

## WP3: Iterative amendment of the testing strategy

WP3 is a central work package of the ACuteTox project, which aims to reduce step by step the outliers from the *in vitro/in vivo* correlations, by examining the underlying reasons for deviations, such as the quality of *in vivo* and *in vitro* data, lack of information on ADME and/or target organ specificity. Consequently, WP3 interacts intensively with all the other WPs to identify a set of assays that will provide valuable alerts and corrector factors. The selected assays will be further introduced into a testing strategy.

The objectives in WP3 are the following:

1. Provide a database platform (Acutoxbase) to facilitate storage of SOPs, transfer of data from all partners (*in vivo* human and animal data, *in vitro* experimental data) and statistical analyses of larger data sets.
2. Identify outliers from the *in vivo-in vitro* comparisons of data obtained from WP1 and WP2.
3. Support with competence in multivariate modelling as performed in the MEIC (Multicenter Evaluation of In Vitro Cytotoxicity) project (Ekwall et al., 1998, 2000) and to carry out modelling of new data generated in WP1, WP2, and WP4-7.
4. Adapt *in vitro* methods to robotic platforms for high-throughput screening (HTS).

### 1. Acutoxbase

A pilot version of Acutoxbase was opened to all ACuteTox partners in March 2006.

#### The database consists of the following sections:

**Chemicals section** – contains the list of 97 ACuteTox reference chemicals with information about the provider (company, Cat number), a set of physico-chemical properties and a short description of the chemical (common use, reported target organ toxicities, some information on human biokinetics, etc.).

**Animal *in vivo* section** - contains oral acute toxicity data from animal experiments collected from literature on the 97 reference chemicals.

**Human poisoning cases** - gathers available data on human acute poisoning cases.

***In vitro* section** – is the largest part of the database. An accurately designed, standardized template enables to report practically any experiment performed *in vitro*, including detailed information about the protocol used, the conditions in which the particular experiment was carried on, and the raw data. The *in vitro* part contains also a section where the SOPs for all *in vitro* assays used in this project, and truthfully revised by experts in the field, are stored.

**Kinetics** - includes data from *in vitro* kinetics experiments and *in silico* modeling.

**Reports** – enables to generate summaries of all data in form of Excel files.

At the beginning of 2009 Acutoxbase contained a full set of data regarding the selected 97 reference chemicals, including molecular structure, physicochemical properties and summary descriptions. Moreover, 2204 data from acute oral toxicity studies *in vivo*, 2902 data sets from *in vivo* human blood poisoning reports, 10.300 files from *in vitro* experiments and 90 SOPs were introduced into the database. In summary, Acutoxbase has proven to be a very useful platform for data management and data exchange in such a large international project as ACuteTox (Kinsner-Ovaskainen A. et al 2009).

### 2. The robotic platform for HTS

One of the endeavours of the ACuteTox project is to provide an *in vitro* testing strategy composed of methods that are amendable to robotic HTS platforms and thus, could be easily

automised. In WP3, the 3T3/NR uptake and HepG2/MTT assays have successfully been adapted to two commercially available HTS robotic platforms.

### 3-4. Multivariate modelling and identification of outliers

*In vitro-in vivo* modelling of LC<sub>50</sub> values for humans and LD<sub>50</sub> values for rats have been performed using data from the 3T3/NRU assay. The model shows a number of outliers (17 in the comparison with human LC<sub>50</sub> values and 16 in the comparison with rat LD<sub>50</sub> values) (Table 1).

Furthermore, the *in vitro-in vivo* linear regression analysis has shown that the *in vitro* basal cytotoxicity assays correlated better with human peak lethal blood concentrations (LC<sub>50</sub>) than with LD<sub>50</sub> rat values. From this analysis it was also concluded that the *in vitro-in vivo* correlation based on mol/l and mol/kg was better than g/l and g/kg.

During 2007-08, 57 compounds (including outliers and non-outliers) were tested in WP4-7. The *in vitro – in vivo* modelling of LC<sub>50</sub> blood concentration values for humans and LD<sub>50</sub> values, including 75 *in vitro* assays, and data for the 57 chemicals were performed in 2008. Six of the 75 *in vitro* assays were basal cytotoxicity tests (see results **WP2**) and the remaining tests were organ target specific tests for neurotoxicity, nephrotoxicity, hepatocytotoxicity etc (see results **WP4-7**). Partial least squares regression was used for the comparison. With the aim to find subsets of *in vitro* tests with good predictive capability, the influence of variable (assay) reduction was studied. The results showed that small batteries of a few *in vitro* tests give better correlations than batteries with many tests, both for models based on LD<sub>50</sub> rat ( $R^2=0,59$  and  $Q^2=0,57$ ) and LC<sub>50</sub> human ( $R^2=0,71$  and  $Q^2=0,69$ ). The assays contributing to the best models were mainly basal cytotoxicity tests and the target organ specific tests did only improve  $R^2$  and  $Q^2$  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  $\pm 0,75$  log unit. For the best LD<sub>50</sub> 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, 17 $\alpha$ - Ethynylylestradiol, Cadmium chloride, Carbamazepine. For LC<sub>50</sub> 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 Pentachlorophenol, Isopropyl alcohol, 2,4 dichlorphenoxyacid Dichlorvos, Diquat dibromide and Acetylsalicylic acid.

The result that small batteries of basal cytotoxicity tests predict acute human toxicity better than individual tests or models based on many test are similar to the results 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. In 2009 a biostatistician was subcontracted to perform other statistical analysis using the raw data submitted by the partners to Acutetoxbase (see news from the project and results of WP9).

**Table 1. Outliers (in bold) identified by comparing mean values of 3T3/NRU assay with LD<sub>50</sub> and human LC<sub>50</sub> values**

|    | Reference Chemicals                 | Outliers (rat oral LD <sub>50</sub> ) | Outliers (human LC <sub>50</sub> ) | Comments               |
|----|-------------------------------------|---------------------------------------|------------------------------------|------------------------|
| 1  | <b>acetaminophen</b>                |                                       | <b>x</b>                           |                        |
| 2  | acetylsalicylic acid                |                                       |                                    |                        |
| 3  | <b>atropine sulfate monohydrate</b> |                                       | <b>x</b>                           |                        |
| 4  | caffeine                            |                                       |                                    |                        |
| 5  | carbamazepine                       |                                       |                                    |                        |
| 6  | colchicine                          | No animal data                        |                                    | Variable in vitro data |
| 7  | cycloheximide                       |                                       | No human data                      | Variable in vitro data |
| 8  | diazepam                            |                                       |                                    |                        |
| 9  | <b>digoxin</b>                      | <b>x</b>                              | <b>x</b>                           | Variable in vitro data |
| 10 | isopropyl alcohol                   |                                       |                                    |                        |
| 11 | <b>malathion</b>                    |                                       | <b>x</b>                           |                        |
| 12 | mercury (II) chloride               |                                       |                                    |                        |
| 13 | <b>pentachlorophenol</b>            |                                       | <b>x</b>                           | Poor human data        |
| 14 | <b>phenobarbital</b>                | <b>x</b>                              |                                    | Poor human data        |
| 15 | sodium lauryl sulfate               |                                       |                                    |                        |
| 16 | sodium valproate                    |                                       |                                    |                        |
| 17 | <b>5-fluorouracil</b>               |                                       | <b>x</b>                           | Variable in vitro data |
| 18 | benzene                             |                                       | No human data                      |                        |
| 19 | tert-butylhydroperoxide             |                                       | No human data                      |                        |
| 20 | acrylaldehyde (acrolein)            |                                       | No human data                      |                        |
| 21 | cadmium (II) chloride               |                                       |                                    | Poor human data        |

|           |                                |                |                      |                        |
|-----------|--------------------------------|----------------|----------------------|------------------------|
| 22        | phenanthrene                   |                | No human data        |                        |
| 23        | pyrene                         |                | No human data        |                        |
| 24        | 1,2,3,4-tetrachlorobenzene     |                | No human data        |                        |
| 25        | pentachlorobenzene             |                | No human data        |                        |
| 26        | hexachlorobenzene              |                | No human data        | Variable in vitro data |
| 27        | benz(a)anthracene              | No animal data | No human data        | No in vivo data        |
| 28        | amiodarone hydrochloride       |                |                      | Poor human data        |
| 29        | (±)-verapamil hydrochloride    |                |                      | Poor human data        |
| 30        | rifampicine                    |                |                      |                        |
| 31        | tetracycline hydrochloride     |                | No human data        |                        |
| 32        | orphenadrine hydrochloride     |                |                      |                        |
| <b>33</b> | <b>nicotine</b>                | <b>x</b>       | <b>x</b>             | Poor human data        |
| <b>34</b> | <b>lindane</b>                 |                | <b>x</b>             | Poor human data        |
| bis 29    | (±)-verapamil hydrochloride    |                |                      |                        |
| <b>35</b> | <b>D-amphetamine sulfate</b>   | <b>x</b>       | <b>No human data</b> | Restricted             |
| <b>36</b> | <b>methadone hydrochloride</b> |                | <b>x</b>             | Restricted             |
| 37        | ethanol                        |                |                      |                        |
| <b>38</b> | <b>parathion</b>               | <b>x</b>       | <b>No human data</b> |                        |
| 39        | dichlorvos                     |                | No human data        |                        |
| <b>40</b> | <b>physostigmine</b>           | <b>x</b>       | <b>No human data</b> |                        |
| 41        | glufosinate-ammonium           |                |                      | Poor human             |

|           |  |          |               |                 |
|-----------|--|----------|---------------|-----------------|
|           |  |          |               | data            |
| <b>42</b> | <b><i>cis</i>-diammineplatinum (II) dichloride</b> |          | <b>x</b>      |                 |
| bis<br>21 | cadmium (II) chloride                              |          |               |                 |
| 43        | diethylene glycol                                  |          | No human data |                 |
| <b>44</b> | <b>diquat dibromide</b>                            |          | <b>x</b>      |                 |
| <b>45</b> | <b>ochratoxin A</b>                                | <b>x</b> | No human data |                 |
| bis<br>28 | amiodarone hydrochloride                           |          |               |                 |
| bis<br>31 | tetracycline hydrochloride                         |          | No human data |                 |
| bis<br>30 | rifampicine  |          | No human data |                 |
| <b>46</b> | <b>cyclosporine A</b>                              |          | <b>x</b>      |                 |
| 47        | 17 $\alpha$ -ethynylestradiol                      |          | No human data |                 |
| 48        | sodium fluoride                                    |          |               |                 |
| 49        | paraquat dichloride                                |          |               |                 |
| 50        | glycerol   |          |               |                 |
| 51        | dimethylformamide                                  |          |               | Poor human data |
| 52        | iron II sulfate                                    |          |               |                 |
| 53        | amitriptyline hydrochloride                        |          |               |                 |
| 54        | ethylene glycol                                    |          |               |                 |
| 55        | methanol   |          |               |                 |
| 56        | phenol   |          |               | Poor human data |
| <b>57</b> | <b>sodium chloride</b>                             |          | <b>x</b>      |                 |
| 58        | xylene   |          |               |                 |
| <b>59</b> | <b>potassium cyanide</b>                           | <b>x</b> |               |                 |
| 60        | lithium sulfate                                    |          |               |                 |

|           |                                       |          |          |                 |
|-----------|---------------------------------------|----------|----------|-----------------|
| 61        | theophylline                          |          |          |                 |
| 62        | haloperidol                           |          |          |                 |
| 63        | propranolol hydrochloride             |          |          |                 |
| 64        | arsenic trioxide                      |          |          |                 |
| 65        | thioridazine hydrochloride            |          |          |                 |
| <b>66</b> | <b>thallium sulphate</b>              | <b>x</b> |          |                 |
| <b>67</b> | <b>warfarin</b>                       | <b>x</b> |          | Poor human data |
| 68        | chloroform                            |          |          |                 |
| 69        | isoniazid                             |          |          |                 |
| 70        | dichloromethane                       |          |          |                 |
| 71        | hexachlorophene                       |          |          |                 |
| 72        | chloroquine diphosphate               |          |          |                 |
| 73        | 5,5-diphenylhydantoin                 |          |          |                 |
| 74        | chloramphenicol                       |          |          |                 |
| 75        | potassium chloride                    |          |          |                 |
| 76        | chloral hydrate                       |          |          |                 |
| <b>77</b> | <b>2,4-dichlorophenoxyacetic acid</b> |          | <b>x</b> |                 |
| 78        | meprobamate                           |          |          |                 |
| 79        | sodium pentobarbital                  |          |          |                 |
| <b>80</b> | <b>strychnine</b>                     | <b>x</b> | <b>x</b> |                 |
| 81        | glutethimide                          |          |          |                 |
| 82        | maprotiline                           |          |          |                 |
| 83        | disopyramide                          |          |          |                 |
| 84        | diphenhydramine                       |          |          |                 |
| 85        | chlormethiazole                       |          |          |                 |
| 86        | quinidine sulfate dihydrate           |          |          |                 |
| 87        | procainamide hydrochloride            |          |          |                 |
| <b>88</b> | <b>codeine</b>                        |          | <b>x</b> | Restricted      |
| 89        | chlorpromazine hydrochloride          |          |          | Poor human data |

|           |                        |          |                      |                 |
|-----------|------------------------|----------|----------------------|-----------------|
| 90        | paraldehyde            |          |                      | Poor human data |
| <b>91</b> | <b>sodium selenate</b> | <b>x</b> |                      |                 |
| 92        | acetonitrile           |          |                      |                 |
| 93        | sodium bicarbonate     |          |                      |                 |
| 94        | acrylamide             |          |                      |                 |
| <b>95</b> | <b>formaldehyde</b>    | <b>x</b> | <b>No human data</b> | Not testable    |
| <b>96</b> | <b>(-)-epinephrine</b> | <b>x</b> | <b>No human data</b> |                 |
| 97        | acetaldehyde           |          |                      |                 |

### References:

Ekwall, B. et al (1998) MEIC evaluation of acute systemic toxicity: Part VI. The prediction of human toxicity by rodent LD50 values and results from 61 *in-vitro* methods. *ATLA* 26: 617-658.

Ekwall B. et al (2000). MEIC evaluation of acute systemic toxicity. Part VIII. Multivariate partial least squares evaluation, including the selection of a battery of cell line tests with a good prediction of human acute lethal peak blood concentrations for 50 chemicals. *ATLA* 28, 201-234.

Kinsner-Ovaskainen A., Rzepka R., Rudowski R., Coecke S., Cole T., Prieto P. (2009) Acutoxbase, an innovative database for in vitro acute toxicity studies. *Toxicol. In Vitro*, 23:476-85.