Preliminary (Phase I) Results of a Validation Study to Evaluate the Reliability and Relevance of two In Vitro Cytotoxicity Assays for Predicting Rodent and Human Acute Systemic Toxicity

S. Casati[1], J.A. Strickland[2,3], M.W. Paris[2,3], W.S. Stokes[2], R.R. Tice[2,3], J. Haseman[4], A.P. Worth[5], H. Raabe[6], C. Cao[7], R. Clothier[8], J. Harbell[6], R. Curren[6], M.L. Wenk[9], M.K. Vallant[4], G. Mun[6], M. Clear[6], G.O. Moyer[6], J. Madren-Whalley[7], C. Krishna[7], M.Owen[8], N. Bourne[8]

[1]European Centre for the Validation of Alternative Methods (ECVAM) JRC, Ispra, Italy; [2]NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), RTP, NC USA 27709; [3]ILS, Inc., RTP, NC USA, [4]National Institute of Environmental Health Sciences (NIEHS), RTP, NC USA; [5]European Chemicals Bureau (ECB) JRC Ispra Italy; [6]Institute for In Vitro Sciences, Gaithersburg, MD USA, [7]U.S. Army Edgewood Chemical Biological Center, APG, MD USA; [8]Univ. of Nottingham, Nottingham, UK; [9]BioReliance Corp., Rockville, MD USA

INTRODUCTION

The oral LD50 (median lethal dose) test has been conducted since the twenties for the acute toxicity ranking of substances. Although three methods that reduce the use of animals now substitute for the classical LD50 test, acute systemic toxicity testing still uses a large proportion of the total number of animals used in regulatory toxicology.

In October 2000, ICCVAM and NICEATM organised an international workshop (ICCVAM 2001a) to review the state-of-the-art of *in vitro* cytotoxicity assays for assessing acute toxicity. One of the workshop recommendations was that two basal cytotoxicity assays were sufficiently developed and standarsised to be evaluated for their usefulness to improve starting dose selection in *in vivo* studies, and to evaluate their potential to correlate with human lethal concentrations (ICCVAM 2001b). Subsequently NICEATM and ECVAM started a joint multi-laboratory validation study.

STUDY OBJECTIVES

•To optimise and standardise the two test method protocols

•To assess the ability of the two assays to estimate rodent oral LD50 values across the Globally Harmonised System categories of acute oral toxicity, and human lethal concentrations.

•To evaluate the accuracy in prediction for the starting dose in *in vivo* testing.

•To determine the reduction in the number of animals that would be obtained by predicting the starting doses for *in vivo* acute toxicity testing.

•To generate a high quality *in vitro* database to be used for the evaluation of other methods to be part of an *in vitro* testing strategy for acute systemic toxicity.

IN VITRO CYTOTOXICITY ASSAYS

- **The mouse fibroblast (BALB/c) 3T3 NRU**
- **The normal human keratinocyte (NHK) NRU**

The **neutral red uptake** (NRU) cytotoxicity assay is a viability staining procedure (Borenfreund and Puerner, 1984). Determination of the amount of retained NR in cells exposed to test compounds (Riddell et al., 1986), compared with control cells, enables the relative toxicity of test chemicals to be assessed.

CHEMICALS

72 chemicals are being tested coded in both assays. Chemical selection followed the following criteria:

• Representative of five Globally Harmonised System (GHS) categories of acute toxicity (OECD 2001) as well as non-toxic chemicals.

•Representative of chemicals regulated by the various regulatory authorities.

•Availability of acute oral rodent toxicity data and oral human toxicity data.

STUDY PHASES

Phase Ia: Laboratory evaluation phase – Completed
Establishment of the positive control chemical (sodium lauryl sulfate [SLS]) reference range (IC50 ± 2.5 SD; N>10) for each cell type, to be used to assess the quality performance in subsequent phases.

Phase Ib: Laboratory evaluation phase- Completed
Testing of three coded chemicals (N≥3) → protocol refinement

Phase II: Laboratory qualification phase- Ongoing
Testing of nine coded chemicals (N≥3) → protocol finalisation

Phase III: Testing phase
Each lab tests 60 coded chemicals in each assay (N=3)

PREDICTION MODELS

RODENT

The prediction model evaluated will be the Registry of Cytotoxicity (RC) regression between cytotoxicity values (IC_{50}) and rodent acute oral LD50 values of 347 chemicals (Halle, 1998; 2003).

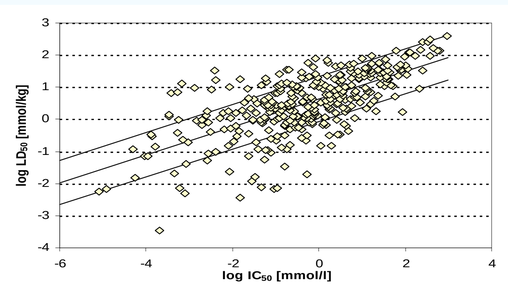


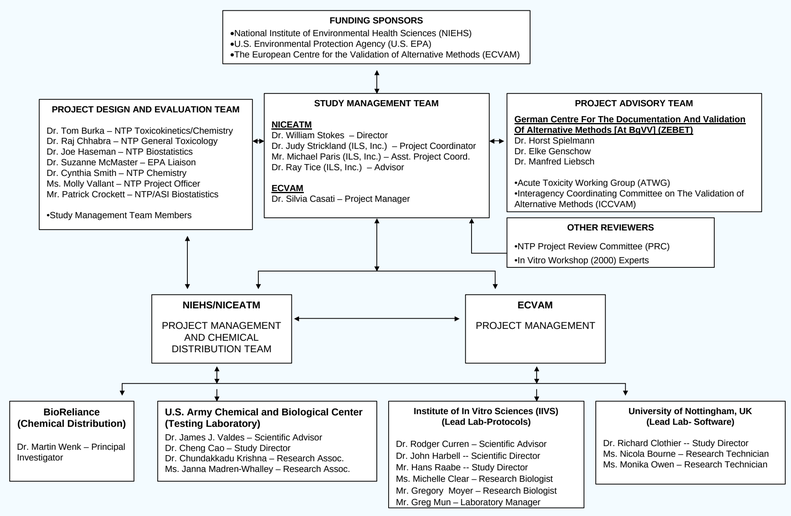
Figure 1. RC regression model

The thicker line represents the fit of the data to a linear regression (r=0.67). The two additional lines represent the empirical boundaries of the prediction interval (± log 5). The equation of the regression line is: log LD50 = 0.435 × log IC_{50X} + 0.625

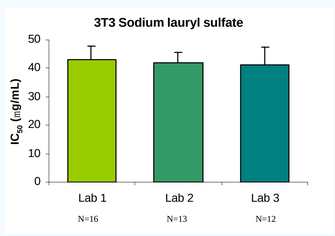
HUMAN

The feasibility of developing a preliminary human prediction model will be evaluated by using the *in vitro* results, obtained in both tests, for the 12 chemicals tested in Phase I and II, and the corresponding human sublethal and lethal blood concentrations (MEMO database; Ekwall et al. 1998). *In vitro* data for Phase III Chemicals will be used to validate the model.

PROJECT MANAGEMENT



PHASE I RESULTS



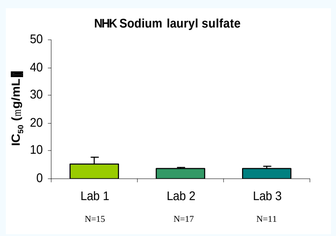
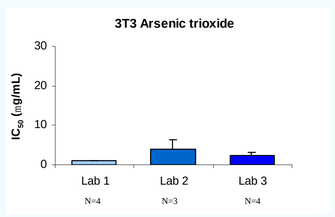


Figure 2. Toxicity of sodium lauryl sulfate to 3T3 cells and NHK cells. Data shown represent the mean IC_{50} value (concentration that inhibits cell viability by 50%.) ± SD for each laboratory.



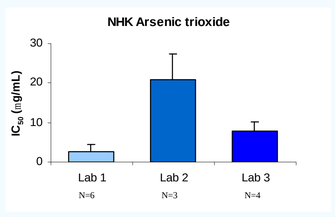
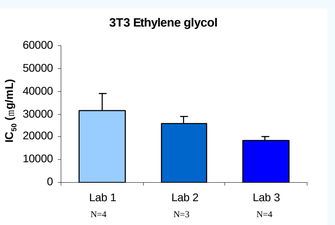


Figure 3. Toxicity of arsenic trioxide to 3T3 cells and NHK cells. Data shown represent the mean IC_{50} value (concentration that inhibits cell viability by 50%.) ± SD for each laboratory.



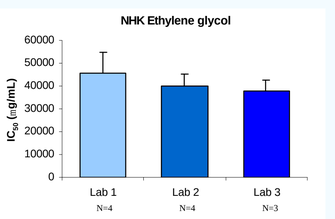
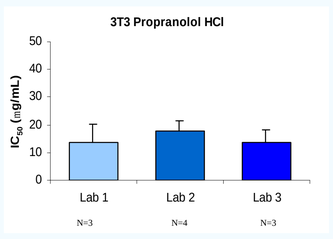


Figure 4. Toxicity of ethylene glycol to 3T3 cells and NHK cells. Data shown represent the mean IC_{50} value (concentration that inhibits cell viability by 50%.) ± SD for each laboratory.



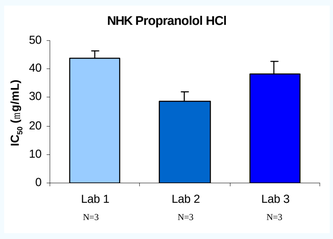


Figure 5. Toxicity of propranolol HCl to 3T3 cells and NHK cells. Data shown represent the mean IC_{50} value (concentration that inhibits cell viability by 50%.) ± SD for each laboratory.

Preliminary results (phases Ia and Ib) showed the following :

•The NHK cells proved to be more sensitive to the positive control SLS whereas the 3T3 cells were more sensitive to the other test compounds.

•The interlaboratory comparison of the endpoint measured (IC50 values) for each chemical showed no statistically significant difference at $p < 0.05$ (analysis of variance for random effects).

•Results for 3T3 cells were more reproducible than NHK results. The average coefficient of variation (CV= SD/mean) for the three test chemicals was 0.34 for the 3T3 cells and 0.41 for the NHK cells.

•The higher interlaboratory variability for arsenic trioxide can be explained by the difficulty in dissolving the chemical.

Phase I results provided the means to further standardise the protocols for phase II testing. Results for study II will be used to finalise the protocols for study phase III.

REFERENCES

Borenfreund E, J Puerner J. 1984. A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/ NR-90). J. Tissue Culture Meth. 9: 7-9.

Ekwall B, Clemedson C, Crafoord B, Ekwall B, Hallander S, Walum E, Bondesson I. 1998. MEIC evaluation of acute systemic toxicity. Part V. Rodent and human toxicity data for the 50 reference chemicals. ATLA 26: 571-616.

Halle W. 1998. Toxizitätsprüfungen in Zellkulturen für eine Vorhersage der akuten Toxizität (LD_{50}) zur Einsparung von Tierversuchen. Life Sciences/ Lebens-wissenschaften, Volume 1, 94 pp., Jülich: Forschungszentrum Jülich.

Halle W. 2003. The Registry of Cytotoxicity: Toxicity testing in cell cultures to predict acute toxicity (LD50) and to reduce testing in animals. ATLA 31: 89-198.

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). 2001a. Report of the international workshop on *in vitro* methods for assessing acute systemic toxicity. NIH Publication 01-4499. Research Triangle Park, NC:National Institute for Environmental Health Sciences. http//:iccvam.niehs.nih.gov/

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). 2001b. Guidance document on using *in vitro* data to estimate *in vivo* starting doses for acute toxicity. NIH publication 01-4500. Research Triangle Park, NC:National Institute for Environmental Health Sciences. http//:iccvam.niehs.nih.gov/

MEMO Database (MEIC Monographs). http://www.cctoxconsulting.a.se/meicinvivo.htm

OECD (Organisation for Economic Co-operation and Development). 2001. Series on testing and assessment, No. 33: Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures. OECD Guidelines for the Testing of Chemicals, vol. 1, no. 5, pp. 1-249 (249). http://www.sourceoecd.org/

Riddell RJ, Panacer DS, Wilde SM, Clothier RH, Balls M. 1986. The Importance of exposure period and cell type in in vitro cytotoxicity tests. ATLA 14: 86-92.

Institute for Health and Consumer Protection

For information, please contact:

ECVAM
Silvia Casati
silvia.casati@jrc.it

NICEATM
Judy Strickland
strickl2@niehs.nih.gov

JOINT RESEARCH CENTRE
EUROPEAN COMMISSION