Protocol Optimization During a Validation Study to Evaluate *In Vitro* Cytotoxicity Assays for Estimating Rodent Acute Systemic Toxicity.

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Introduction

Acute oral toxicity testing is used to characterize the risk of hazard 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 provides details and examples on how to execute such an approach.

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the European Centre for the Validation of Alternative Methods (ECVAM) designed and initiated a three-phase multi-laboratory validation study using the *Guidance Document* approach. The study also evaluates another workshop recommendation to compare the ability of two *in vitro* basal cytotoxicity test methods (rodent or human cells) to estimate rodent LD_{50} data and human mortality. This poster highlights the value of a phased approach that allows data evaluation and protocol optimization prior to each subsequent phase.

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

The NRU cytotoxicity assay procedure is a cell survival/viability assay based on the ability of viable cells to incorporate and bind neutral red (NR), a weak cationic supravital dye that readily penetrates cell membranes by non-ionic diffusion and predominately 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 produced by toxic substances cause decreased uptake and binding of NR, making it possible to distinguish between viable, damaged, or dead cells via spectrophotometric measurements.

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.

[1]modification of Borenfreund and Puerner (1984); protocol of Riddell et al. (1986).

Study Implementation

Seventy-two coded chemicals (12 per Globally Harmonized System [GHS] class) covering a wide range of toxicity were tested in two NRU assays using BALB/c 3T3 mouse fibroblast cells and human normal human keratinocyte (NHK) cells.

- **Phase Ia: Laboratory Evaluation Phase - Completed Nov 2002**
  Development of Positive Control Database for Each Laboratory
  For each cell type and each laboratory: performed replicate tests of the positive control (PC) chemical (sodium laurel sulfate [SLS]), calculated mean IC_{50} ± 2 standard deviations, and used IC_{50} values as acceptance criteria for PC performance in future assays where the PC is run with each test run. Revised 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
  Each laboratory tested the same three coded chemicals of varying toxicities and generated three replicate acceptable tests/chemical for each cell type. Refined protocols and repeated, if necessary, until acceptable intra-/inter-laboratory reproducibility was achieved.
- **Phase II: Laboratory Qualification Phase -Completed Nov 2003**
  Additional Chemical Testing/Evaluation of Protocol Refinements
  Each laboratory tested the same nine coded chemicals covering the full range of GHS toxicity categories (three replicate acceptable tests/chemical for each cell type). Assured that corrective actions taken in Phase I produced the desired results. Further refined protocols and re-tested if needed to achieve acceptable results. Finalized protocols for Phase III.
- **Phase III: Laboratory Testing Phase - Completed Jan 2005**
  Testing 60 Coded Chemicals using Optimized Protocols
  Each laboratory tested the same 60 coded chemicals covering the full range of GHS toxicity categories (three replicate acceptable tests/chemical for each cell type) using the final protocols.

Major Steps in Performance of the NRU Assays

(1) 3T3 cells or NHK cells are seeded into 96-well plates to form a sub-confluent monolayer (24 h - 3T3 cells, 48-72 h - NHK cells)
(2) Culture medium is removed (for 3T3 cells only)
(3) Cells are exposed for 48 h to the test chemical in treatment medium over a range of eight (8) concentrations
(4) Cells are evaluated microscopically for morphological alterations
(5) Treatment medium is removed; cells are washed once with Dulbecco's Phosphate Buffered Saline (D-PBS); NR dye medium is added (3T3 cells: 25 µg/mL dye; NHK cells: 33 µg/mL dye); plates are incubated for three (3) h
(6) NR medium is discarded; cells are washed once with D-PBS; NR desorbing fixative is added to the plates
(7) Plates are shaken for 20 min
(8) NR absorption is measured at optical density (OD) 540 ± 10 nm
(9) NR uptake is calculated as % viability with respect to control cells to define IC_{20}, IC_{50}, and IC_{80} chemical concentrations (µg/mL)

Technical Challenges, Resolutions, and Protocol Revisions

Technical Challenge	Resolutions/Revisions
PHASE Ia: Laboratory Evaluation Phase	
50 µg/mL NR dye caused crystal formation in both assays (increased blank optical density [OD]).	NR dye concentration reduced to 33 µg/mL.
3T3 cell growth slower than expected.	Passaged 3T3 cells 2-3 times after thawing before chemical application. Emphasized cell confluency rather than incubation time as a measure of cell growth.
NHK cells occasionally died in a ring pattern near the periphery of the wells.	Eliminated step 2 of the NHK assay (change of medium prior to addition of chemical).
Precipitates occasionally observed in the 3T3 medium but not in the NHK medium at the same concentrations during test article solubility testing. May be attributed to the serum in the 3T3 medium rather than insoluble chemical.	Dissolved chemical in 3T3 medium w/o Newborn Calf Serum (NCS). For chemical exposure, added the dissolved 2X chemical to 10% NCS medium to reach final 5% NCS and 1X chemical concentrations.
NHK cell adherence in 80-cm^2 culture flasks unsatisfactory for one lab.	Coated culture flasks with fibronectin/collagen to promote adherence.
PHASE Ib: Laboratory Evaluation Phase	
33 µg/mL NR dye caused crystal formation in 3T3 assay. 25 and 33 µg/mL NR concentrations tested at 2 - 3 h exposure durations. SLS toxicity tested to determine whether sensitivity differed under these conditions (see Figure 1). Observed crystals only at 33 µg/mL NR for 3 h.	Used 25 µg/mL NR for 3 h incubation in 3T3 NRU assays. Filtered NR medium, maintained at 37°C, and applied to cells within 15 min after removing from 37°C. Observed plates in both assays for crystal formation before desorbing NR.
Use of solubility procedures failed to dissolve arsenic trioxide.	Increased duration of heating the solution.
One laboratory used different brand of growth medium that failed to support NHK cell proliferation under the conditions of the assay system, resulting in several assay failures.	Delayed testing until a new lot of medium from the original supplier became available.
High error rates and test failures were observed at one laboratory. Compiled error rates for all laboratories to illustrate that low error rates were achievable.	Weeklong training session for all technical personnel enhanced harmonization of techniques between the laboratories. Standardized: use of multi-channel repeater pipetter; use of 8-channel reservoirs; rinse time during cell subculturing; use of plate protection from light during NR extraction; timeframe for collecting the OD measurements. Changed seeding density for 3T3 assay. Used 25-cm^2 NHK culture flasks without fibronectin/collagen for start-up of cryopreserved cells.
PHASE II: Laboratory Qualification Phase	
Vapors from the highest concentration wells of 2-propanol contaminated the vehicle control (VC) and the lower concentration wells. (see Figure 2).	Used plate permeable sealers to test volatile chemicals.
Unusual dose-response curves for aminopterin and colchicine. Toxicity plateaued > 0% viability regardless of test chemical concentration (see Figure 3). The Hill function fit was poor and r^2 values often failed the acceptance criterion.	To obtain a better model fit, the Bottom parameter was unconstrained (previously used Bottom = 0). The Hill function calculation was rearranged to calculate the concentration corresponding to the IC_{50}.
Lithium carbonate was insoluble in the 3T3 medium.	Added an additional solubility procedure for stirring in an incubator for 3 h and a requirement for performing 3 definitive tests for insoluble chemicals before testing ceases for the particular chemical. Revision for Phase III.
Some lots of NHK medium/supplements didn't support adequate growth for the NRU assay.	Acceptable medium and supplement lots were screened and recommended for use. Phase III included prequalification of NHK media.
Adjust for potential chemical interference with dye.	Added test chemical to the blank wells used to subtract background from test chemical concentration ODs.
PHASE III: Laboratory Testing Phase	
Some chemicals produced biphasic curves.	The IC_{80} and IC_{50} are calculated from the initial toxicity part of the curve.
Acceptance criteria for Hill equation curve fit.	Rescinded requirement for test articles to fit Hill equation with $r^2 \geq 0.90$. Hill equation used to characterize test article response curve shape, rather than establish acceptance criterion. The PC acceptance criterion was modified to $r^2 \geq 0.85$.

Test Acceptance Criteria

CRITERION	Phase Ia	Phase Ib	Phase II	Phase III
IC_{50} range for the positive control (SLS)[a]	NA	IC_{50} ± 2 SDs	IC_{50} ± 2.5 SDs	IC_{50} ± 2.5 SDs
One cytotoxicity point on each side of the IC_{50} value	between 10 and 90% viability[b]	between 10 and 90% viability	≥10.0% and ≤ 50.0% and ≥50.1% and ≤ 90.0%	> 0% and ≤ 50.0% and >50.0% and <100%
Left and right mean of VCs do not differ by more than 15% from all VCs mean	Yes	Yes	Yes	Yes
Hill Function (Coefficient of Determination)	$R^2 > 0.9$ or $0.8 < R^2 < 0.9$[c]	$R^2 > 0.9$ or $0.8 < R^2 < 0.9$[c]	$R^2 \geq 0.90$ or $0.8 \leq R^2 < 0.90$[c]	$R^2 \geq 0.85$ for SLS only (not a criterion for test chemicals)
$OD_{540±10\ nm}$ range for VCs	≥ 0.3 and ≤ 1.1[d]	≥ 0.3 and ≤ 0.8 (3T3)[e] ≥ 0.6 and ≤ 1.7 (NHK)[e]	NA	NA

[a] IC_{50} is determined from data collected in the previous phase for each individual laboratory
[b] Added after commencement of Phase Ia
[c] R^2 values in this range were evaluated on a case by case basis for acceptability by the SMT
[d] rescinded after commencement of Phase Ia
[e] rescinded after commencement of Phase Ib
NA not applicable

References

Borenfreund E, Puerner J. 1984. J. Tissue Culture Meth. 9: 7-9.

Ekwall B, *et. al.* 1998. ATLA 26:571-616

Halle W. 1998. Life Sciences/ Lebens-wissenschaften, Volume 1, 94 pp., Jülich: Forschungszentrum Jülich.

ICCVAM 2001a. NIH Publication 01-4499. http://iccvam.niehs.nih.gov/

ICCVAM 2001b. NIH Publication 01-4500. http://iccvam.niehs.nih.gov/

Riddell R J, *et. al.* 1986. Food Chem. Tox. 24: 469-471

Spielmann H, *et. al.* 1999. ATLA 27: 957-966.

Lessons Learned/Conclusions

- Regardless of the expertise of the testing facilities with an assay, each participating laboratory must demonstrate that a test method protocol performs as expected prior to beginning a validation study. A team meeting of those conducting the assays is vital to ensure conformity and proficiency with the assay.
- It is important during test method protocol optimization to include substances representative of the range of expected toxicity, solubility, volatility, and other relevant physical/chemical properties to maximize the likelihood that the protocol will yield reproducible results for a broad range of test substances.
- Differences in test results attributable to differences in assay execution were generally biologically insignificant. This is illustrated by the consistency of IC_{50} values for SLS within and among labs through several protocol changes (see Figure 4). The 3T3 values were realtively consistent throughout the study and the NHK values became more consistent when cell culture methods were revised for one laboratory. This suggests that the NRU assay is a robust assay for chemicals that have toxicity profiles that conform to the Hill equation.

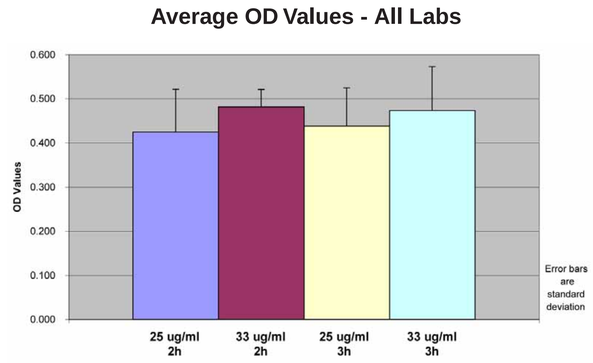

Figure 1. Optical Density of SLS 3T3 NRU Assay with NR Concentration and Duration.

96-WELL PLATE MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
C	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
D	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
E	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
F	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
G	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

RAW ABSORBANCE DATA (OD_{550})

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.046	0.043	0.047	0.045	0.044	0.044	0.044	0.046	0.045	0.045	0.046	0.046
B	0.043	0.045	0.048	0.049	0.047	0.045	0.114	0.193	0.353	0.495	0.559	0.045
C	0.044	0.042	0.048	0.048	0.043	0.049	0.077	0.199	0.292	0.435	0.572	0.044
D	0.044	0.044	0.048	0.048	0.048	0.048	0.073	0.178	0.304	0.440	0.542	0.047
E	0.044	0.045	0.051	0.046	0.044	0.045	0.063	0.180	0.300	0.427	0.504	0.048
F	0.043	0.047	0.050	0.056	0.052	0.049	0.092	0.199	0.317	0.451	0.509	0.045
G	0.042	0.048	0.054	0.049	0.047	0.046	0.147	0.218	0.370	0.468	0.542	0.046
H	0.043	0.043	0.045	0.043	0.047	0.044	0.047	0.045	0.042	0.045	0.044	0.044
	Conc. (ug/mL)	0	200000	111111	61728	34294	19052	10584	5880	3267	0	

Test 1 - WITHOUT PLATE SEALER

RAW ABSORBANCE DATA (OD_{550})

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.055	0.052	0.052	0.052	0.050	0.051	0.053	0.051	0.055	0.052	0.049	0.046
B	0.053	0.711	0.058	0.057	0.054	0.055	0.141	0.320	0.542	0.710	0.768	0.049
C	0.051	0.703	0.059	0.060	0.051	0.053	0.131	0.311	0.598	0.723	0.797	0.050
D	0.053	0.717	0.059	0.058	0.054	0.054	0.135	0.319	0.620	0.723	0.788	0.051
E	0.053	0.699	0.059	0.059	0.056	0.054	0.157	0.320	0.633	0.714	0.768	0.049
F	0.051	0.738	0.061	0.058	0.055	0.053	0.131	0.315	0.622	0.727	0.785	0.050
G	0.050	0.705	0.058	0.058	0.056	0.055	0.150	0.325	0.633	0.689	0.771	0.052
H	0.051	0.050	0.052	0.053	0.051	0.050	0.052	0.050	0.048	0.052	0.053	0.050
	Conc. (ug/mL)	0	200000	111111	61728	34294	19052	10584	5880	3267	0	

Test 2 - WITH PLATE SEALER

Figure 2. NHK NRU Test Data for a Volatile Chemical, Acetonitrile, With/Without Plate Sealer

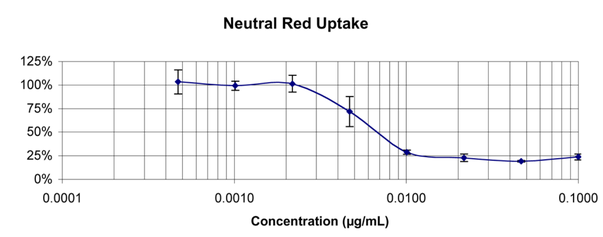

Figure 3. Representative Dose-Response for Aminopterin in a NHK Definitive Assay. %Viability Plateaued at the Highest Doses.

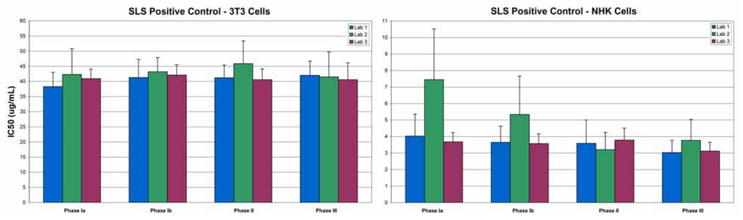

Figure 4. Stability of SLS Over Time.
Bars show mean IC_{50} (µg/mL) + 1 SD for 3T3 and NHK NRU assays. Testing Period: August 2002 to January 2005. In Phases Ia and Ib, Lab 2 seeded cryopreserved NHKs into 80-cm^2 flasks with fibronectin/collagen coating. In Phases II and III, Lab 2 used 25-cm^2 flasks without fibronectin/collagen coating.

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Current Validation Study Protocols and Information Available at:
http://iccvam.niehs.nih.gov/methods/invitro.htm