# Phase I and II Results of a Validation Study to Evaluate In Vitro Cytotoxicity Assays for Estimating Rodent and Human Acute Systemic Toxicity

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Introduction
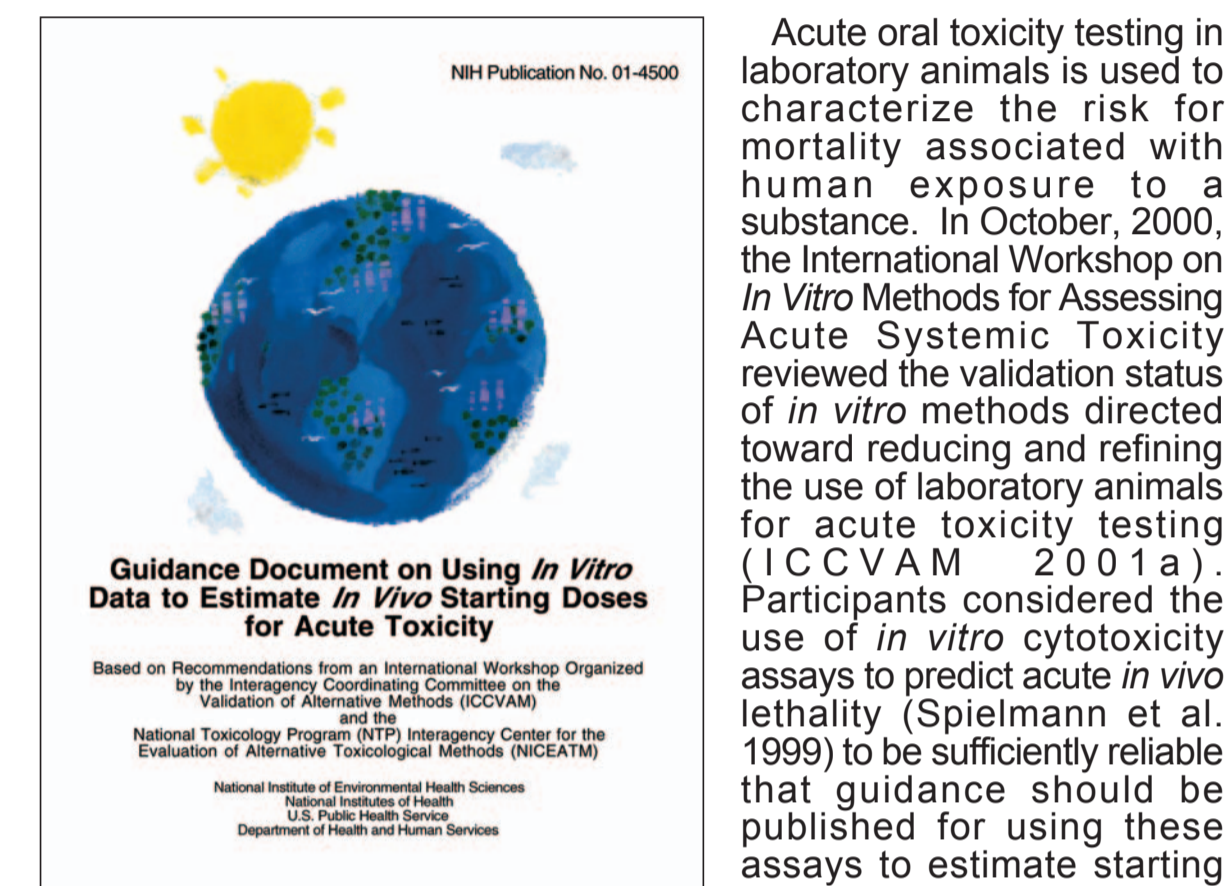
Acute oral toxicity testing in laboratory animals is used to characterize the risk for mortality associated with human exposure to a substance. In October, 2000, the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity reviewed the validation status of *in vitro* methods directed toward reducing and refining the use of laboratory animals for acute toxicity testing (ICCVAM 2001a). Participants considered the use of *in vitro* cytotoxicity assays to predict acute *in vivo* lethality (Spielmann et al. 1999) to be sufficiently reliable that guidance should be published for using these assays to estimate starting doses for acute oral lethality tests (ICCVAM 2001b). The recommended guidance, illustrated on the left, provides details and examples on how to execute this approach.

The validation study organized by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the European Centre for Validation of Alternative Methods (ECVAM) implements the *Guidance Document* approach as well as another workshop recommendation to evaluate the ability of *in vitro* data from rodent and human cells to predict rodent *in vivo* LD50 data and human *in vivo* mortality data. This study will determine the extent that *in vitro* cytotoxicity tests can reduce the number of animals necessary for an acute oral toxicity test, and will assist in identifying additional *in vitro* tests necessary to accurately predict *in vivo* LD50 values. This poster reports the results of Phases Ib and II of the validation study.

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 *in vitro* cytotoxicity assays for estimating rodent oral LD50 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 deaths) 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* database that can be used to support investigation of other methods necessary to improve the accuracy of *in vitro* assessments of acute systemic toxicity.

Study Design

The bases for the selection of neutral red uptake (NRU) assays using mouse fibroblast (BALB/c) 3T3 cells and normal human keratinocyte (NHK) (Strickland et al. 2003) and for the selection of the 72 test chemicals (Strickland et al. 2002) were reported previously.

Selection of Phases Ib and II Chemicals

The following criteria were used to select 12 of the 72 test chemicals for testing in Phases Ib and II:
- Two chemicals to represent each of the five toxicity categories in the GHS classification of oral toxicity and two chemicals with unclassified toxicity (i.e., nontoxic) (UN 2003);
- Log LD50 (mmol/kg) values within 0.699 of the RC prediction (i.e., NOT an "RC outlier") as recommended by the *Guidance Document* (ICCVAM 2001b) for evaluating a cytotoxicity test for use with the RC prediction model;
- Preferably tested in the MEIC study, since human toxicity data are available.

If more than one chemical in a GHS category met the above criteria, chemicals were chosen so as to be closest to the RC prediction and/or to represent the range of toxicity in each GHS category

Prediction Models

Rodent. The approach for predicting starting doses for acute oral lethality tests, described by the *Guidance Document* (ICCVAM 2001b) is based on the linear regression analysis of rodent *in vivo* oral LD50 and *in vitro* IC50 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):

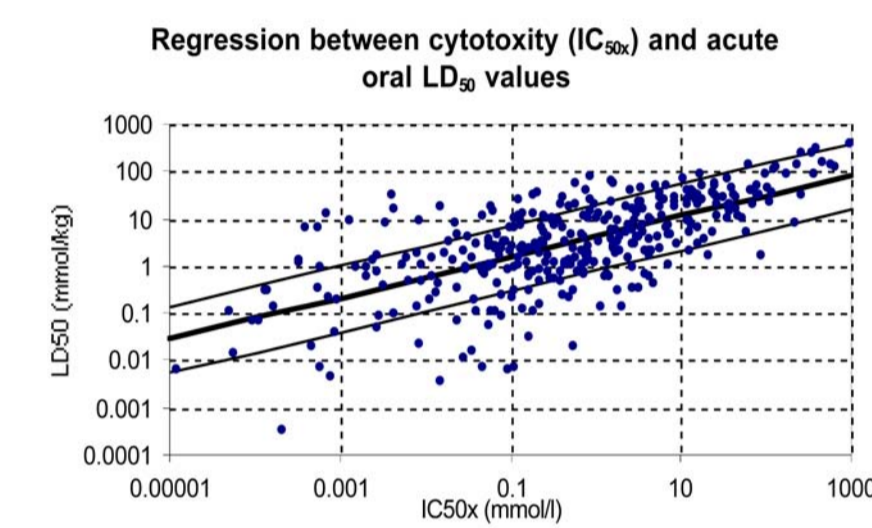$$\log LD_{50} \, (mmol/kg) = 0.435 \log IC_{50} \, (mM) + 0.625$$

Figure 1. Registry of Cytotoxicity regression between cytotoxicity (IC50x) and rodent acute oral LD50 values for 347 chemicals.

The heavy line shows the fit of the data to a linear regression model, log (LD50) = 0.435 x log (IC50x) + 0.625; r=0.67. IC50x values are the geometric means of multiple endpoints and cell types. The thinner lines show the empirical FG = log 5 acceptance interval (0.699) for the prediction model that is based on the anticipated precision of LD50 values from rodent studies (Halle 1998).

Human. The feasibility of developing a human prediction model will be evaluated using the *in vitro* results for the 12 chemicals tested in Phases Ib and II with the corresponding human sublethal and lethal blood concentrations from the Multicentre Evaluation of In Vitro Cytotoxicity (MEIC) study (MEMO database; Ekwall et al. 1998) or from more recently collected human data (for nonMEIC chemicals). The predictive ability of the preliminary human prediction model will be assessed with the *in vitro* data from Phase III chemicals.

Table 1

Table 1. Chemicals Tested in Phases Ib and II

Chemical	CASRN	RC Ref. No.	MEIC Ref. No.	Rodent Oral LD50[1] (mg/kg)	Observed-Predicted log LD50[2]
LD50 < 5 mg/kg					
Aminopterin	54-62-6	3	NA	3	-0.652
Sodium selenate	13410-01-0	NA	NA		NA
LD50 > 5 - < 50 mg/kg					
Colchicine	64-86-8	6	60	6	-0.593
Arsenic trioxide	1327-53-3	153	26	20	-0.591
LD50 > 50 - < 300 mg/kg					
Cadmium chloride	10108-64-2	81	NA	88	-0.336
Sodium fluoride	7681-49-4	106	14	180	-0.109
LD50 > 300 - < 2000 mg/kg					
DL-Propranolol HCl	350-60-90	54	23	470	-0.023
Lithium carbonate	544-13-2	327	20	1187	-0.256
LD50 > 2000 - < 5000 mg/kg					
Potassium chloride	7447-40-7	346	50	2602	0.085
Chloramphenicol	56-75-7	91	45	3393	0.441
LD50 > 5000 mg/kg					
2-Propanol	67-63-0	128	10	5843	0.396
Ethylene glycol	107-21-1	360	7	8567	0.321

Notes: [1]Used in the RC (Halle 1998). [2]Available only for chemicals included in the RC. CASRN = Chemical Abstracts Services Registry Number; NA – not applicable.

Study Phases

Phase Ia: Laboratory Evaluation – Completed Nov 2002
Development of positive control database for each laboratory (N=3) (Strickland et al. 2003)

- Perform at least 10 replicate NRU tests of the positive control chemical (sodium laurel sulfate [SLS]) with each cell type.
- Calculate mean IC50x + 2 standard deviations for each cell type for each lab.
- Establish acceptance criteria for positive control performance in future assays.

Technical challenges:
- Formation of NR dye crystals in both assays at 50 µg/mL
  - Troubleshooting efforts explored incubating NR medium overnight, centrifuging, filtering, reducing NR dye concentration, reducing NR incubation duration
  - *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 evaluating various brands of 96-well plates and eliminating change of medium prior to chemical treatment
  - *Resolution:* Eliminated change of medium prior to chemical treatment

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.

Technical challenges:
- Formation of NR dye crystals in the 3T3 assay at 33 µg/mL
  - Troubleshooting efforts explored reducing NR dye concentration and incubation duration
  - *Resolution:* Reduced NR dye concentration to 25 µg/mL

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.

Technical challenges:
- Due to its volatility, 2-propanol contaminated the nearby vehicle controls
  - Troubleshooting efforts explored reducing contamination by using film plate sealers and mineral oil for reducing volatile contamination
  - *Resolution:* Film plate sealers, which produced more consistent results, will be used or volatile chemicals in Phase III to prevent contamination of nearby wells
- Some lots of NHK media and/or supplements did not support adequate cell growth in the NRU assay
  - *Resolution:* NHK media and supplements will be prequalified prior to use in PhaseIII

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

Figure 2.

Phases Ib and II Preliminary Results: IC50 values for 12 Coded Chemicals
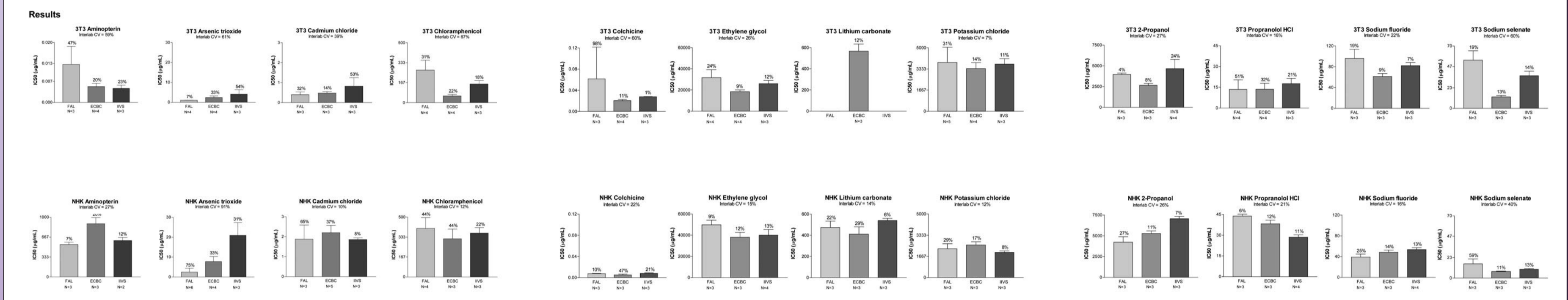
Results

Top panel shows 3T3 NRU results. Bottom panel shows NHK NRU results. Error bars show standard deviation. Numbers above error bars are intralaboratory coefficient of variation (CV). The mean intralab CV was 23% for 3T3 assays. The interlab CV for the 3T3 assay was 40% and that for the NHK assay was 25%. Due to the relative insolubility of lithium carbonate in the 3T3 medium, only one laboratory obtained IC50 data for that assay. Random effects analysis of variance (ANOVA) shows no significant difference among the laboratories at p< 0.05; however, analyses for the following chemicals/assays were not calculable: colchicine, potassium chloride, and propranolol HCl for the 3T3 assay; and cadmium chloride, chloramphenicol, and potassium chloride for the NHK assay. Labs: FAL – FRAME Alternatives; ECBC – US Army Edgewood Chemical Biological Center; IIVS – Institute for In Vitro Sciences.

Figure 3.

Phase Ib and II Preliminary IC50 Values on RC Regression
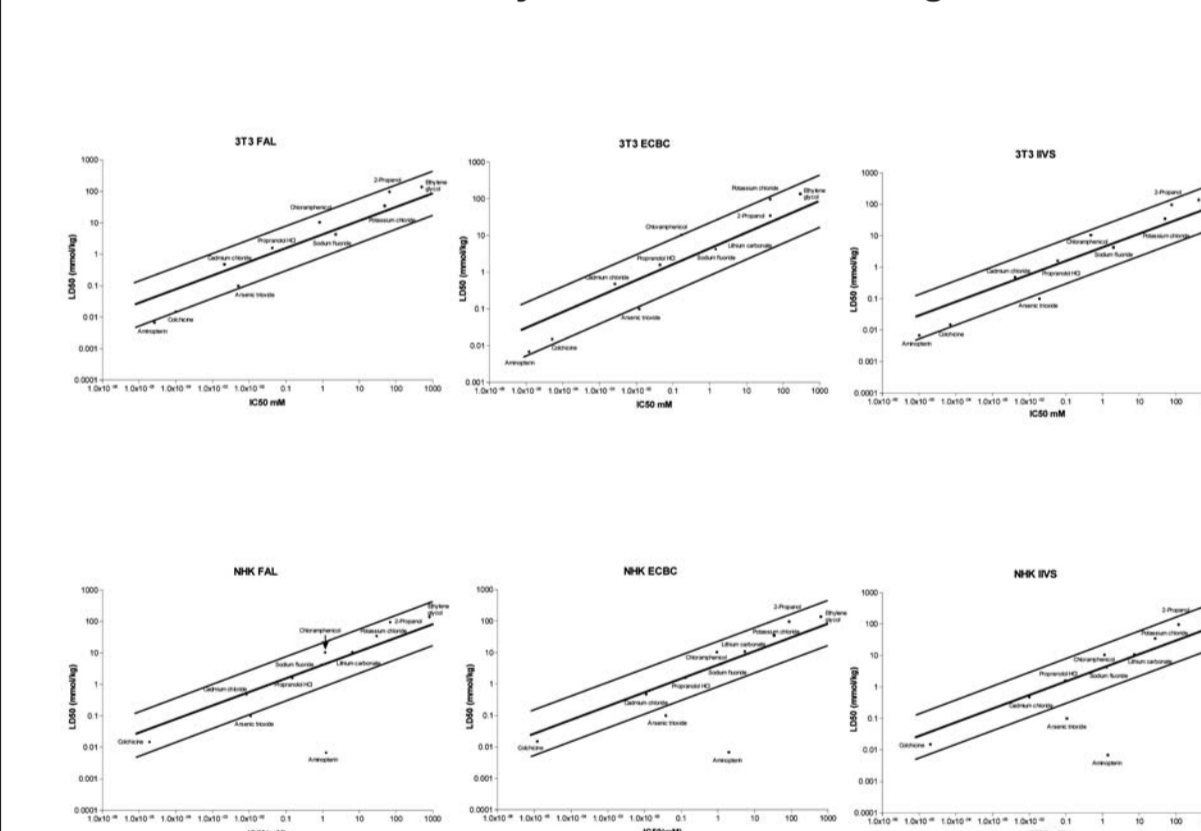
Log IC50 results are graphed with log LD50 values used in the RC (shown in Table 1). Top panel shows 3T3 NRU results while bottom panel shows NHK NRU results. Bold line shows RC regression. Lighter lines show RC prediction interval. Sodium selenate is not shown because it was not included in the RC. Comparison of the regressions is shown in Table 2.

Table 2.

Comparison of Lab/Assay Results with RC Regression

Regression	Slope	y intercept	r[2]	Comparison with RC Slopes (p-value)	Intercepts (p-value)
RC	0.435	0.625	0.452	NA	NA
FAL 3T3	0.592	0.722	0.958	0.114	0.899
FAL NHK	0.545	0.402	0.619	0.327	0.305
ECBC 3T3	0.579	0.771	0.937	0.134	0.883
ECBC NHK	0.525	0.383	0.576	0.422	0.254
IIVS 3T3	0.582	0.706	0.949	0.132	0.875
IIVS NHK	0.545	0.357	0.588	0.341	0.211

Comparison of slopes and intercepts showed that no lab or assay specific results were significantly different from the RC regression (i.e., p >> 0.05).

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

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

Conclusions

- Even assays in use for 20+ years must be tested and optimized underrent laboratory conditions (see "Technical Challenges" under "Study Phases").
- Intralaboratory reproducibility was the same for both assays (mean CV= 23%), but interlaboratory reproducibility was better for the NHK assay (mean CV = 25% vs 40% for 3T3).
- Judging by goodness of fit r[2] values, the 3T3 assay provided a better linear regression (i.e., IC50 was a better predictor of LD50) (see Table 2). This may be due to the five fold difference in the sensitivity of NHK cells to aminopterin (see Figure 3).
- Phases Ib and II of the validation study confirmed that cytotoxicity results from individual cytotoxicity assays do not differ from the RC regression (see Table 2), which was based on pooled cytotoxicity data from multiple endpoints and various cell types. Phase III results will determine how many animals may be saved using this approach for estimating starting doses for LD50 tests.

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