Design and Phase 1A Results of a Validation Study to Evaluate In Vitro Cytotoxicity Assays for Predicting Rodent and Human Acute Systemic Toxicity

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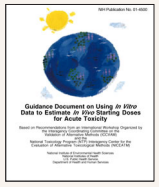
Introduction



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 from *in vitro* test methods using rodent or human cells to predict rodent *in vivo* LD_{50} data and human mortality. A goal of this study is 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 pain, suffering, and deaths) in animal use that would result from using *in vitro* cytotoxicity assays to estimate the starting dose for *in vivo* acute toxicity tests.
- 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.

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):

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

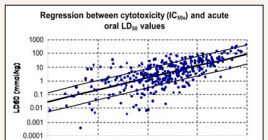


Figure 1. Registry of Cytotoxicity regression between cytotoxicity (IC_{50}x) and rodent acute oral LD_{50} values for 347 chemicals. The heavy line shows the fit of the data to a linear regression model, $\log (LD_{50}) = 0.435 \times \log (IC_{50x}) + 0.625$; r=0.67. The thinner lines show the empirical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

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 normal human keratinoctyes (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.

Implementation

Seventy-two coded chemicals covering a wide range of toxicity will be tested in neutral red uptake (NRU) assays using mouse fibroblast (BALB/c) 3T3 cells and human normal keratinocytes (NHK).

Study Phases

Phase Ia: Laboratory Evaluation Phase – Completed Nov 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} ± 2 standard deviations for each cell type for each lab as acceptance criteria for positive control performance in future assays.

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Phase Ib: Laboratory Evaluation Phase – Completed May 2003

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-/inter-laboratory 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 with each cell type using the final protocols.

The Neutral Red Uptake (NRU) Cytotoxicity Assay[1]

The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of toxic substances result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells.

Healthy proliferating mammalian cells, when properly maintained in culture, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of NR after chemical exposure, thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

Cell cultures are grown in 96-well microtiter plates and exposed to test chemical. After the predetermined incubation time, the test chemical is removed and NR solution is applied to the cells. The cells are incubated again, the excess NR solution is removed, and NR is eluted from the cells. The NRU is determined by using a microtiter plate reader/spectrophotometer to measure the optical density (at a wavelength of 540 ± 10 nm) of the eluted NR dye in the 96-well plate. A calculation of cell viability expressed as NRU is made for each concentration of a test chemical by using the mean NRU of six replicate values (minimum of four acceptable replicate wells) per test concentration. The cell viability value is compared with the mean NRU of all vehicle control (VC) values (provided VC values have met the VC acceptance criteria). Relative cell viability is then expressed as percent of untreated VC.

[1]see Borenfreund and Puerner (1984).

Assay Procedures

(1) BALB/c 3T3 cells or NHK cells are seeded into 96-well plates to form a semi-confluent monolayer (24 h for 3T3 cells, 48-72 h for NHK cells)

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(2) Remove culture medium

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(3) Cells are then exposed for 48 h to the test chemical in treatment medium over a range of 8 concentrations

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(4) Microscopic evaluation of morphological alterations

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(5) Remove treatment medium; wash once with D-PBS; add Neutral Red (NR) medium (50 μg/mL NR dye); incubate for 3 h.

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(6) Discard NR medium; wash once with D-PBS; add NR desorbing fixative

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(7) Shake plate for 20 minutes

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(8) Detect NR absorption at optical density (OD) 540nm

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(9) Calculation NR uptake as % viability at IC_{20}, IC_{50}, and IC_{80}

Figure 2. Major steps in performance of the NRU assays.

Technical Challenges

- NR dye crystals formed in both assays when used at 50 μg/mL
  - Troubleshooting efforts explored incubating medium overnight, centrifuging, filtering, reducing concentration of NR dye
  - ***Resolution***: Reduced NR dye concentration to 33 μg/mL
- Slower than expected cell growth for 3T3 cells
  - ***Resolution***: Passaged 3T3 cells 2-3 times after thawing before chemical application.
- NHK cells often died in a ring around the center of wells
  - Troubleshooting efforts included using various brands of 96-well plates and eliminating step 2 of the assay
  - ***Resolution***: Eliminated step 2 of the assay (change of medium prior to addition of chemical).
- Difficulty in obtaining optical density (OD) readings for vehicle control cells within the range recommended in the *Guidance Document* and proposed as test acceptance criteria in the protocols
  - Analyzed OD data from all laboratories for both cell types and provided new OD ranges as guidelines for each cell type
  - Analyzed cell responsiveness (i.e., dose response data) and the ability of each test to pass the other acceptance criteria
  - ***Resolution***: Eliminated OD range as a test acceptance criterion

Lessons Learned/Conclusions

- No matter how often an assay has been used, it must be proven in each lab before beginning a validation study. A preliminary study (e.g., 2 SLS tests/cell type/lab) should precede the full study.
- NRU is a fairly robust assay. Even with protocol changes, the IC_{50} for SLS was consistent within and among labs.

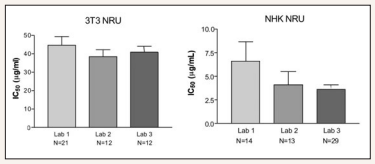
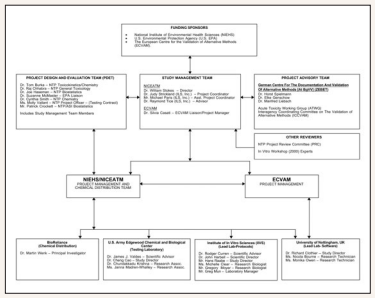


Figure 3. Phase Ia results for SLS.
IC_{50} – concentration of SLS that inhibits cell viability by 50%. Error bars show standard deviation. Analysis of variance for random effects indicates that there is no significant difference among the laboratories at p< 0.05.
[Each laboratory is using its IC_{50} ± two standard deviations as the test acceptance criteria for positive control performance during the Phase Ib testing of three coded chemicals.]



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