Data Collection and Analysis for an *In Vitro* Cytotoxicity Validation Study

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Introduction

Participants at an October 2000 workshop convened by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods (NICEATM) recommended validation of *in vitro* screening methods for predicting rodent toxicity as a means to reduce and refine the use of animals in acute toxicity testing (ICCVAM 2001). NICEATM and the European Centre for the Validation of Alternative Methods (ECVAM) subsequently designed a multi-laboratory validation study to evaluate two *in vitro* basal cytotoxicity tests for estimating rodent oral LD_{50} values and human lethal concentrations.

Twelve coded chemicals with oral toxicities across the five Globally Harmonized System (GHS; UN 2003) categories of acute oral toxicity, as well as unclassified toxicity, were tested in Phases I and II of the study to allow optimization of the cytotoxicity protocols and data collection and evaluation procedures before testing 60 coded chemicals in Phase III. This poster presents the evolution of the data analysis procedures over the study phases.

Methods

Figure 1 defines the tasks performed for each laboratory phase.

Microsoft® Excel™ templates were distributed to the participating laboratories for the collection of raw data, documentation of the materials and procedures used, simple graphical analysis, and transformation of the data to the proper format for GraphPad Prism® 3.0 software. Prism® was used to calculate the concentrations associated with 20%, 50%, and 80% viability (i.e., EC_x or IC_xvalues) with 95% confidence limits using a Hill function nonlinear regression analysis. The Hill function is a four-parameter logistic model relating the test chemical concentration to response in a sigmoidal shape (see Figure 2). Prism® templates were distributed to the laboratories to automate this analysis and provide a graphical display of the data and fitted model.

Study Phases

- **Phase Ia: Laboratory Evaluation Phase - Completed Nov 2002**
  Development of Positive Control Database for Each Laboratory

  For each cell type and laboratory: test positive control (PC) chemical (sodium laurel sulfate [SLS]) at least 10 times, calculate mean IC_{50} ± 2 standard deviations (SD), as acceptance criteria for PC performance in future assays. Revise protocols as needed to achieve reproducibility within and across laboratories.
- **Phase Ib: Laboratory Evaluation Phase - Completed May 2003**
  Limited Chemical Testing for Possible Protocol Refinement

  For each cell type and laboratory: test the same three coded chemicals of varying toxicities in three replicate tests. Refine the protocols and repeat, if necessary, for acceptable intra-/inter-laboratory reproducibility.
- **Phase II: Laboratory Qualification Phase - Completed Nov 2003**
  Additional Chemical Testing/Evaluation of Protocol Refinements

  For each cell type and laboratory: Test the same nine coded chemicals covering the full range of GHS acute oral toxicity categories in three replicate tests. Assure that Phase I revisions produce the desired results. Refine protocols and re-test if needed to achieve acceptable results. Finalize protocols for Phase III.
- **Phase III: Laboratory Testing Phase - Completed Jan 2005**
  Testing with Final Optimized Protocols

  For each cell type and laboratory: Test the same 60 coded chemicals covering the full range of GHS toxicity categories in three replicate tests.

Figure 1. Testing required by each laboratory phase.

Results

Figures 2 and 3 show the Prism® analyses and explain the features and changes made during the study. Figure 2 shows an example of the Hill function analysis to calculate the IC_{50} (concentration corresponding to 50% viability) using the Prism® template for Phases I and II. Figure 3 shows an example of the Prism® Hill function analysis for Phase III. Table 1 shows the development of the test acceptance criteria as the study proceeded.

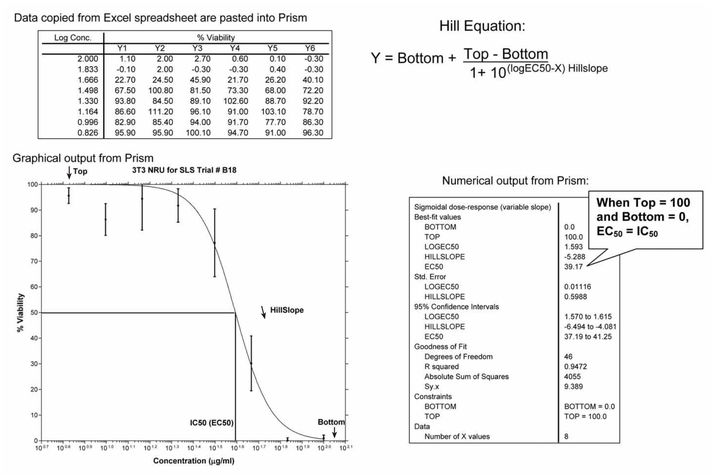

Figure 2. Data format, Hill function, results table, and graphical analysis performed by Prism® for Phases I and II.

Example shows IC_{50}, but IC_{20} and IC_{80} were also calculated. Hill function illustration shows Top = 100 and Bottom = 0. Y= response, X = logarithm of concentration, Bottom = minimum response, Top = maximum response, $logEC_{50}$ = logarithm of X at the response midway between Top and Bottom, and HillSlope = steepness of the curve.

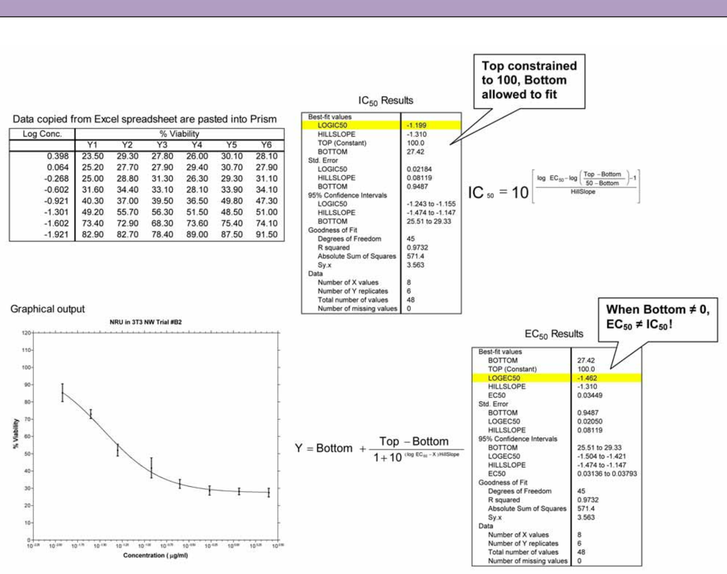

Figure 3. Data format, Hill function results table, and graphical analysis performed by Prism for Phase III.

Challenge: Endpoint of interest is IC_{50} – 50% viability (in relation to VC of 100%), but EC_{50} of Hill function = midpoint between top and bottom of curve (not necessarily 100 and 0, respectively).

Thus, $EC_{50} \neq IC_{50}$ when top = 100 but bottom ≠ 0. Rearranged Hill equation to IC_{50}=10^[(logEC_{50})-log[((Top-Bottom)/(Y-Bottom))-1]/Hill Slope] so that IC_{50} = 50% viability when Bottom is fit by the model while Top is constrained to 100. Figure compares EC_{50} to IC_{50}.

Table 1. Test Acceptance Criteria for Each Phase

Criterion	Study Phases: Phase Ia	Phase Ib	Phase II	Phase III
Mean absolute OD_{540} of VCs = 0.3 -1.1	I, R	NA	NA	NA
Corrected mean VC OD_{540} = 0.60 -1.70 for NHK; 0.30 - 0.80 for 3T3 cells	NA	I, R	NA	NA
Left and right mean VCs ≤ 15 % different from mean of all VCs	I	I	I	I
Preferably three points between 10 & 90%	I, R	NA	NA	NA
At least one point ≥ 10.0% and ≤ 50.0% viability **and** one point > 50.0% and ≤ 90.0% viability	P	I	I,R	NA
At least one point > 0% and ≤ 50.0% viability **and** one point > 50.0% and < 100% viability	NA	NA	P	I
SLS IC_{50} = ± 2 SD of the historical mean established by the Test Facility	NA	I	NA	NA
SLS IC_{50} = ± 2.5 SD of the historical mean established by the Test Facility	NA	NA	I	I
$R^2 \geq 0.90$. If $0.80 \leq R^2 < 0.90$, the SMT evaluated and determined acceptability. $R^2 < 0.80$ was unacceptable	NA	P	I	NA

I – applied initially; R – applied initially, but rescinded; P – instituted during testing or after testing ended; NHK – normal human epidermal keratinocytes; 3T3 – BALB/c 3T3 fibroblasts; NA – not applicable; SLS – sodium laurel sulfate (positive control); SD – standard deviation; SMT – Study Management Team; VC – vehicle control; OD_{540} – optical density at 540 nm; R^2 – coefficient of determination for Hill function

Development of Test Acceptance Criteria

Phase Ia - Rescinded the criteria for vehicle control (VC) optical density (OD) upon post-hoc analysis (cells exhibited adequate sensitivity with no signs of senescence). Accepted one point on either side of the IC_{50} as sufficient; rescinded the criteria for three points between 10 and 90% viability. Correlated visual fit to R^2 values from the Hill function analysis to develop a criterion: $R^2 > 0.9$ acceptable, $R^2 < 0.8$ unacceptable, and R^2 between 0.8 & 0.9 evaluated visually.

Phase Ib - Initially applied VC OD acceptance range based on Phase Ia data, but did not reject tests that were outside the range. Retrospectively applied the criterion for the R^2 values developed in Phase Ia.

Phase II - VC OD values used as target ranges rather than criteria. Midphase review altered other criteria to increase the number of acceptable tests without affecting the IC_{50} quality: (1) accepted points > 0 - < 100% viability with at least one point on either side of the IC_{50}, and (2) expanded the SLS acceptance range to mean IC_{50} from Phase I ± 2.5 SD. Since one chemical did not fit the Hill model well (i.e., $R^2 < 0.8$) because it did not produce 100% toxicity regardless of concentration, the Bottom parameter was unconstrained to get a better fit. But when Bottom ≠ 0, the calculated $EC_{50} \neq$ 50% viability. Reanalyzed to calculate concentration at 50% viability using a rearranged Hill equation: X=10^[(logEC_{50})-log[((Top-Bottom)/(Y-Bottom))-1]/Hill Slope]. Y= response (i.e., 50%), X = logarithm of concentration at 50% response, Bottom = minimum response, Top = maximum response, $logEC_{50}$ = logarithm of X at the response midway between Top and Bottom, and HillSlope describes the steepness of the curve.

Phase III - Criteria set to correspond with lessons learned in Phases I and II. No acceptance criterion for R^2 since it was inappropriate for a test acceptance criterion. Chemicals that disrupt the cell cycle, may not fit the Hill function well even when the tests are functioning perfectly. Retained R^2 criterion for SLS since its good fit to the Hill function was well documented. Final criteria for Phase III:

- Left and right mean VCs ≤ 15% different from mean of all VCs
- At least one point > 0% and ≤ 50.0% viability **and** one point > 50.0% and < 100% viability
- Positive control, SLS, IC_{50} = ± 2.5 SD of the historical mean and $R^2 \geq 0.85$

Lessons Learned

- Those who conduct the experiments should meet to discuss and confirm practical data collection/analysis techniques and methods.
- Experience with data under current laboratory conditions must be obtained before making final decisions on acceptance criteria and data analysis techniques
- Validation studies should include several small phases for standardization and optimization of the protocols. This offers the flexibility to incorporate lessons learned with study progress before using a large proportion of study resources.
- Electronic submission of data on standardized forms is rapid and allows efficient data collection and analysis, but it must be reviewed for accuracy just like paper techniques.

Acknowledgments

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