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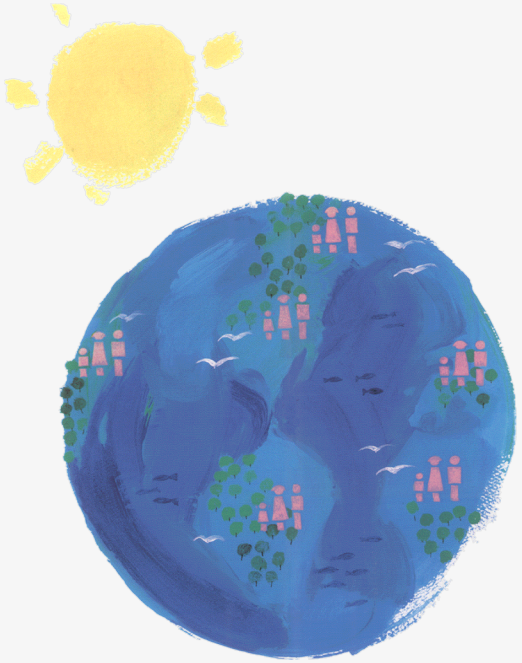
National Toxicology Program
Interagency Center for the Evaluation of Alternative Toxicological Methods

ICCVAM

Interagency Coordinating Committee on the Validation of Alternative Methods



Current ICCVAM Recommendations for the Use of *In Vitro* Test Methods to Estimate Acute Systemic Toxicity

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Acute Chemical Safety Testing: Advancing *In Vitro* Approaches and Humane Endpoints for Systemic Toxicity Evaluations

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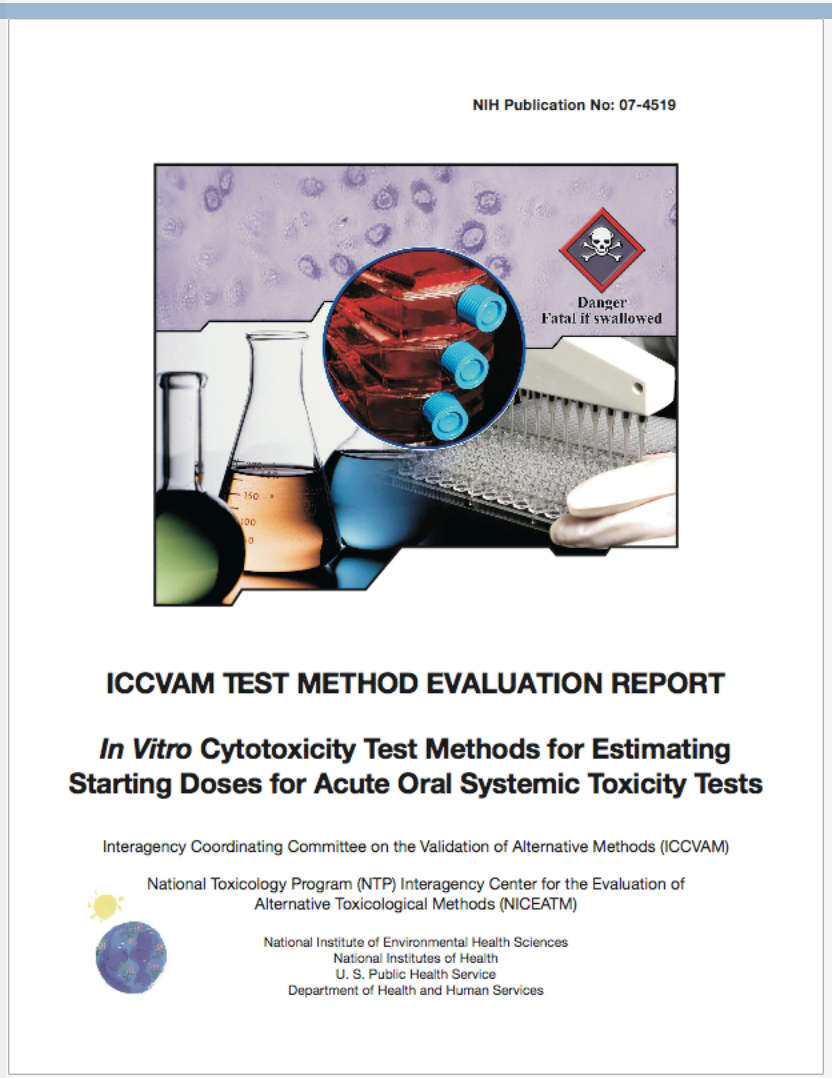
Background

- A NICEATM/ECVAM-sponsored validation study (2002-2005) evaluated two *in vitro* neutral red uptake (NRU) basal cytotoxicity assays by testing 72 substances
  - BALB/c 3T3 (clone A31) mouse fibroblast NRU test method
  - Normal human keratinocyte (NHK) NRU test method.
- The objectives of the study were to:
  - determine the extent that NRU test methods could estimate rodent acute oral LD_{50} values to be used to set the starting doses for *in vivo* acute oral toxicity tests
  - develop high quality *in vivo* acute oral lethality and *in vitro* NRU cytotoxicity databases
  - further standardize and optimize the *in vitro* NRU basal cytotoxicity protocols to maximize test method reliability (intralaboratory repeatability, intra- and inter-laboratory reproducibility)
- An independent peer review panel was convened (May 2006) to evaluate the test methods and the draft ICCVAM test method recommendations.

ICCVAM Test Method Evaluation Report



- Recommended:

1. Test Method Uses
2. Standardized Protocols
3. Future Studies
4. Performance Standards

ICCVAM Recommendations: Test Method Uses - 1

1. *The 3T3 and NHK NRU test methods are not sufficiently accurate to predict acute oral toxicity for the purpose of regulatory hazard classification.*
2. *For the purposes of acute oral toxicity testing, the 3T3 and NHK NRU test methods may be used in a weight-of-evidence approach to determine the starting dose for the current acute oral toxicity protocols (i.e., the Up-and-Down Procedure [UDP] and Acute Toxic Class [ATC]).*

ICCVAM Recommendations: Test Method Uses - 2

3. *Consistent with the U.S. Government Principles on the Use of Animals in Research, Testing, and Education[1], and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals[2], in vitro basal cytotoxicity test methods as part of a weight-of-evidence approach to estimate the starting dose for acute oral in vivo toxicity test methods* ***should be considered and used where appropriate before testing is conducted using animals****.*

   - *For some types of substances, this approach will reduce the number of animals needed.*
   - *In some testing situations, the approach may also reduce the numbers of animals that die or need to be humanely killed.*

[1]Interagency Research Animal Committee. 1985. U.S. Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. Federal Register 50, No. 97, May 20.
[2]PHS. 2002. Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals. Office of Laboratory Animal Welfare. Washington, DC:National Institutes of Health.

ICCVAM Recommendations: Test Method Uses - 3

4. *The starting doses for substances with certain toxic mechanisms that are not expected to be active in 3T3 or NHK cells (e.g., those that are neurotoxic or cardiotoxic) will likely be underpredicted by these in vitro basal cytotoxicity test methods.*

   - *Therefore, the results from basal cytotoxicity testing with such substances may not be appropriate for estimating starting doses.*

ICCVAM Recommendations: Test Method Uses - 4

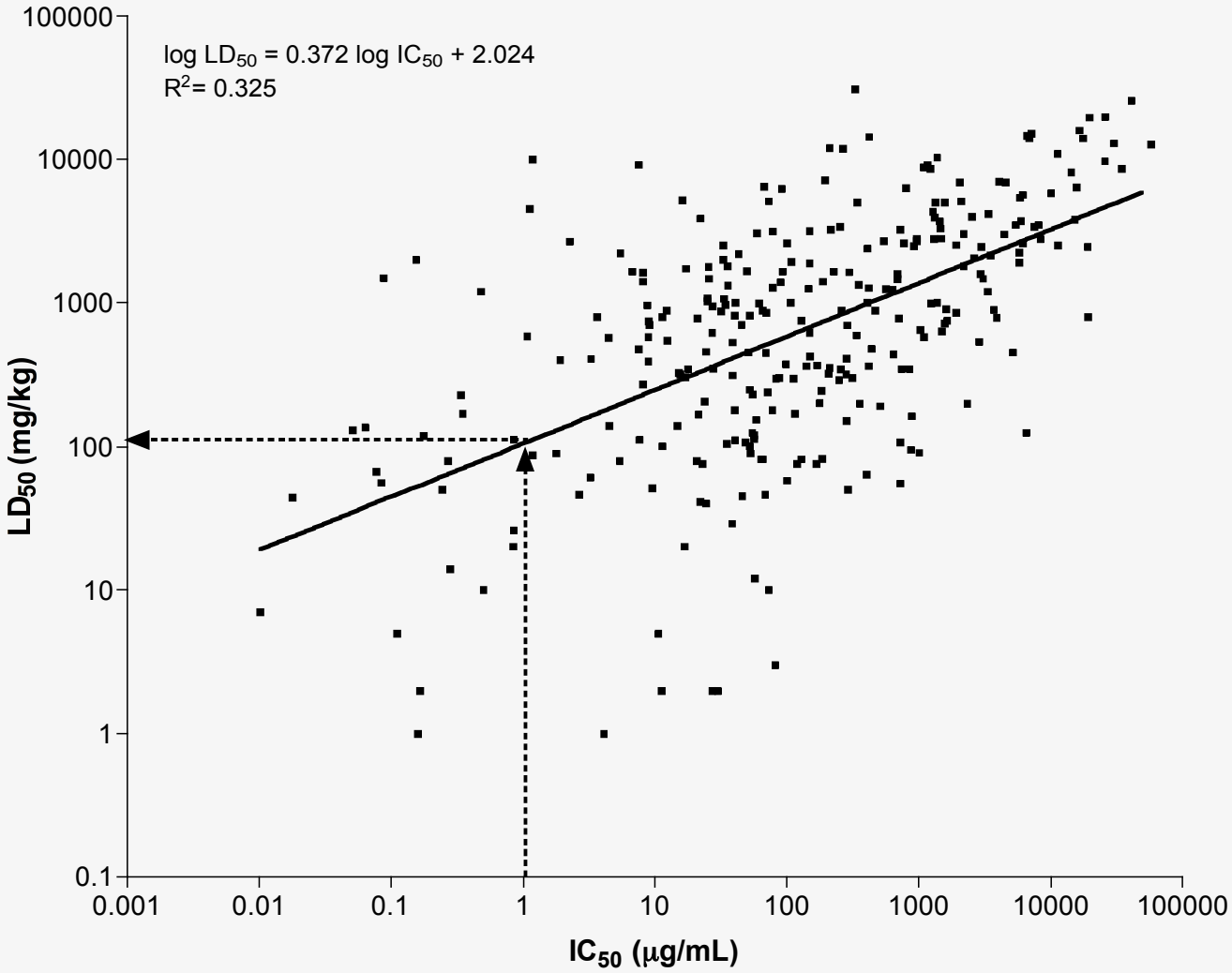
5. *The regression formula used to determine starting doses for test substances with known molecular weights and high purity should be the revised Registry of Cytotoxicity (RC) millimole regression line based on substances with rat LD_{50} data, with IC_{50} values in mmol/L and LD_{50} values in mmol/kg[1].*

   - *The regression formula used to determine starting doses for mixtures, test substances with low or unknown purity, or test substances with unknown molecular weights should be the revised RC regression line, based on substances with rat LD_{50} data, with IC_{50} values in μg/mL and LD_{50} values in mg/kg[2].*

[1] $\log LD_{50}$ (mmol/kg) = 0.439 $\log IC_{50}$ (mmol/L) + 0.621

[2] $\log LD_{50}$ (mg/kg) = 0.372 $\log IC_{50}$ (μg/mL) + 2.024

Prediction of LD_{50}



ICCVAM Recommendations: Test Method Uses - 5

6. *The performance of other in vitro basal cytotoxicity test methods that are based on similar scientific principles and that measure or predict the same biological response (i.e., basal cytotoxicity and the rat acute oral LD_{50} value, respectively) should be demonstrated to meet or exceed the accuracy and reliability of the 3T3 and NHK NRU test methods.*

   - Performance standards are provided in the test method evaluation report to expedite evaluation of other *in vitro* methods.

ICCVAM Recommendations: Test Method Uses - 6

7. *Compared to the NHK NRU test method, the 3T3 NRU test method appears to be less labor intensive and less expensive to conduct; therefore, the 3T3 NRU cytotoxicity test method is recommended for general use.*

   - *Although the 3T3 NRU test method was less reproducible than the NHK NRU test method, it produced slightly higher animal savings and accuracy for prediction of GHS[1] acute oral toxicity category using the IC_{50} and the revised RC regressions evaluated for the prediction of LD_{50}.*

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

ICCVAM Recommendations: Protocols

- 3T3 NRU Test Method Protocol

http://iccvam.niehs.nih.gov/methods/acutetox/invidocs/phIIIprot/3t3phIII.pdf

- NHK NRU Test Method Protocol

http://iccvam.niehs.nih.gov/methods/acutetox/invidocs/phIIIprot/nhkphIII.pdf

ICCVAM Recommendations: Future Studies and Activities - 1

1. *Additional data should be collected using the 3T3 NRU basal cytotoxicity test method to evaluate its usefulness for predicting the rodent acute oral toxicity of chemical mixtures.*
2. *To supplement the high quality validation database started by this study, additional high quality comparative in vitro basal cytotoxicity data should be collected when rat acute oral toxicity testing is conducted.*

ICCVAM Recommendations: Future Studies and Activities - 2

3. *Additional efforts should be conducted to identify in vitro tests and other methods necessary to achieve accurate acute oral hazard classification (without animals). Studies should be conducted to investigate the potential use of in vitro cell-based test methods that incorporate mechanisms of action and evaluations of ADME (absorption, distribution, metabolism, excretion) to provide improved estimates of acute toxicity hazard categories.*
    - *Methods should be developed to extrapolate from in vitro toxic concentrations to equivalent doses in vivo.*

ICCVAM Recommendations: Future Studies and Activities - 3

4. *The in vivo database of reference substances used in this validation study should be used to evaluate the utility of other non-animal approaches to estimate starting doses for acute oral toxicity tests (e.g., widely available software that uses quantitative structure-activity relationships [QSAR]).*

5. *Standardized procedures to collect information pertinent to an understanding of the mechanisms of lethality should be included in future rat acute oral toxicity studies.*

    - *Such information will likely be necessary to support the further development of predictive mechanism-based in vitro methods.*

ICCVAM Recommendations: Future Studies and Activities - 4

6. *An expanded list of reference substances with estimated rat acute oral LD_{50} values substantiated by high quality in vivo data (including data currently held by industry) should be developed for use in future in vitro test method development and validation studies.*

Animal Use Example: Standard Up-and-Down Procedure (UDP)

Example 1

New chemical with no basis for LD_{50} (but actual LD_{50} is > 5000 mg/kg). Perform the standard Up-and-Down Procedure (UDP) starting with default dose of 175 mg/kg.

Animal	Dose (mg/kg)[1]	Test Time	Outcome
1	175	48 hr	Lives
2	550	48 hr	Lives
3	1750	48 hr	Lives
4	5000	48 hr	Lives
5	5000	48 hr	Lives
6[2]	5000	48 hr	Lives

[1]Default doses

[2]Testing stops when 3 successive animals live at the upper limit dose of 5000 mg/kg.

6 animals used; 12 days of testing

STOP; classify as LD_{50} > 5000 mg/kg

Animal Savings Using *In Vitro* Cytotoxicity Testing to estimate starting dose for the UDP

Example 2

Perform cytotoxicity test and in vitro data predicts LD_{50} is > 5000 mg/kg. Proceed to the Up-and-Down Procedure (UDP) and start with dose of 5000 mg/kg rather than the default starting dose of 175mg/kg

Animal	Dose (mg/kg)[1]	Test Time	Outcome
1	5000	48 hr	Lives
2	5000	48 hr	Lives
3[2]	5000	48 hr	Lives

[1]Default doses
[2]Testing stops when 3 successive animals live at the upper limit dose of 5000 mg/kg.

3 animals used; 6 days of testing
STOP; classify as LD_{50} > 5000 mg/kg

Use of *in vitro* cytotoxicity test method would result in 50% reduction in animals (3 vs. 6) and 50% reduction in time (6 days vs. 12 days)

Acute Oral Toxicity Hazard Classification Distribution of Industrial Chemicals in the EU

The *in vitro* approach will save animals, since most substances are in the non-harmful category

Spielmann et al. (1999) states:

- Since 1982, 90% of 1115 industrial chemicals from the European Union (EU) have an oral LD_{50} value > 200 mg/kg and are therefore neither toxic nor very toxic according to the EU regulations
- 75% are not harmful (i.e., LD_{50} > 2000 mg/kg and need not be classified in the EU).

LD_{50} mg/kg	Toxicity Class	Number of Chemicals	Chemicals (%)
≤ 25	1 Very toxic	0	0
> 25 - 200	2 Toxic	35	3.1
> 200 - 2000	3 Harmful	235	21.1
> 2000	0 Unclassified	845	75.8

Spielmann H, Genschow E, Liebsch M, Halle W. 1999. Determination of the starting dose for acute oral toxicity (LD50) testing in the up and down procedure (UDP) from cytotoxicity data. Altern Lab Anim **27**:957-966.

ICCVAM Recommendations - Summary

1. Laboratories should consider the two in vitro cytotoxicity methods before conducting any in vivo acute toxicity study.
   - Use the *in vitro* test where determined appropriate to assist with the setting or confirming of starting doses.
2. Submit both the *in vitro* and *in vivo* data to NICEATM-ICCVAM.
   - Data will be assessed to further determine usefulness and limitations of the approach.
   - Chemical identification is preferred but not required.

References

- ICCVAM. 2006. ICCVAM Test Method Evaluation Report: *In Vitro* Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Tests. NIH Publication No. 07-4519. Research Triangle Park, NC:National Institute for Environmental Health Sciences.
- ICCVAM. 2006. Background Review Document*: In Vitro* Cytotoxicity Test Methods for Estimating Acute Oral Systemic Toxicity. NIH Publication No. 07-4518. Research Triangle Park, NC:National Institute for Environmental Health Sciences.
- Spielmann H, Genschow E, Liebsch M, Halle W. 1999. Determination of the starting dose for acute oral toxicity (LD50) testing in the up and down procedure (UDP) from cytotoxicity data. Altern Lab Anim **27**:957-966.
- ICCVAM-NICEATM website (http://iccvam.niehs.nih.gov)