ECVAM Scientific Advisory Committee (ESAC): Shadow Panel for the ICCVAM Peer Review of *In Vitro* Acute Toxicity Test Methods

André Guillouzo (France)
Manfred Liebsch (Germany)
Katalin Horvath (Hungary)
Michael Ryan (Ireland)
Mykolas Maurica (Lithuania)
Julia Fentem (ECETOC)

5 May 2005

Dr. William Stokes
Director of NICEATM
NIEHS, MD EC-17
P.O. Box 12233
Research Triangle Park, NC, 27709
USA

Submitted per Email to: niceatm@niehs.nih.gov

Comments on "Draft *In Vitro* Acute Toxicity Test Methods BRD" issued 28 March 2006

Dear Dr Stokes

upon issue of the above mentioned BRD on the outcome of the ICCVAM-ECVAM basal cytotoxicity test methods validation study, the ECVAM Scientific Advisory Committee (ESAC) has on it's 24th Meeting decided to form an "ESAC shadow panel" to facilitate a transparent communication process between ICCVAM and ECVAM, jointly responsible for this study.

The six ESAC panel members (representing five EU Member Countries and one NGO, see header of this letter) have communicated in writing and finally agreed in a teleconference to comment on the BRD in a tiered manner: In the current letter we will address only general comments that we have agreed upon to be most important. We will then at the public NIH Peer Review Meeting on 23 May 2006 in Bethesda add more specific comments.

The ESAC panel identified the following issues to be most important and necessary to comment on:

1. Preceding the validation study, experts of an international ICCVAM/NICEATM Workshop on *in vitro* methods for prediction of acute systemic toxicity, in October 2000 recommended to use standard basal cytotoxicity tests plus Halle's linear RC prediction model (after a Guidance Document had been produced), without any further validation for the determination of starting doses in *in vivo* testing for acute oral toxicity studies according to OECD TG 425 and OECD TG 423 (1). For a possible full replacement of *in vivo* systemic toxicity tests, the experts recommended conduct of an experimental validation study including advanced *in vitro* ADME test systems addressing biokinetics by modelling bioavailability through barriers and metabolism of test chemicals. Although in 2001 the Guidance Document had been drafted by Workshop participants, and approved and published by ICCVAM (2), in the current experimental validation study only basal cytotoxicity tests and Halle's RC prediction model were investigated. <u>The ESAC panel therefore questions why the recommendations of the experts (1)(2) have not been considered</u>.

2. As a scientific principle, validation should be hypothesis testing, i.e. verification or falsification of hypothesis(es) clearly defined in the study objectives. However, the ESAC panel regards the objectives of the current validation study a mixture of partly conflicting goals:

   (a) validate the RC prediction model (which is based on collected *in vitro* data from various literature sources) by experimentally generating high quality *in vitro* data with two standard tests in three laboratories and comparing them with high quality *in vivo* data from broader sources than just one LD_{50} value from RTECS/NIOSH.

   (b) assess the boundaries of applicability for basal cytotoxicity tests by challenging the RC prediction model with test chemicals with specific modes of action, not well characterised by basal cytotoxicity (see comment No.3 below).

   (c) assess predictive performance for predicting rat LD_{50} point measures and derived GHS toxicity classes

   (d) assess usefulness of the basal cytotoxicity tests in connection with the RC prediction model for determining staring doses

   (e) optimise during the course of the study the two standard *in vitro* assays

   In particular, conflicting objectives (a) and (b) have resulted in an unclear study design.

3. The ESAC panel regards the selection of the 72 test chemicals inappropriate to achieve the main goal of the study (verification or falsification of the RC prediction model). In a draft paper of the ICCVAM Acute Toxicity Working Group (ATWG) of December 2001 on the study design it was stated "***chemicals will be chosen so as to represent the range of toxicity in each GHS category, and/or so that the entire set of chemicals has no more chemicals that were more than half log (i.e., 0.699) from the RC regression (proportionally) than the entire RC database (referred to as "RC outliers" by the authors of the RC database)***". However, the authors of the RC data base (ZEBET) got aware in spring 2004 that the final selection of test chemicals not only contained a higher percentage of outliers, but also of these 21 RC outliers 19 were below the acceptance boundaries of the regression (false negative predictions), and only 2 chemicals were above the boundaries (false positives). In a letter dated 1 March 2004, ZEBET commented on this unbalanced selection of chemicals and stated that it will be impossible to meet the main study objective of validating the RC prediction model (**ATTACHMENT 1**). The ESAC panel shares this statement.

4. **Variability of *in vitro* data**: During phases I and II of the validation study, the *in vitro* test protocols were optimised. The necessity and success of this "optimisation" may be questioned, given that the test acceptance criteria had to be loosened for the final testing phase. The 72 reference substance data differed in 2 cases with 5 orders of magnitude (!) and in 23 cases with 1-2 orders of magnitude. The ESAC panel therefore recommends to compare this outcome with other interlaboratory validation studies that have used the 3T3 NRU standard protocol.

5. **Variability of *in vivo* reference data**: The enormous efforts of ICCVAM for acquisition of multiple *in vivo* LD50 data per test chemical including the application of defined acceptance criteria to these data are appreciated. However, in all current and most of the past validation studies the variability of the *in vivo* data was analysed as a means to assess the performance of the alternative methods. The ESAC panel is missing this type of analysis in the BRD. To depict that in particular in the toxic range the confidence interval of one LD_{50} may span over 2-3 GHS toxicity classes we have attached a figure visualising mean LD_{50} values and confidence intervals (**ATTACHMENT 2**). At the same time, this figure shows the relation between LD50 values used in the RC (NIOSH 1983) in relation to the new values used in the current validation study.

6. ***In vitro* / *in vivo* predictivity**: The evaluation of predictive capacity of the two *in vitro* assays is highly biased by the unbalanced selection of chemicals. A good example for this bias is **Figure 6-1**, where in the range of 0.001 to 1.0 mmol almost all *in vitro* data are underpredictions, while in the range of >1.0 up to 1000 mmol only overpredictions occur. Even with a simple eyeball assessment the regression shown in Figure 6-1 has an extremely poor fit with the data points. As a consequence, all secondary calculations, as e.g. the contingency tables for prediction of the GHS classes are influenced by the bias in the chemical selection, so that even the strength of the prediction model (correct prediction of the absence of toxicity) is lost. The ESAC panel therefore recommends a thorough discussion of the influence of chemical selection on the study outcome.

7. The current validation study outcome needs to be discussed in relation to the already many existing studies (e.g. of the MEIC programme). Therefore, the ESAC panel recommends to relate the results of the current study to the existing information, interpret and discuss appropriately.

8. In view of the issues addressed above, the ESAC panel regards the drafted ICCVAM recommendation to change the regression of the RC as too early. In the same context, the ESAC panel regards the draft definition of minimum performance standards as much too early.

The ESAC panel is looking forward to constructive discussions with ICCVAM of the points raised in this comment.

Sincere regards

André Guillouzo (France)
Manfred Liebsch (Germany)
Katalin Horvath (Hungary)
Michael Ryan (Ireland)
Mykolas Maurica (Lithuania)
Julia Fentem (ECETOC)

Enclosures:

ATTACHMENT 1: letter of ZEBET dated 01 March 2004

ATTACHMENT 2: analysis of variability of *in vivo* reference data used

(1) NIEHS (National Institute of Environmental Health Sciences). 2001a. Report of the international workshop on *in vitro* methods for assessing acute systemic toxicity. NIH Publication 01-4499. NIEHS, Research Triangle Park, North Carolina.
http://iccvam.niehs.nih.gov/methods/invidocs/finalall.pdf

(2) NIEHS (National Institute of Environmental Health Sciences). 2001b. Guidance document on using *in vitro* data to estimate *in vivo* starting doses for acute toxicity. NIH publication 01-4500. NIEHS, Research Triangle Park, North Carolina.
http://iccvam.niehs.nih.gov/methods/invidocs/guidance/iv_guide.pdf