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

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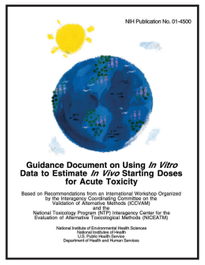
Abstract

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and NICEATM convened an international workshop in October 2000 to evaluate the validation status of *in vitro* methods for predicting acute systemic toxicity. Workshop participants recommended that two *in vitro* cytotoxicity methods should be further evaluated to determine their usefulness for predicting rodent and human acute toxicity. The NICEATM and ECVAM subsequently designed a multi-laboratory validation study to evaluate the relevance and reproducibility of two neutral red uptake assays using one rodent cell line and one human cell type. Seventy-two coded chemicals representing 12 chemicals from each of five hazard classification categories and 12 unclassified chemicals will be tested in each of three laboratories. The study will proceed in three phases. Twelve chemicals will be tested in Phases I and II, followed by 60 chemicals in Phase III. The Registry of Cytotoxicity prediction model will be used to evaluate the prediction of rodent oral LD_{50} tests. Prediction of human toxicity will be evaluated using a prediction model based on human poisoning data. This study will further characterize the usefulness and limitations of these basal cytotoxicity tests for predicting acute systemic toxicity and for reducing and replacing animal use. Supported by NIEHS contract N01-ES-85424.

Introduction



Acute oral toxicity testing is used to identify and characterize the potential hazards associated with a particular chemical. In October, 2000, the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity reviewed the validation status of *in vitro* methods and approaches directed toward reducing and refining the use of laboratory animals for acute toxicity testing (ICCVAM 2001a). One approach 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 starting doses 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 recommendation from the workshop to compare rodent and human *in vitro* data with one another, with rodent *in vivo* data, and with human *in vivo* data so as to further the development of simple 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 relevance 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 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* basal cytotoxicity database for acute toxicity resulting from chemicals that act by various mechanisms.
- To provide a database that can be used to support investigation of other methods to improve the accuracy and usefulness of *in vitro* assessments of acute systemic toxicity.

Planning

Selection of *In Vitro* Cytotoxicity Assays

Neutral red uptake (NRU) assay using mouse fibroblast (BALB/c) 3T3 cells.

- Development of rodent *in vitro* model recommended by workshop participants (ICCVAM 2001a); 3T3 NRU specifically recommended in the *Guidance Document* (ICCVAM 2001b).
- Highly reproducible in several validation studies.
- Database on responsiveness of cells is available.
- Amenable to 96-well plate culture.
- Commercially available.

NRU assay using normal human keratinoctyes (NHK).

- Development of human *in vitro* model recommended by workshop participants (ICCVAM 2001a); NHK NRU specifically suggested in the *Guidance Document* (ICCVAM 2001b).
- Reproducible in several validation studies.
- Database on responsiveness of cells is available.
- Amenable to 96-well plate culture and not easily dislodged.
- Early passage, nontransformed cells with normal human cellular targets for chemical toxicity.
- Commercially available.

Chemical Selection and Identification of Reference LD_{50} Values

Seventy-two chemicals were selected[1] 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 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 the chemicals regulated by the various U.S. regulatory agencies.
- Availability of human toxicity data and/or human exposure potential.

LD_{50} reference values for each chemical were selected after evaluation of the primary data sources identified by literature and database searches[2]. Reference values were selected based on a weight of evidence approach that included the following major factors:

- The use of healthy 8-12 week old adult rats from a commonly recognized strain/stock.
- Documentation of experimental parameters such as the method of administration, doses used, number of animals at each dose, number of deaths at each dose.
- Oral administration by gavage is favored over administration in food or capsules.
- Measure of variability for the LD_{50}.

[1]See poster entitled "Selection of Reference Chemicals for the Validation of *In Vitro* Cytotoxicity Assays for Predicting *In Vivo* Acute Systemic Toxicity" by Strickland et al. for more information on chemical selection.

[2]See poster entitled "Establishment of LD_{50} Reference Values for Chemicals used in Validation Studies of *In Vitro* Acute Toxicity Assays" by Paris et al. for more information on the selection of reference values.

Implementation

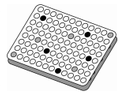
Chemical Distribution

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

Study Phases

Phase Ia: Laboratory Evaluation Phase

Development of positive control database



- Peform 10 replicate tests of the positive control chemical (i.e., sodium laurel sulfate [SLS]) with each cell type.
- Calculate mean IC_{50} and 95% confidence interval for each cell type for each lab.
- Establish acceptance criteria for positive control performance in future assays.

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 variation is achieved.

Phase II: Laboratory Qualification Phase

- Each lab tests nine blinded chemicals from all 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.

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}s and *in vitro* IC_{50}s for 347 chemicals in the Registry of Cytotoxicity (RC) (Halle 1998):

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

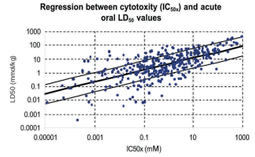


Figure 1. Registry of Cytotoxicity regression between cytotoxicity (IC_{50}) and rodent acute oral LD_{50} values for 347 chemicals. The heavy line shows the fit of the data to a linear regression model, log (LD_{50}) = 0.435 x log (IC_{50}) + 0.625; r=0.67. The thinner lines show the empirical F_5 = 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 regression of LD_{50} (mmoles/kg) on IC_{50} (mM) and compare results to the RC prediction model.
- For each cell type, use IC_{50} data to predict starting doses 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.
- Compare reduction in animal use for each cell type.
- Refine prediction model using most appropriate rodent LD_{50} values to determine whether the regression: (a) is significantly different from the RC prediction model, and (b) significantly improves the correlation between the LD_{50} and the IC_{50}.

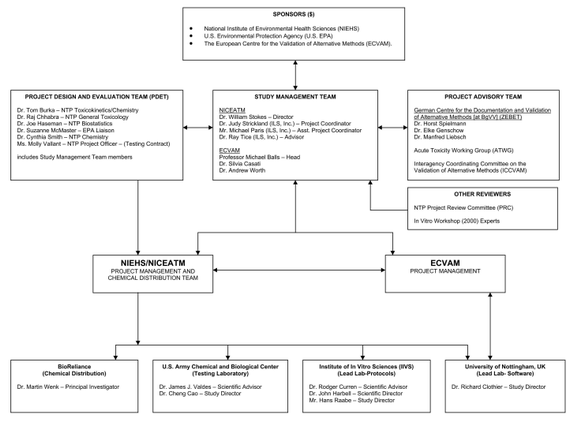
Human Prediction Model

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 and selected 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 or redevelop the prediction model.
- The results for the 60 Phase III chemicals will be evaluated by an independent statistician to assess the predictivity of the prediction model and the accuracy of its prediction.

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



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