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

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Introduction

Participants at a 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) in October 2000 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 2001a). 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 Harmonised System (GHS; UN 2003) categories of acute oral toxicity, as well as unclassified toxicity, were tested in the first two phases to allow optimization of the cytotoxicity protocols and data collection and evaluation procedures before testing the majority of the chemicals. The optimized standardized protocols and data collection procedures are now being used to evaluate the remaining 60 coded chemicals in Phase III of the study. This poster presents the evolution of the data collection and analysis procedures over the study phases.

Methods

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$_X$ values) 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 1). Prism® templates were distributed to the labs to automate this analysis and provide a graphical display of the data and fitted model.

Figure 1
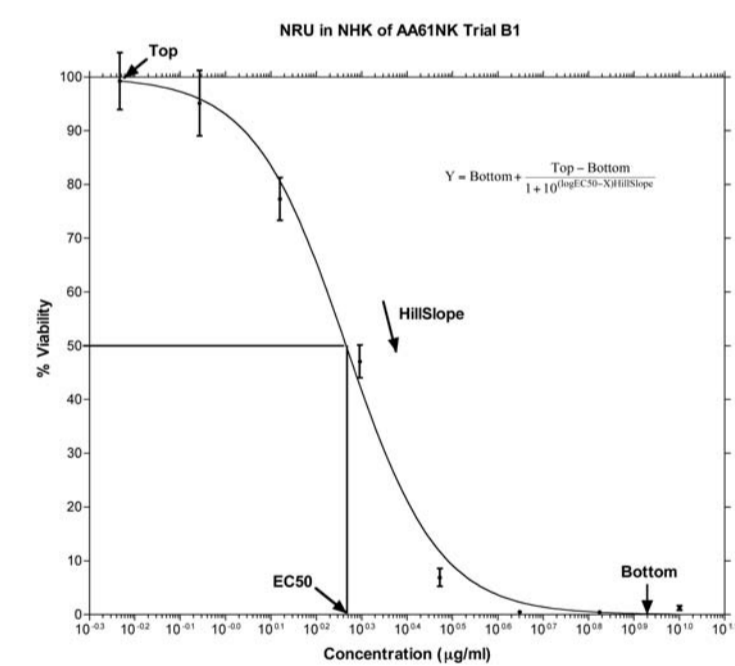
Hill function illustration shows Top = 100 and Bottom = 0. Y= response, X is the logarithm of concentration, Bottom is the minimum response, Top is the maximum response, logEC$_{50}$ is logarithm of X at the response midway between Top and Bottom, and HillSlope describes the steepness of the curve.

Results

Figure 2 defines the tasks performed for each laboratory phase. Table 1 shows the development of the test acceptance criteria as the study proceeded. Figures 3-5 show the Prism and Excel templates used and explain the various features and changes that occurred throughout the study. Figure 3 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 4 shows the Excel™ spreadsheet template for data collection and analysis. Balloons highlight the features. Figure 5 shows an example of the Prism® Hill function analysis for Phase III.

Figure 2. Study Phases

Phase Ia: Laboratory Evaluation – Completed Nov 2002
Development of positive control database for each laboratory (Strickland et al. 2003)
- Perform at least 10 replicate NRU tests of the positive control chemical (sodium lauryl sulfate [SLS]) with each cell type.
- Calculate mean IC50 ± 2 SD for each cell type for each lab.
- Establish acceptance criteria for positive control performance in future assays.

Phase Ib: Laboratory Evaluation – 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/interlaboratory reproducibility is achieved.

Phase II: Laboratory Qualification – Completed Nov 2003
- 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

Phase III: Laboratory Testing Phase
- Each lab tests 60 coded chemicals three times using the final protocol for each assay.

Testing required by each laboratory phase. Test acceptance criteria for each phase are shown in Table 1.

Development of Test Acceptance Criteria

Phase I - The post hoc analysis by the Study Management Team (SMT) rescinded the criteria for vehicle control (VC) optical density (OD) since the cells exhibited adequate sensitivity with no signs of senescence. The SMT considered one point on either side of the IC$_{50}$ sufficient to describe the IC$_{50}$ and thus rescinded the criteria for three points between 10 and 90% viability. Although there was no criterion for curve fit, graphical evaluation resulted in the rejection of 14 tests for inadequate fit. Fit was correlated to the r^2 values from the Hill function analysis to develop a criterion. The SMT considered $r^2 > 0.9$ acceptable, $r^2 < 0.8$ unacceptable, and r^2 between 0.8 & 0.9 were evaluated visually for fit.
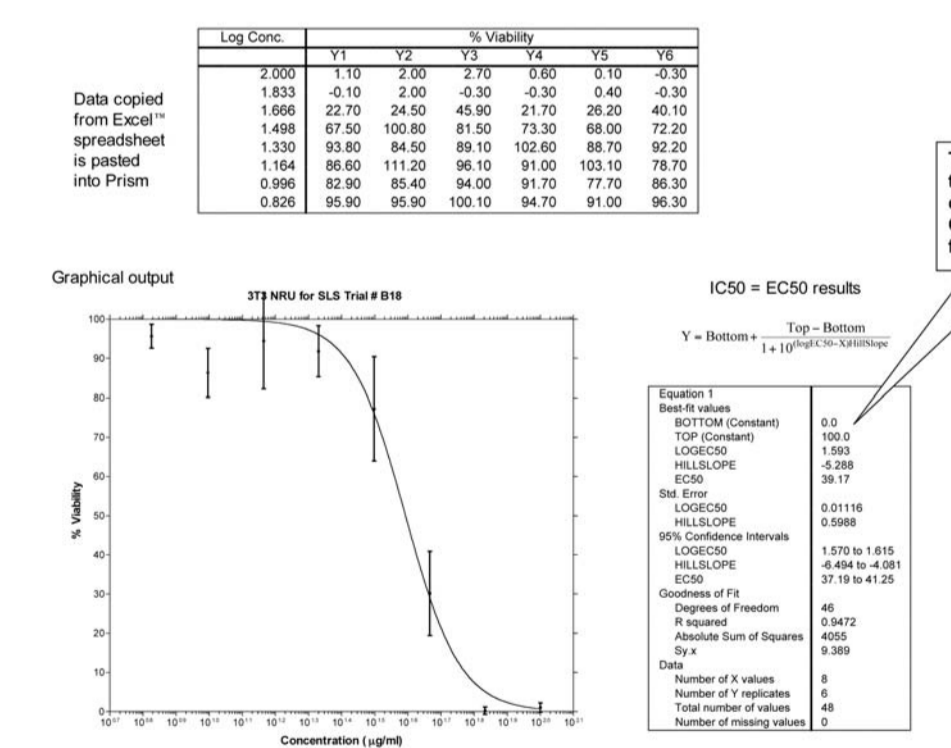
Phase Ib - Initially applied a VC OD acceptance range based on Phase Ia data, but the post-hoc analysis by the SMT did not reject tests that were outside the range. The SMT retrospectively applied the criterion for the r^2 values developed in Phase Ia.

Phase II - Rather than using criteria for VC OD, target ranges were used. A midphase SMT review determined that two criteria changes would increase the number of acceptable tests without affecting the quality of the IC$_{50}$s: (1) accept points from > 0 - < 100% viability with at least one point on either side of the IC$_{50}$, and (2) expand the SLS acceptance range to 2.5 standard deviations (SD) on either side of the mean IC$_{50}$ from Phase I (because a larger database tends to narrow the acceptable range). Since one chemical did not fit the Hill model well (i.e., $r^2 < 0.8$) because it did not produce 100% toxicity no matter how high the concentration, the Bottom parameter of the Hill model was unconstrained to get a better fit. But when the Bottom ≠ 0, then EC$_{50}$ is not necessarily equal to 50% viability, our measure of interest. Such tests were reanalyzed by calculating 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 is the logarithm of concentration at 50% response, Bottom is the minimum response, Top is the maximum response, logEC$_{50}$ is logarithm of X at the response midway between Top and Bottom, and HillSlope describes the steepness of the curve.

Phase III - Criteria were set to correspond with lessons learned in Phases I and II. There is no acceptance criterion for r^2 for test chemicals since experience indicated that it was a good data analysis criterion but inappropriate for a test acceptance criterion. Not all chemicals are expected to fit the Hill function well even when the tests are functioning perfectly (by other measures). Retained r^2 criterion for SLS since its good fit to the Hill function was well documented. The final criteria for Phase III are:
- 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 $r^2 ≥ 0.85$.

Table 1

Test Acceptance Criteria

Criterion	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	NA	NA	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	NA	NA	NA
SLS IC$_{50}$ = ± 2.5 SD of the historical mean established by the Test Facility	NA	NA	I	I
$r^2 ≥ 0.90$. If 0.80 ≤ r^2 < 0.90, the Study Management Team evaluated and determined acceptability. r^2 < 0.80 was unacceptable.	NA	P	NA	NA

I – applied initially; R – applied initially, but rescinded; P – instituted during testing or after testing ended; NA – not applicable; SD – standard deviation
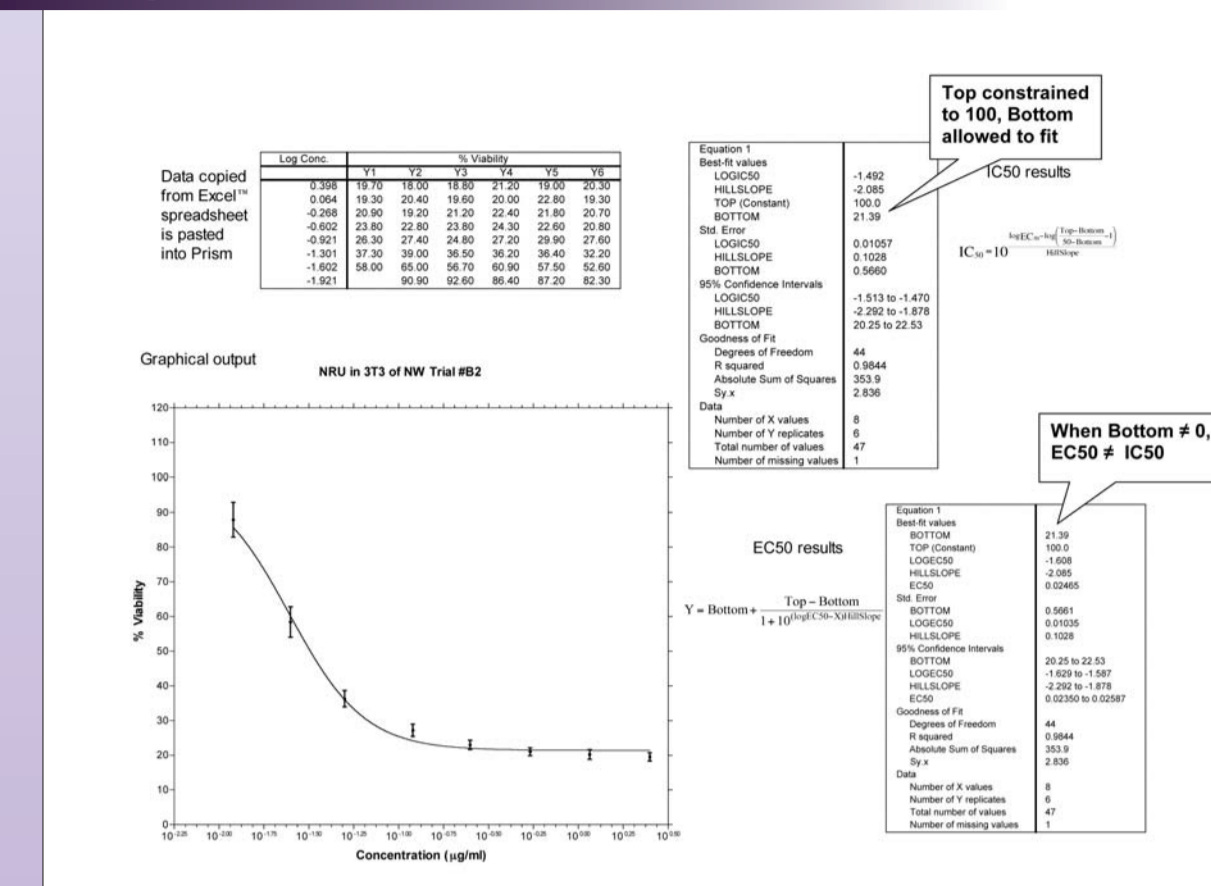
Figure 3

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. Bottom was constrained to 0 and Top was constrained to 100. HillSlope and IC$_X$ were fit by the model.

Figure 4.

Excel™ spreadsheet for data collection and analysis for Phase III. Balloons highlight changes from Phase I. The major changes in Phase II were (a) the addition of a test for outliers within the six replicates at each concentration, and (b) the addition of test chemical to the blank cells on either end of each column of test chemical, which necessitated a change in the calculation of absorbance. The major change for Phase III was the addition of linear interpolation, the standard method for IC$_{50}$ calculation, for comparison with the Hill function results.

Figure 5.

Data format, Hill function results table, and graphical analysis performed by Prism for Phase III. Rearranged the Hill equation to IC$_{50}$=10^{([logEC50)-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. EC$_X$ values are also calculated as a comparison to IC$_X$ values.

Lessons Learned

- Experience with data under current laboratory conditions must be obtained before making final decisions on acceptance criteria and data analysis techniques

- A large study such as this should include several small phases for standardization and optimization of the protocols. This offers the flexibility to incorporate lessons learned with study progress before investing a large proportion of study resources.

- Electronic submission of data is rapid and allows relatively rapid collation and analysis, but it must be reviewed and analyzed for accuracy just like paper techniques.

References

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Phase III Neutral Red Uptake Protocols for 3T3 and NHK cells are available at:

http://iccvam.niehs.nih.gov/methods/invitro.htm