give better predictive capabilities both for models based on LD50 rat (R2=0,59 and Q2=0,57) and LC50 human (R2=0,71 and Q2=0,69). The variables contributing to the best models were mainly basal cytotoxicity tests and the target organ specific tests did only improve R2 and Q2 with about 0.02, compared models based on only basal cytotoxicity tests.

A principal component analysis based on four basal cytotoxicity tests and eight target organ specific tests showed that the information content is similar in all tests and could be summarized in two principal components that described 88% of the variance in the data.

The models contained many chemicals that deviated more than +-0,75log unit. For the best LD50 model, the most deviating chemicals with negative residuals (-0,75 to -2 log units are Strychnine, Physostigmine, Warfarin, Sodium selenate, Parathion, Nicotine, Cycloheximide, and Epinephrine and with positive (0,75 to 1,5 log units) residuals are Amiodarone, Sodiumlauryl sulphate, 17a- Ethynylylestradiol, Cadmium chloride, Carbamazepine. For LC50 for humans the most deviating chemicals with negative residuals are Colchicine, Nicotine, Lindane, Atropine, Acetonitrile, Strychnine, Malathion, Cyclosporine, Parathion and the chemicals with positive residuals are Pentachlorphenol, Isopropyl alcohol, 2,4 dichlorphenoxyacid Dichlorvos, Diquat dibromide and Acetylsalicylic acid.

The results that small batteries of basal cytotoxicity tests are favourable to individual tests and models based on many test are similar to those obtained in the MEIC study.

Based on the results from the Statistical analyses presented above, the ACuteTox Consortium draw the conclusion that further analysis of data is needed in order to select methods for the testing strategy. For that reason, a brainstorming meeting was held with an Expert group. The main recommendation from this meeting was that additional analysis and data mining of ACuteTox data are needed and should be carried out by an independent expert. As a result of a call for tender a contractor has been selected and statistical analyses will start in the beginning of 2009.

The evaluation of data from **WP7.1** (3-8) showed that no single neuronal endpoint (AChE activity measurements, $GABA_A$ receptor function, CMP, CASP3, RS, GUp, NF-H, GFAP and MBP) dramatically improved correlation to the human lethal blood concentration (used as an estimate of the target tissue concentration of acute systemic toxicity) as compared to the general cytotoxicity measured in the 3T3-NRU assay (WP2) when analysed by linear regression. However, the neurotoxic endpoints identified several “alerts”, defined as a displayed effect in the neuronal endpoints at lower concentrations than in the 3T3-NRU test. Hence, the combination of general cytotoxicity data (pIC50) with neurotoxicity data (pNTC) gave a better prediction of the pLC50 than pIC50 alone. All neuronal endpoints identified a few chemicals with higher predicted activity (over-estimated toxicity) than the pLC50. This can in some cases can be explained by a restricted passage over the blood brain barrier, which illustrates the importance to integrate biokinetic information in the prediction of systemic toxicity by the use of the in vitro methodology.

Preliminary results (**P6**) indicate that integration of kinetic information, such as absorption (9), protein binding, lipophilicity and clearance with cytotoxicity test results could improve the prediction of acute systemic toxicity (**WP5**).

P9 in collaboration with **P21** has developed a novel assay of cytokine secretion by using human whole blood, based on the detection by bead-based flow cytometry of three relevant inflammatory cytokines; IL-1β, IL-6 and TNF-α upon stimulation of leucocytes by a bacterial product, lipopolysaccharide (**WP4**). In this way, the effect of toxic exposure on immune function may be examined through a more relevant method than the in vitro models based upon activation induced by a mitogenic agent, such as phytohemagglutinin.

By performing miniaturized assays (grouped as Cytomic Panel for Cytotoxicity Screening and Cytomic Panel for Oxidative Stress Screening) with three human cell lines (HepG2 hepatoma, SH-SY5Y neuroblastoma and A.704 kidney adenocarcinoma) **P9** showed that the cytomic assays correlated excellently with in vivo human toxicity, lower with in vitro and very poor with rodent toxicities (**WP4**). The suitability of these assays for classification, according to the Global Harmonization System (GHS) was assessed. The result showed that the cytomic assays do not separate clearly compounds belonging to toxic classes (GHS classes 1-5) but seem to reveal compounds labeled as non-toxic by GHS. It was concluded that cytomics is a promising analytical system for ACuteTox and for similar in vitro cell-based toxicological studies.

The results from testing 57 compounds in **WP7.2** indicated that TEER is a more sensitive indicator of nephrotoxicity than the Alamar blue assay (**P18** and **P25**).

The above described methods have also been included in the PLS-analyses described above.

P3 has assisted a subcontractor to further develop the Phototox software in order to fit the needs of the ACuteTox project, such as comparing dose-response curves from three different cell cultures (**WP6** and **7.3**). A first version of the software was developed during 2008. Experimental data can automatically be imported to the software from any type of Excel sheets. For each compound tested in the study, the software calculates automatically final results such as % viability and ICs. In addition, the software allows calculating the relative IC50 of the test compound considering the IC50 values of low and high reference compounds. This will enable better comparisons of IC50 between compounds. The IC50 of the 57 ACuteTox reference compounds as well as some additional bioactivable compounds tested by **P3** (cyclophosphamide monohydrate, tamoxifen, chlorpromazine and amphetamine sulphate) have been recalculated using the first version of Acusoft

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

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