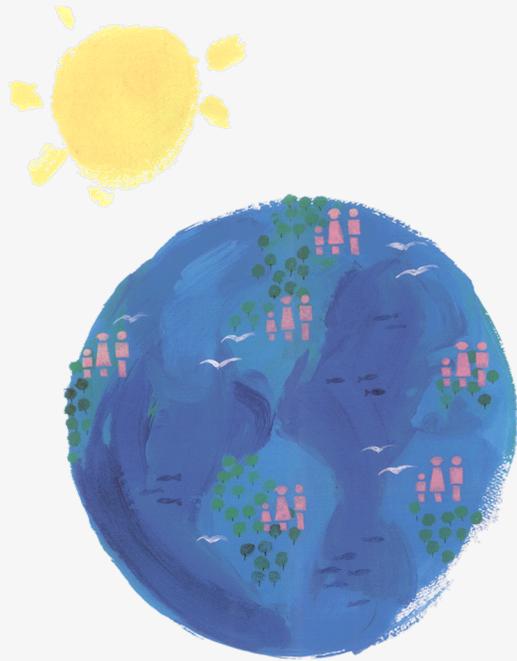


NICEATM

*National Toxicology Program
Interagency Center for the Evaluation of
Alternative Toxicological Methods*

ICCVAM

*Interagency Coordinating Committee on
the Validation of Alternative Methods*



ICCVAM *In Vitro* Acute Toxicity Peer Panel Evaluation

Judy Strickland, Ph.D., DABT

Test Method Overview

May 23, 2006

**National Institutes of Health (NIH)
Matcher Conference Center
Bethesda, Maryland**



Objectives of the Validation Study

- Further standardize and optimize two *in vitro* neutral red uptake (NRU) cytotoxicity protocols to maximize intra- and inter-laboratory reproducibility
- Assess the accuracy of the two standardized *in vitro* cytotoxicity test methods for estimating rodent oral LD₅₀¹ values across the GHS (UN 2005) categories of acute oral toxicity
- Estimate the reduction and refinement in animal use from using *in vitro* basal cytotoxicity assays to identify starting doses for *in vivo* acute toxicity tests
- Generate high quality *in vivo* lethality and *in vitro* cytotoxicity databases that can be used to support the investigation of other *in vitro* test methods necessary to improve the prediction of acute systemic toxicity

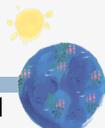
¹Median lethal dose.

UN. 2005. Globally Harmonized System of Classification and Labeling of Chemicals (GHS), First Revised Edition. [ST/SG/AC.10/30/Rev.1]. United Nations, New York and Geneva.



Study Design Overview

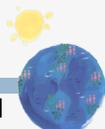
- 72 coded substances covering the complete range of acute oral toxicity based on the GHS hazard classification
- 3 Laboratories: 1 E.U., 2 U.S.
- 2 Cell types: Neutral Red Uptake Assays
 - BALB/c 3T3 murine fibroblast cell line
 - Normal human keratinocytes (NHK)
- Start: August 2002; finish: January 2005



Priority for Selection of Reference Substances

- Human toxicity/exposure
 - MEIC (42/72 substances)
 - EDIT (17/72 substances)
 - TESS (46/72 substances) [poison control centers]
 - NTP (54/72 substances)
 - U.S. EPA HPV Program (18/72 substances)
- Rodent toxicity data available
- Relatively nonvolatile

MEIC = Multicentre Evaluation of *In Vitro* Cytotoxicity
EDIT = Evaluation-Guided Development of *In vitro* Tests
TESS = Toxic Exposure Surveillance System
NTP = U.S. National Toxicology Program
HPV = High Production Volume program



Categorization Based on Reference LD₅₀

LD₅₀ < 5 mg/kg

(N=7)

Cycloheximide
Disulfoton
Epinephrine bitartrate
Phenylthiourea
Physostigmine
Sodium selenate
Triethylenemelamine

5 < LD₅₀ ≤ 50 mg/kg

(N=12)

Aminopterin
Arsenic III trioxide
Busulfan
Colchicine
Digoxin
Endosulfan
Mercury II chloride
Parathion
Potassium cyanide
Sodium arsenite
Strychnine
Thallium I sulfate

50 < LD₅₀ ≤ 300 mg/kg

(N=12)

Cadmium II chloride
Dichlorvos
Diquat dibromide
Fenpropathrin
Hexachlorophene
Lindane
Paraquat
Phenobarbital
Nicotine
Sodium dichromate dihydrate
Sodium I fluoride
Verapamil HCl

300 < LD₅₀ ≤ 2000 mg/kg

(N=16)

Acetylsalicylic acid
Amitriptyline
Atropine sulfate
Caffeine
Chloral hydrate
Cupric sulfate * 5 H₂O
Glutethimide
Haloperidol
Lithium carbonate
Meprobamate
Phenol
Procainamide HCl
Propranolol HCl
Sodium oxalate
Triphenyltin hydroxide
Valproic acid

2000 < LD₅₀ ≤ 5000 mg/kg

(N=12)

Acetaminophen
Acetonitrile
5-Aminosalicylic acid
Boric acid
Carbamazepine
Carbon tetrachloride
Chloramphenicol
Lactic acid
Potassium I chloride
Sodium chloride
Trichloroacetic acid
Xylene

LD₅₀ > 5000 mg/kg

(N=13)

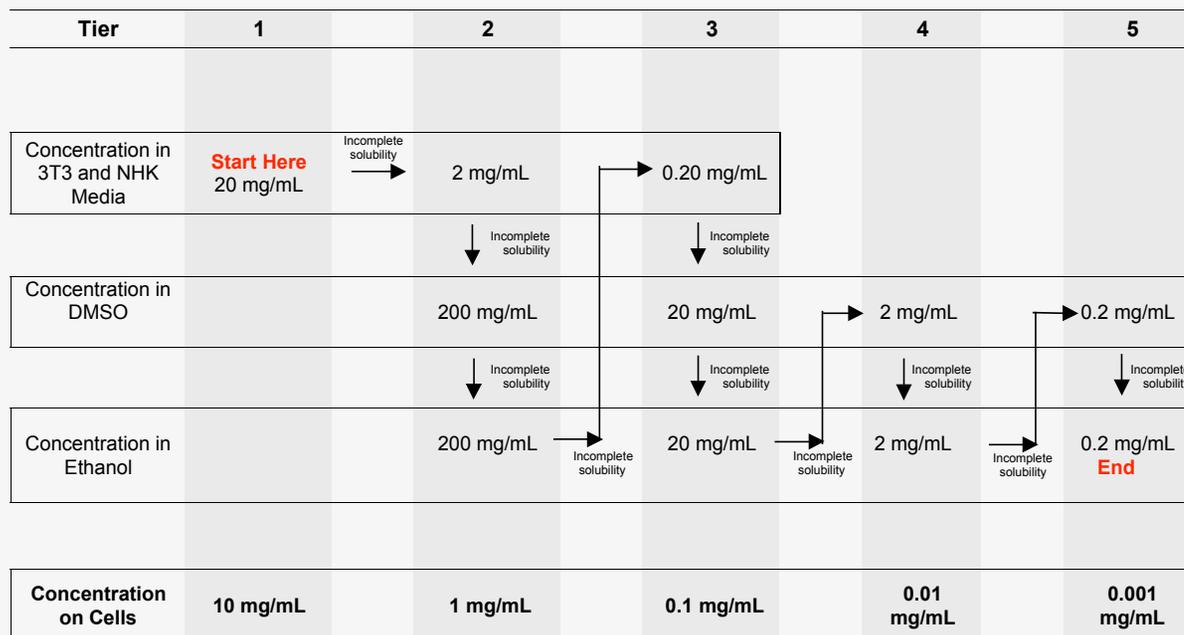
Citric acid
Dibutyl phthalate
Diethyl phthalate
Dimethylformamide
Ethanol
Ethylene glycol
Gibberellic acid
Glycerol
Methanol
2-Propanol
Propylparaben
Sodium hypochlorite
1,1,1-Trichloroethane



Steps for *In Vitro* Testing (1)

- Laboratory receives test substance and performs solubility test

Flow Chart for Determination of Reference Substance Solubility in Medium, DMSO, or Ethanol

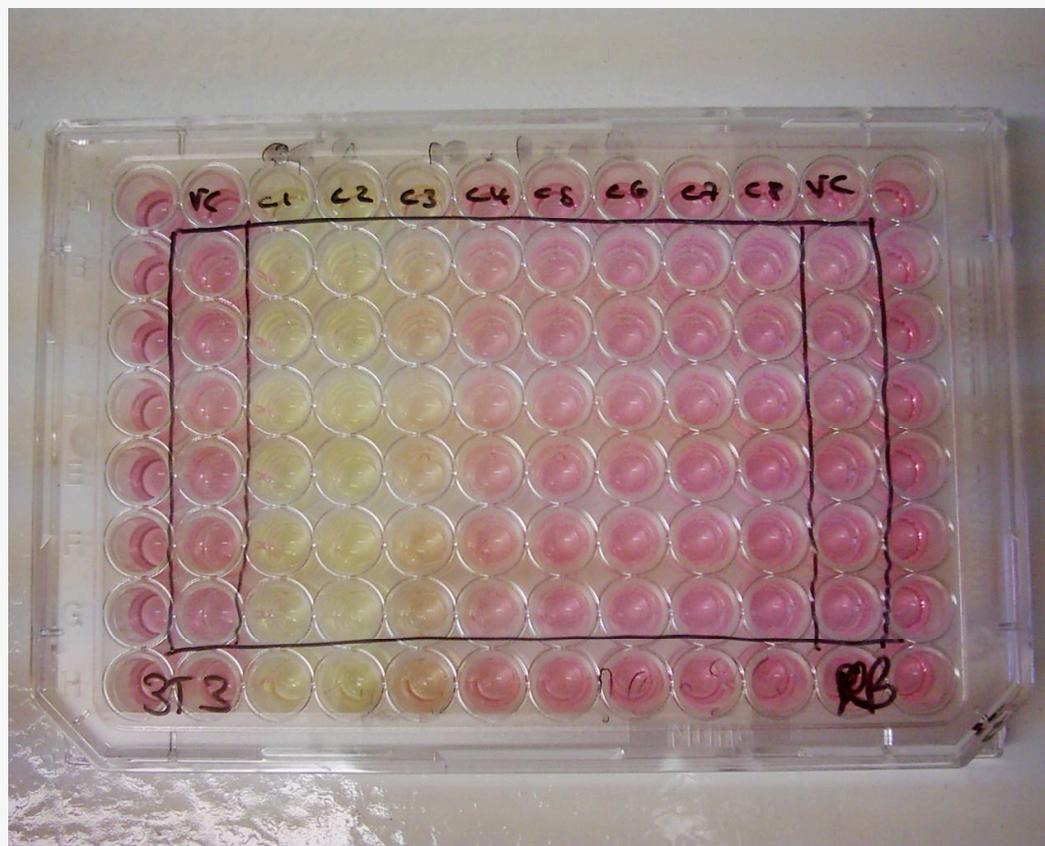


Steps for *In Vitro* Testing (2)

- *In vitro* cytotoxicity tests are performed in 96-well microtiter plates (8 concentrations, 6 wells/concentration)



3T3 Cells in Test Plate

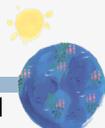


Rows 1, 8 & Columns 1, 12 = blanks
VC = vehicle control
C1 = test chemical concentration 1 (highest)
C8 = test chemical concentration 8 (lowest)
[courtesy of FRAME Alternatives Laboratory]

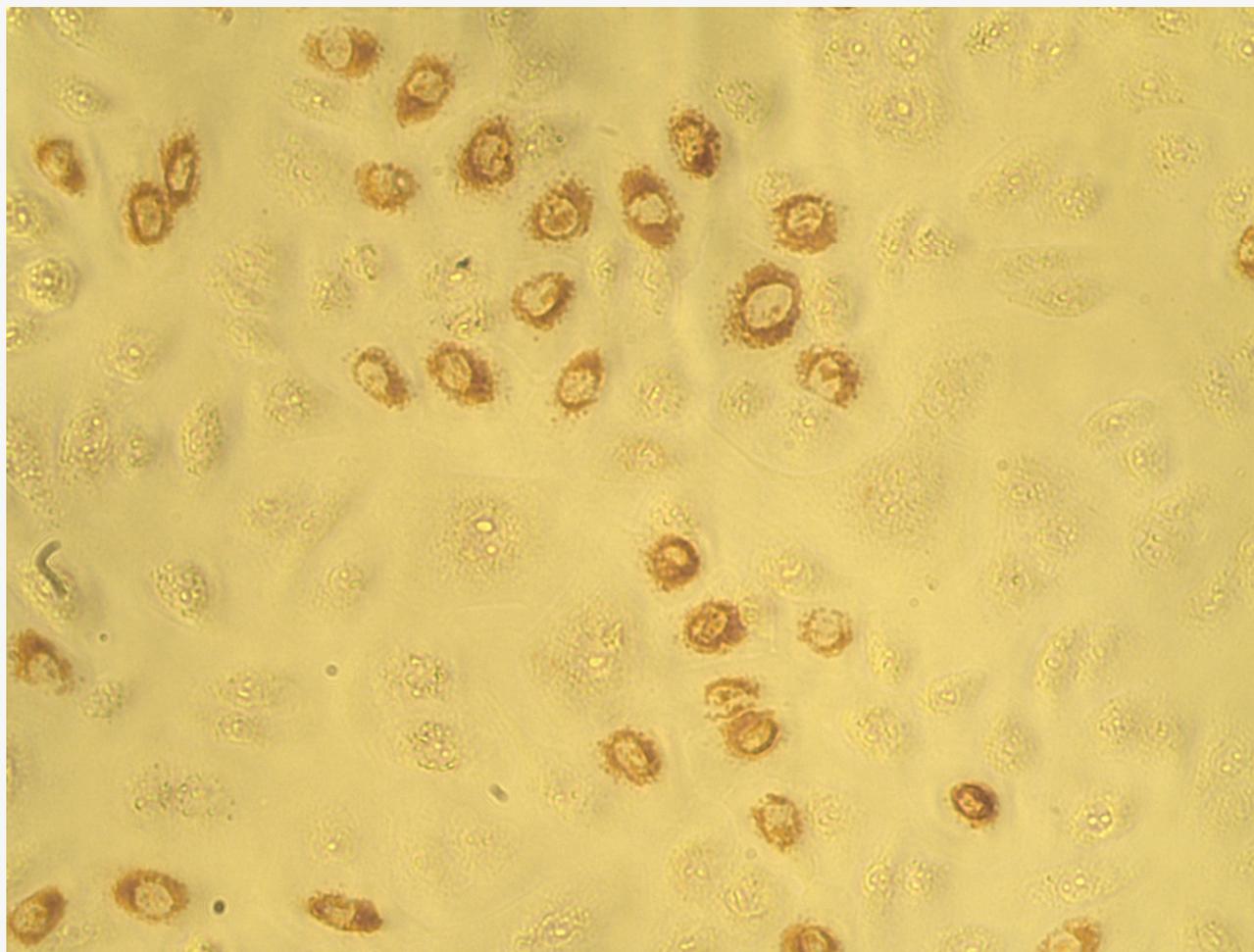
Steps for *In Vitro* Testing (3)

- Laboratory first performs a range finding NRU assay using log dilutions for the test substance
- Laboratory performs a definitive concentration-response NRU assay using a dilution factor smaller than the range finder (e.g., 1.47) to cover the IC_{50} and to have cytotoxicity points on each side of the IC_{50} (minimum of 3 definitive tests on different days for each test substance)
- Positive control (sodium lauryl sulfate [SLS]) tested in same manner as definitive test on separate plate

¹Concentration at 50% inhibition.



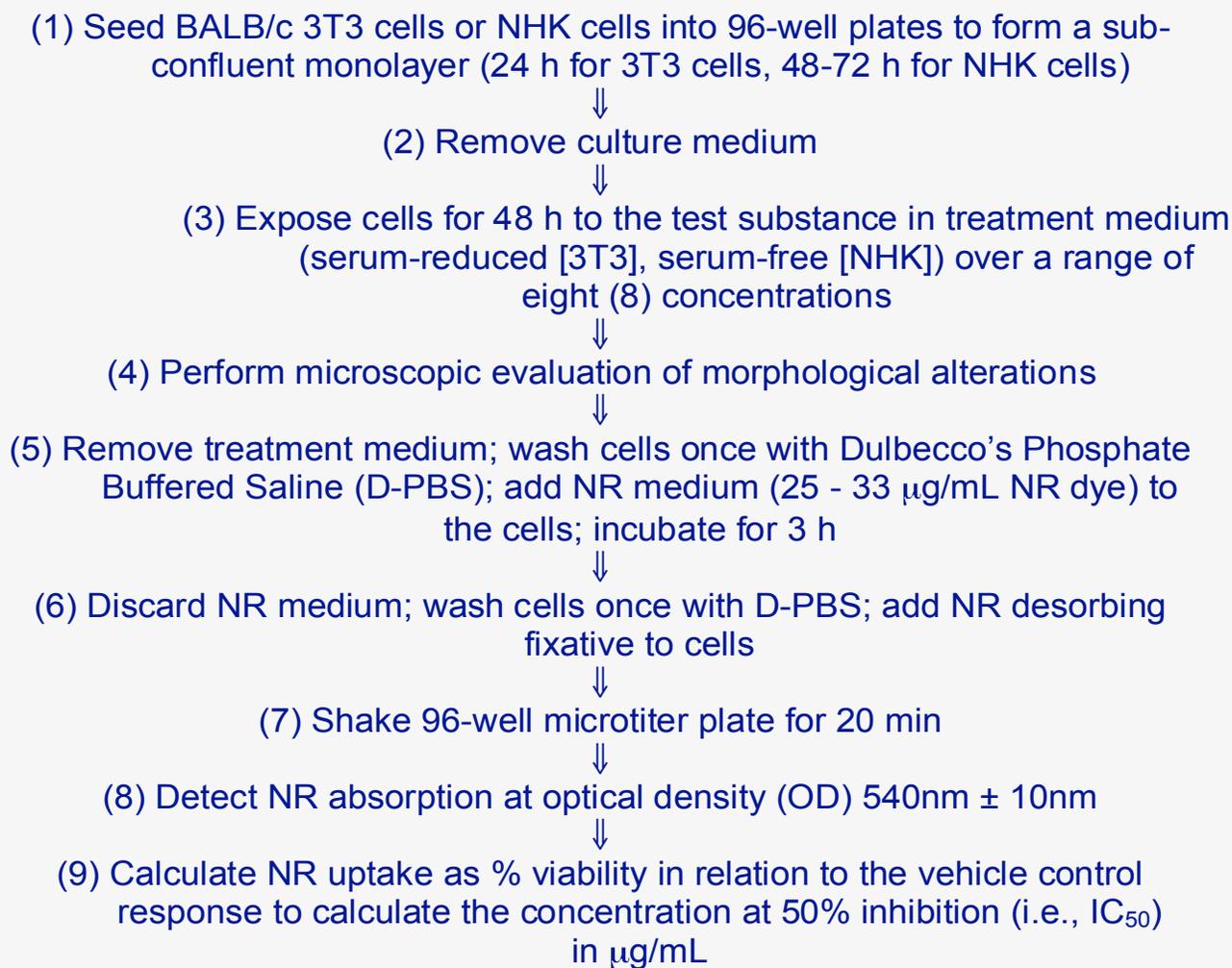
NHK Cells in NRU Assay



[courtesy of FRAME Alternatives Laboratory]



Flow Chart of the NRU Assay



Test Acceptance Criteria¹

- All acceptance criteria must be met for a test to be acceptable.
 1. The positive control (PC [SLS]) IC_{50} must be within 2.5 standard deviations of the historical mean established by the Test Facility, and must meet criteria 2 and 3, and have an $r^2 \geq 0.85$ for the Hill model fit (i.e., from PRISM[®] software)
 2. The left and right mean of the vehicle controls (VCs) do not differ by more than 15% from the mean of all VCs
 3. At least one cytotoxicity point $> 0\%$ and $\leq 50.0\%$ viability and at least one cytotoxicity point $> 50.0\%$ and $< 100\%$ viability must be present
- Exception: If a test has only one point between 0 and 100 % and the smallest dilution factor (i.e., 1.21) was used and all other test acceptance criteria were met, then the test will be considered acceptable.

¹Used in Phase III of the validation study



Determination of IC₅₀

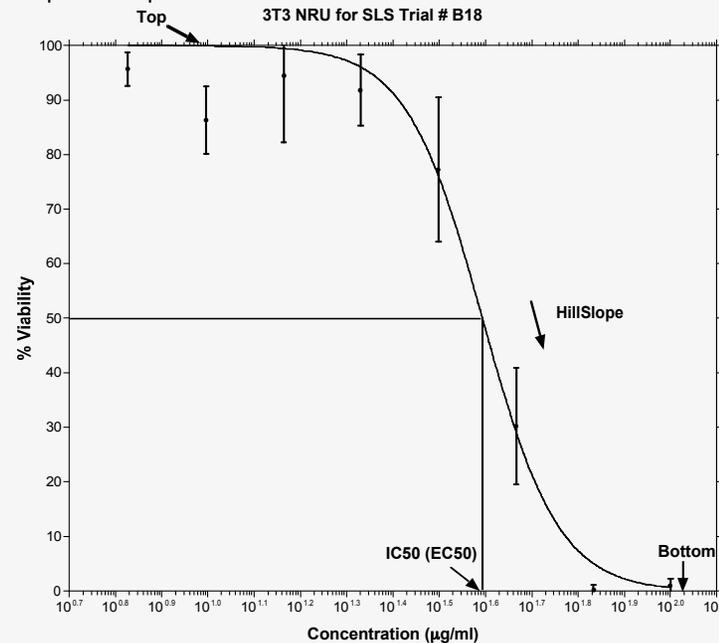
Data copied from Excel spreadsheet are pasted into Prism

Log Conc.	% Viability					
	Y1	Y2	Y3	Y4	Y5	Y6
2.000	1.10	2.00	2.70	0.60	0.10	-0.30
1.833	-0.10	2.00	-0.30	-0.30	0.40	-0.30
1.666	22.70	24.50	45.90	21.70	26.20	40.10
1.498	67.50	100.80	81.50	73.30	68.00	72.20
1.330	93.80	84.50	89.10	102.60	88.70	92.20
1.164	86.60	111.20	96.10	91.00	103.10	78.70
0.996	82.90	85.40	94.00	91.70	77.70	86.30
0.826	95.90	95.90	100.10	94.70	91.00	96.30

Hill Equation:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log \text{EC}_{50} - X) \text{Hillslope}}}$$

Graphical output from Prism



Numerical output from Prism:

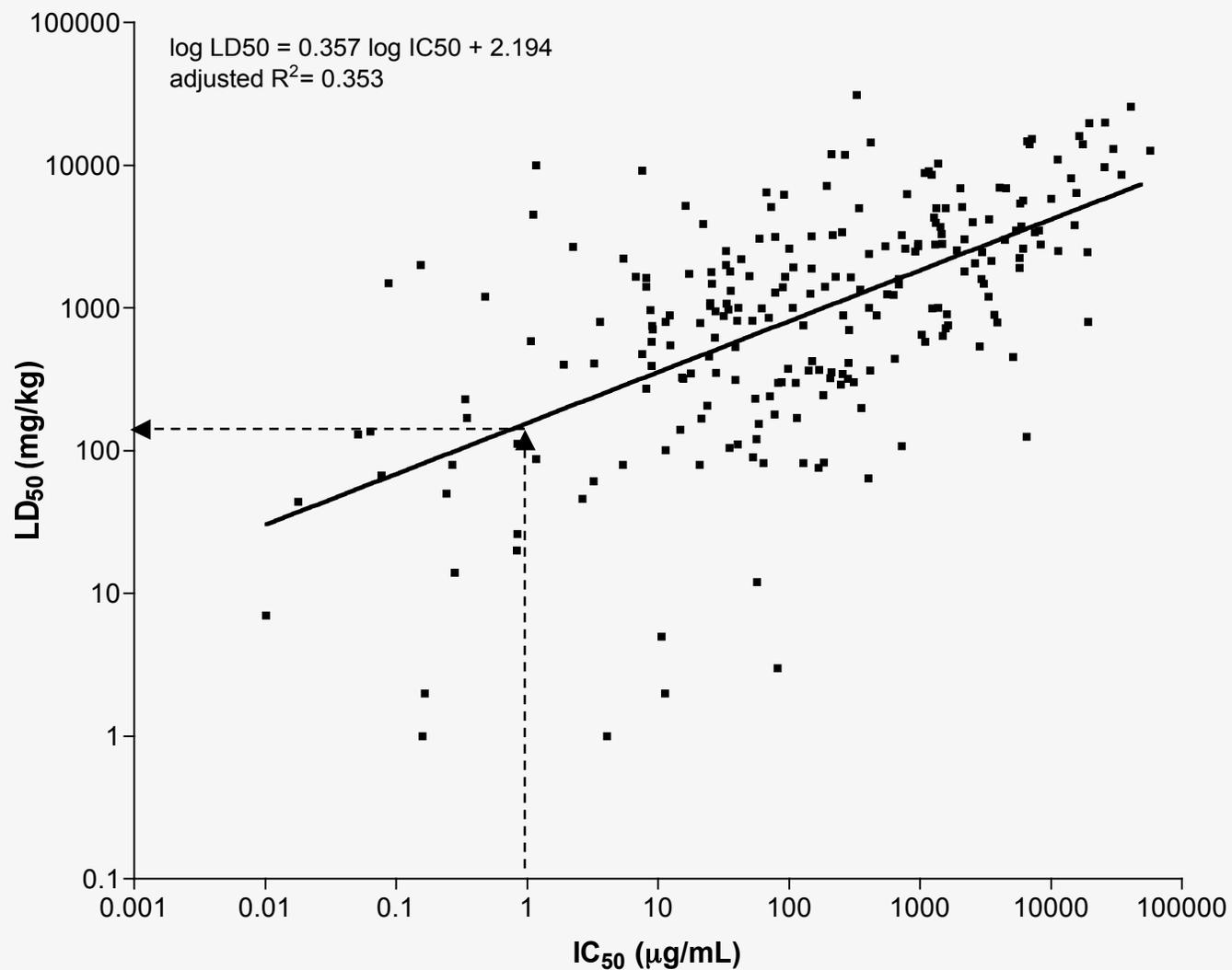
Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	1.593
HILLSLOPE	-5.288
EC50	39.17
Std. Error	
LOGEC50	0.01116
HILLSLOPE	0.5988
95% Confidence Intervals	
LOGEC50	1.570 to 1.615
HILLSLOPE	-6.494 to -4.081
EC50	37.19 to 41.25
Goodness of Fit	
Degrees of Freedom	46
R squared	0.9472
Absolute Sum of Squares	4055
Sy.x	9.389
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8

When Top = 100 and Bottom = 0, EC₅₀ = IC₅₀

Hill function illustration shows Top = 100 and Bottom = 0. Y= response, X = logarithm of concentration, Bottom = minimum response, Top = maximum response, logEC₅₀ = logarithm of X at the response midway between Top and Bottom, and HillSlope = steepness of the curve.

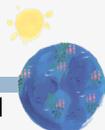


Prediction of LD₅₀



Determination of Starting Dose

- Calculate predicted LD_{50} using IC_{50} in the regression formulas
- Up-and-Down Procedure (UDP)
 - Use next lower default dose
 - Default doses: 1.75, 5.5, 17.5, 55, 175, 550, 1750, 5000 mg/kg
- Acute Toxic Class (ATC) Method
 - Use next lower fixed dose
 - Fixed doses = 5, 50, 300, 2000 mg/kg
- Predicted LD_{50} for cadmium chloride = 16 mg/kg, starting dose = 5.5 mg/kg for UDP and 5 mg/kg for ATC



Simulation Modeling of *In Vivo* Testing

- Calculated animal use to test each substance
 - Using default starting dose (175 mg/kg for the UDP and 300 mg/kg for the ATC)
 - Using NRU-determined starting dose



Animal Use for the UDP

Toxicity Category ¹ (mg/kg)	N	With Default Starting Dose	With NRU- Based Starting Dose	Animals Saved ²
3T3 NRU Test Method				
LD ₅₀ ≤ 5	4	11.68 ± 0.17	11.26 ± 0.55	0.42 (3.6%)
> 5 < LD ₅₀ ≤ 50	7	9.05 ± 0.13	9.03 ± 0.55	0.02 (0.3%)
> 50 < LD ₅₀ ≤ 300	5	7.82 ± 0.18	7.84 ± 0.15	-0.02 (-0.2%)
> 300 < LD ₅₀ ≤ 2000	9	8.81 ± 0.35	7.81 ± 0.06	1.00* (11.4%)
> 2000 < LD ₅₀ ≤ 5000	9	10.84 ± 0.07	8.62 ± 0.23	2.22* (20.5%)
> 5000	12	9.59 ± 0.27	7.71 ± 0.40	1.88* (19.6)%
NHK NRU Test Method				
LD ₅₀ ≤ 5	4	11.55 ± 0.23	11.90 ± 0.32	-0.35 (-3.0%)
> 5 < LD ₅₀ ≤ 50	7	9.28 ± 0.25	8.30 ± 0.28	0.98* (10.6%)
> 50 < LD ₅₀ ≤ 300	5	7.87 ± 0.20	8.03 ± 0.24	-0.16 (-2.0%)
> 300 < LD ₅₀ ≤ 2000	9	8.76 ± 0.33	7.86 ± 0.06	0.90* (10.3%)
> 2000 < LD ₅₀ ≤ 5000	9	10.82 ± 0.07	8.84 ± 0.26	1.98* (18.3%)
LD ₅₀ > 5000	13	9.52 ± 0.28	7.75 ± 0.43	1.77* (18.6%)

¹Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005)

²Difference between mean animal use with default starting dose and mean animal use with NRU-based starting dose. * notes statistically significant differences by the Wilcoxon signed rank test.



Study Management Team (SMT)

■ NICEATM

- **William S. Stokes, D.V.M., DAACLAM (NIEHS)**
Director; Project Officer
- **Raymond Tice, Ph.D. (NIEHS)**
Deputy Director; Asst. Project Officer
- **Judy Strickland, Ph.D., DABT (ILS, Inc.)**
Sr. Staff Toxicologist
- **Michael Paris (ILS, Inc.)**
Sr. Project Coordinator/Technical Writer

■ ECVAM

- **Thomas Hartung, M.D., Ph.D.**
Head of Unit
- **Silvia Casati, Ph.D.**
Task Leader



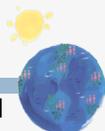
Validation Study Laboratories

- **BioReliance Corp. (Chemical Distribution)**
Gaithersburg, MD
 - Martin Wenk, Ph.D. – Principal Investigator

- **U.S. Army Edgewood Chemical and Biological Center (ECBC)**
Edgewood, MD
 - Cheng Cao, Ph.D. – Study Director

- **Fund for the Replacement of Animals in Medical Experiments (FRAME) Alternatives Laboratory (FAL)**
University of Nottingham, United Kingdom
 - Richard Clothier, Ph.D. – Study Director

- **Institute for In Vitro Sciences (IIVS)**
Gaithersburg, MD
 - Hans Raabe, M.S. – Study Director



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- Robert Lee, M.S.
- Janine Wilcox

■ Statistical Consultant

Raleigh, NC

- Joseph Haseman, Ph.D.

■ Other Support Staff

Integrated Laboratory Systems (ILS), Inc., RTP, NC

- | | |
|-------------------------------------|------------------------------|
| • Bradley Blackard, M.S.P.H. | Project Manager |
| • Sue Brenzel | Webmaster |
| • Jeff Charles, Ph.D., M.B.A., DABT | Principal Investigator |
| • Linda Litchfield | Meeting Planner/Admin. Asst. |

Molly Vallant

NIEHS, Project Officer

