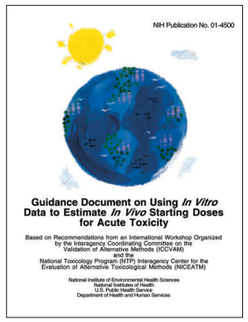
DESIGN OF A VALIDATION STUDY TO EVALUATE *IN VITRO* CYTOTOXICITY ASSAYS FOR PREDICTING RODENT AND HUMAN ACUTE SYSTEMIC TOXICITY

J A Strickland[1], W S Stokes[2], S Casati[3], M W Paris[1], A P Worth[3], H Raabe[4], C Cao[5], R Clothier[6], J Harbell[4], R Curren[4], J Haseman[2] and R R Tice[1]

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

Introduction



Acute oral toxicity testing is used to characterize the risk for mortality associated with human exposure to a substance. In October, 2000, the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity reviewed the validation status of *in vitro* methods directed toward reducing and refining the use of laboratory animals for acute toxicity testing (ICCVAM 2001a). One approach considered was the use of *in vitro* cytotoxicity assays to predict acute *in vivo* lethality (Spielmann et al. 1999). One of the workshop recommendations for reducing and refining the use of animals for lethality assays in the near-term was the publication of guidance for using *in vitro* cytotoxicity assays to estimate the starting dose for acute oral lethality assays (ICCVAM 2001b). The recommended publication, illustrated above, provides details and examples on how to execute such an approach.

This validation study implements the *Guidance Document* approach and another workshop recommendation to compare the ability of toxicity data obtained *in vitro* using rodent or human cells to predict rodent *in vivo* LD_{50} data and human *in vivo* mortality data so as to further the development of *in vitro* predictive models for human acute toxicity.

Study Objectives

- To further standardize and optimize two *in vitro* cytotoxicity protocols in order to maximize intra- and inter-laboratory reproducibility.
- To assess the accuracy of two standardized *in vitro* cytotoxicity assays for estimating rodent oral LD_{50} values and human lethal concentrations across the five Globally Harmonised System (GHS; OECD 2001) categories of acute oral toxicity as well as unclassified toxicities.
- To estimate the reduction and refinement (i.e., reduced deaths) in animal use that would result from using *in vitro* cytotoxicity assays to estimate starting doses for *in vivo* acute toxicity testing.
- To generate a high quality *in vitro* database that can be used to support investigation of other methods necessary to improve the accuracy of *in vitro* assessments of acute systemic toxicity.

Planning

Selection of *In Vitro* Cytotoxicity Assays

Two neutral red uptake (NRU) assays[1] were selected using

1. Mouse fibroblast (BALB/c) 3T3 cells, and
2. Normal human keratinocytes cells.

Rationale:

- Development of both rodent and human *in vitro* models recommended by workshop participants (ICCVAM 2001a)
- Both assays specifically recommended in the *Guidance Document* (ICCVAM 2001b) for prediction of starting doses for acute lethality assays.
- Reproducibility demonstrated in previous validation studies.
- Database on responsiveness of cells is available.
- Amenable to 96-well plate culture.
- Commercially available.

[1]See poster #764 entitled "Assessment of Protocol Variables in Cytotoxicity Assays Utilizing Balb/c 3T3 Cells and Normal Human Keratinocytes" by Curren et al.

Chemical Selection and Identification of Reference LD_{50} Values

Seventy-two chemicals were selected for testing using the following criteria recommended by Workshop participants (ICCVAM 2001a):

- Representative of five GHS categories of acute oral toxicity (OECD 2001) as well as unclassified (i.e., nontoxic) chemicals.

Category	Oral LD_{50}
Category 1	≤ 5 mg/kg
Category 2	> 5 - ≤ 50 mg/kg
Category 3	> 50 - ≤ 300 mg/kg
Category 4	> 300 - ≤ 2000 mg/kg
Category 5	> 2000 - ≤ 5000 mg/kg
Unclassified	> 5000 mg/kg

- Representative of chemicals regulated by the various regulatory authorities.
- Availability of acute oral rodent (preferably rat) toxicity data.
- Availability of acute oral human toxicity data and/or human exposure potential.

LD_{50} reference values for each chemical were determined after evaluation of the primary data sources identified by literature and database searches for development of the refined prediction model[2]. To identify a relatively homogenous dataset of LD_{50} values from gavage administration to adult laboratory rats, studies with the following, less typical, attributes were excluded:

- Determined with rats < 4 weeks of age, or with feral or anesthetized rats
- Oral administration in food or capsules rather than by gavage
- LD_{50} provided as a range rather than a point estimate

The reference value for each chemical was determined by calculating a geometric mean LD_{50} from the remaining LD_{50} values (if multiple values remained).

[2]See poster #764 entitled "Establishment of Rat LD_{50} Reference Values for Chemicals Tested in a Validation Study of *In Vitro* Cytotoxicity Assays" by Paris et al. for more information on the selection of reference values.

Implementation

General Conduct of the Study

- Conducted in compliance with Good Laboratory Practice (GLP) Standards (U.S. Food and Drug Administration, Title 21 CFR Part 58; Environmental Protection Agency, Title 40 CFR Part 160) at U.S. labs, OR,
- Conducted with a GLP-like approach (e.g., laboratory conditions documented, equipment maintenance and calibration routinely performed and documented, study workbooks maintained; all records, documents, raw data, reports, available to the Management Team for audit; final report states whether the methods and results accurately reflect the raw data) at the U.K. lab.

Chemical Distribution

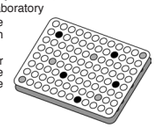
- Chemical samples coded, packaged, and shipped to three participating laboratories by an independent laboratory.
- Received by the laboratory Safety Officers along with data sheets detailing physical description of samples, storage conditions, and health and safety information packet.
- Safety Officer retains health and safety information and passes the coded samples, storage conditions, and physical description of sample to Study Directors.

Study Phases

Phase Ia: Laboratory Evaluation Phase – Completed November 2002

Development of positive control database for each laboratory

- Perform at least 10 replicate tests of the positive control chemical (sodium laurel sulfate [SLS]) with each cell type.
- Calculate mean $IC_{50} \pm 2$ standard deviations for each cell type for each lab to establish acceptance criteria for positive control performance in future assays.



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Phase Ib: Laboratory Evaluation Phase

Limited chemical testing for possible protocol refinement

- Each lab tests the same three coded chemicals of varying toxicities three times with each cell type.
- Refine protocols and repeat, if necessary, until acceptable intra/interlaboratory reproducibility is achieved.

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Phase II: Laboratory Qualification Phase

- Each lab tests nine coded chemicals covering the range of GHS toxicity categories. Three replicate tests/chemical for each assay.
- Assure that corrective actions taken in Phase I have achieved the desired results.
- Further refine protocols and re-test if necessary to achieve acceptable results.
- Finalize protocols for Phase III

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Phase III: Laboratory Testing Phase

- Each lab tests 60 coded chemicals three times using the final protocols for each assay.
- Submit data to Study Management Team for analysis.

Rodent Prediction Model

As the *Guidance Document* (ICCVAM 2001b) describes, the approach is based on the linear regression analysis of rodent *in vivo* oral LD_{50} and *in vitro* IC_{50} values for 347 chemicals in the Registry of Cytotoxicity (RC) developed by ZEBET (German Centre for the Documentation and Validation of Alternative Methods) (Halle 1998):

$$\log LD_{50}\ (\text{mmol/kg}) = 0.435 \log IC_{50}\ (\text{mM}) + 0.625$$

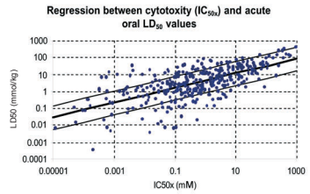
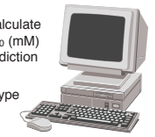


Figure 1. Registry of Cytotoxicity regression between cytotoxicity (IC_{50X}) and rodent acute oral LD_{50} values for 347 chemicals. The heavy line shows the fit of the data to a linear regression model, $\log (LD_{50}) = 0.435 \times \log (IC_{50X}) + 0.625$; r=0.67. The thinner lines show the empirical F_{95} = log 5 acceptance interval for the prediction model that is based on the anticipated precision of LD_{50} values from rodent studies (Halle 1998).

Data Analyses



- For each cell type, use RC LD_{50} data to calculate the regression of LD_{50} (mmoles/kg) on IC_{50} (mM) values and compare results to the RC prediction model.
- Refine the prediction model for each cell type using rodent LD_{50} reference values to determine whether each regression: (a) significantly differs from the RC prediction model, (b) significantly improves the correlation between the LD_{50} and the IC_{50}, (c) significantly differs from the regression for the other cell type.
- For each cell type, use IC_{50} data to predict the starting dose for LD_{50} assays.
- For each cell type, use simulation modeling to calculate the reduction in animal use by employing the predicted starting dose vs a standard starting dose in the Up-and-Down Procedure for estimating the LD_{50}.
- Compare reduction in animal use for each cell type.

Human Prediction Model

To date, a human prediction model based on a single *in vitro* endpoint has not been reported. The feasibility of developing such a model with either 3T3 fibroblast or NHK data will be evaluated by using the *in vitro* results for the 12 chemicals tested in Phases I and II, and the corresponding human sublethal and lethal blood concentrations (MEMO database; Ekwall et al. 1998). Human data for chemicals not included in the MEIC study will be collected from the literature according to the MEMO criteria. If it is possible to develop a preliminary human prediction model on the basis of the data obtained, *in vitro* data for Phase III chemicals will then be used to assess its predictive capacity.

Data Analyses

- For each assay, use data for Phase I and II chemicals to develop a human prediction model.
- If protocol changes are needed between Phase II and III, a portion of the data from Phase III will be used to refine the prediction model.
- The results for the 60 Phase III chemicals will be evaluated to assess the predictivity of the prediction model.

Figure 2. Phase 1a Results: Development of Positive Control Database

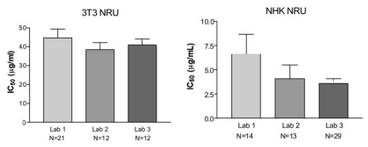
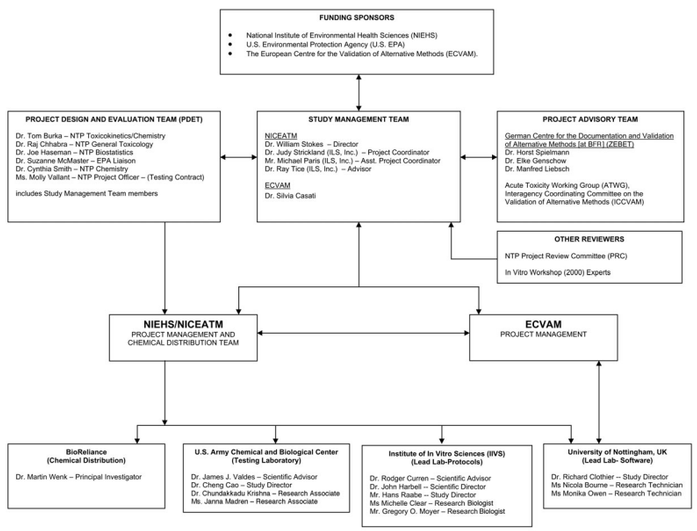


Figure 2. Phase Ia results for SLS.
IC_{50} – concentration of sodium laurel sulfate that inhibits cell viability by 50%. Error bars show standard deviation. Analysis of variance for random effects indicates that there is no significant difference among the laboratories at $p < 0.05$.

Each laboratory will use their $IC_{50} \pm$ two standard deviations as the test acceptance criteria for positive control performance during the Phase Ib testing of three blinded chemicals.

Management of Validation Study on *In Vitro* Methods for Acute Toxicity



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