The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Recommendations for the Use of *In Vitro* Cytotoxicity Test Methods to Reduce Animal Use for Acute Oral Toxicity Testing

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Introduction

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is charged by the ICCVAM Authorization Act of 2000[1] with evaluating the scientific validity of new, revised, and alternative toxicological test methods applicable to U.S. Federal agency safety testing requirements. ICCVAM is also required to provide recommendations to U.S. Federal agencies regarding the usefulness and limitations of such test methods. The ICCVAM test method evaluation report (TMER; *In Vitro Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Tests*) provides the ICCVAM's recommendations for using two *in vitro* basal cytotoxicity methods for estimating starting doses for acute oral systemic toxicity tests.

These recommendations are based on a comprehensive evaluation of the scientific validation status of the test methods by ICCVAM, and take into consideration the comments and recommendations received from an independent expert peer review panel, ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), and the general public.

The Report contains ICCVAM recommendations for:

- Test method uses
- Standardized test method protocols
- Test method performance standards
- Future studies

[1] 42 U.S.C. § 2851-2, 2851-5 (2000) http://iccvam.niehs.nih.gov/about/PL106545.pdf.

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NIEHS National Institute of Environmental Health Sciences

U.S. Department of Health and Human Services National Institutes of Health

ICCVAM The Interagency Coordinating Committee on the Validation of Alternative Methods

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

More information on ICCVAM and NICEATM can be accessed at http://iccvam.niehs.nih.gov/

Timeline for Development of the ICCVAM Test Method Evaluation Report (TMER)

In Vitro Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Tests

Oct 2000
International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity

Sep 2001
Publication of International Workshop Report and *Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses*

Oct 2001
NICEATM and ECVAM begin planning of *in vitro* basal cytotoxicity independent validation study

Aug 2002
NICEATM/ECVAM *in vitro* basal cytotoxicity validation study initiated

Jan 2005
Collection of data for validation study completed

Mar 2006
Draft documents released to an independent scientific peer panel and the public:

- Draft background review document (BRD),
- Draft ICCVAM recommendations on: proposed uses, test method protocols, performance standards, future studies

May 2006
Independent Scientific Peer Review Panel Meeting on the Use of *In Vitro* Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Tests

Jun 2006
Publication of Peer Review Panel Report
(http://iccvam.niehs.nih.gov/methods/acutetox/invidocs/panelrpt/ATpanelrpt.htm)

Aug 2006
Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) comments from the public

Oct 2006
ICCVAM endorses BRD and Test Method Evaluation Report (TMER)

Nov 2006
Publication of *In Vitro* Acute Toxicity Test Methods BRD and ICCVAM TMER

2007
ICCVAM Recommendations forwarded to Federal Agencies for Acceptance Consideration

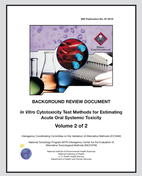
International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity

- No *in vitro* methods had yet been validated for regulatory use
- Recommended validation of *in vitro* basal cytotoxicity test methods to
  - Determine starting doses for acute oral toxicity tests to reduce animal use
  - Generate high quality database of cytotoxicity data that can be used to determine which additional *in vitro* tests will be needed to accurately estimate acute oral toxicity hazard classification categories
- Published Workshop report and *Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses*

NICEATM/ECVAM *In Vitro* Basal Cytotoxicity Validation Study

- Evaluated the usefulness of the BALB/c 3T3 mouse fibroblast (3T3) and normal human epidermal keratinocyte (NHK) neutral red uptake (NRU) test methods to determine starting doses for acute oral toxicity tests (the Up-and-Down Procedure [UDP; EPA 2002; OECD 2001a] and the Acute Toxic Class [ATC] method [OECD 2001b])
- Tested 72 reference substances to determine reproducibility and accuracy for prediction of Globally Harmonized System of Classification and Labelling of Chemicals (GHS; UN 2005) acute oral hazard category
- Determined starting doses and resultant animal savings for the UDP and ATC using computer simulations of animal testing
- Developed a draft background review document (BRD) to publish the results (by ICCVAM and ICCVAM Acute Toxicity Working Group [ATWG])

In Vitro Acute Toxicity Test Methods BRD and ICCVAM Test Method Evaluation Report



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Independent Scientific Peer Review Panel Meeting

- A public meeting of the *In Vitro* Acute Toxicity Test Methods Draft Background Review Document (BRD) for completeness and for any errors or omissions
- Evaluate the extent to which each of the applicable criteria for validation and acceptance have been adequately addressed for the test methods and their specific proposed use
- Comment on the extent to which the draft ICCVAM test method recommendations are supported by the information provided in the Draft BRD

Charges to the Peer Review Panel

- Review the *In Vitro* Acute Toxicity Test Methods Draft Background Review Document (BRD) for completeness and for any errors or omissions
- Evaluate the extent to which each of the applicable criteria for validation and acceptance have been adequately addressed for the test methods and their specific proposed use
- Comment on the extent to which the draft ICCVAM test method recommendations are supported by the information provided in the Draft BRD

Peer Review Panel Conclusions on the Validation Status of the NRU Test Methods

- The applicable validation criteria have been adequately addressed for using these *in vitro* test methods in a weight-of-evidence approach to determine the starting dose for acute oral systemic toxicity test methods.
- The validation study showed that the two NRU test methods evaluated could not be used as a stand-alone replacement for the *in vivo* tests; however, the Panel encouraged future work to develop a tiered testing strategy that includes basal cytotoxicity as part of the overall strategy.
- NRU test methods are useful for estimating starting doses, but not as stand alone tests for hazard classification.

In Vitro Acute Toxicity Peer Review Panel

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ICCVAM Recommended Test Method Protocols

3T3 NRU Test Method and NHK NRU Test Method

General NRU Procedures

BALB/c 3T3 cells or NHK cells are seeded into 96-well plates to form a sub-confluent monolayer (24 h for 3T3 cells; 48-72 h for NHK cells)

↓

Cells are exposed for 48 h to the reference substance in culture medium

↓

Culture medium is removed and neutral red (NR) dye medium is added for a 3 h incubation

↓

NR medium is discarded and NR desorbing fixative is added to the cells

↓

NR absorption is measured at optical density (OD) 540nm ± 10nm (NRU method endpoint)

↓

Percent of vehicle control (VC) at which cell viability or growth is inhibited is calculated to generate an IC_{50} value in µg/mL

↓

The IC_{50} value is used in the regression formula to estimate the rodent acute oral LD_{50} value in mg/kg

ICCVAM Recommendations for Test Method Uses

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. 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 UDP, the ATC method).
3. *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.
4. *In vitro* basal cytotoxicity test methods will likely underpredict the starting doses for substances with toxic mechanisms that are not expected to be active in 3T3 or NHK cells (e.g., those that are neurotoxic or cardiotoxic); therefore, the results for such substances may not be appropriate for use.
5. Use the revised RC millimole regression line based on substances with rat LD_{50} values in mmol/kg and IC_{50} values in mmol/L to determine starting doses for test substances with known molecular weights and high purity. Use the revised RC regression line based on substances with rat LD_{50} values in mg/kg and IC_{50} values in µg/mL to determine starting doses for mixtures, test substances with low or unknown purity, or test substances with unknown molecular weights.
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.
7. The 3T3 NRU test method is recommended for general use because it appears to be less labor intensive and less expensive to conduct than the NHK NRU test method. Although the 3T3 NRU test method was slightly less reproducible than the NHK NRU test method, it produced slightly higher animal savings and accuracy for prediction of GHS acute oral toxicity category using the IC_{50} and the revised RC regressions evaluated for the prediction of LD_{50} values.

Performance Standards: Essential Test Method Components

These test method components consist of essential structural, functional, and procedural elements of an adequately validated test method that should be included in the protocol of a proposed, mechanistically and functionally similar test method. Essential test method components include unique characteristics of the test method, critical procedural details, and quality control measures. Adherence to essential test method components will help to assure that a proposed test method is structurally and functionally similar to the corresponding validated test method.

General Requirements of *In Vitro* Basal Cytotoxicity Test Methods

- The test substance must be soluble in aqueous cell culture medium, dimethyl sulfoxide (DMSO), or ethanol (ETOH).
- The test substance is incubated with the cells for a specified period.
- The test substance is removed and an endpoint indicative of cell viability or cytotoxicity is measured.
- The IC_{50} value is calculated (i.e., the concentration at which cell viability or growth is inhibited by 50% compared to control values). Use the IC_{50} value in the regressions developed to estimate the LD_{50} value in mmol/kg (or mg/kg).

Performance Standards: Essential Test Method Components (Selected)

***In Vitro* Cell Culture Conditions:** Mammalian cell lines (or primary cells); standard culture conditions (e.g., 37 °C ±1 °C, 90% ±10% humidity, 5.0% ±1% CO2/air).

Test Substance Preparation: Dissolve test substances in culture medium, DMSO, or ETOH.

Cytotoxicity Test: Test substance concentrations on cells that produce at least two cytotoxic points greater than 0% and less than 100% viability during exposure from 24 to 72 hours.

Vehicle Controls (VC): Reference for 100% cell growth in the test vessel.

Positive Control (PC): Sodium lauryl sulfate (SLS) or comparable substance that demonstrates that the cell culture system responds with adequate sensitivity to a cytotoxic agent. Should generate a response comparable to an historic IC_{50} range.

Viability Measurements: Use standardized, quantitative methods (e.g., spectrophotometric measurements) to determine cell viability. Use a measurement endpoint that is well established and that has good interlaboratory reproducibility.

IC_{50} Determination: Endpoint values obtained for the test substance used to calculate the percentage of cell viability or growth relative to the VC (arbitrarily set at 100%).

Regression Formula: Use to predict LD_{50} values from IC_{50} values.

- The RC rat-only millimole regression for substances with known molecular weight:
  $\log LD_{50}$ (mmol/kg) = 0.439 log IC_{50} (mM) + 0.621
- The RC rat-only weight regression for mixtures/substances with no known molecular weight:
  $\log LD_{50}$ (mg/kg) = 0.372 log IC_{50} (µg/mL) + 2.024

Performance Standards: Recommended Reference Standards for Evaluating Similar Cytotoxicity Assays

GHS Hazard Category	Reference Substances				
$LD_{50} \leq 5$ mg/kg	Mercury II chloride	Triethylenemelamine	Cycloheximide	Busulfan	Phenylthiourea
$5 < LD_{50} \leq 50$ mg/kg	Dichlorvos	Digoxin	Sodium arsenite	Triphenyltin hydroxide	Sodium dichromate dihydrate
$50 < LD_{50} \leq 300$ mg/kg	Hexachlorophene	Cadmium II chloride	Sodium oxalate	Sodium fluoride	Diquat dibromide monohydrate
$300 < LD_{50} \leq 2000$ mg/kg	Amitriptyline	Propranolol HCl	Atropine sulfate	Acetylsalicylic acid	Carbamazepine
$2000 < LD_{50} \leq 5000$ mg/kg	Acetaminophen	Potassium chloride	Chloramphenicol	Lactic acid	Trichloroacetic acid
$LD_{50} > 5000$ mg/kg	Ethylene glycol	Gibberellic acid	Sodium hypochlorite	Dibutyl phthalate	Glycerol

ICCVAM Recommendations for Future Studies

ICCVAM recommends the following future studies in order to advance the use of *in vitro* methods for assessing acute oral toxicity for regulatory hazard classification purposes:

1. Collect additional data using the 3T3 NRU basal cytotoxicity test method to evaluate its usefulness for predicting the rodent acute oral toxicity of chemical mixtures.
2. Collect additional high quality comparative *in vitro* basal cytotoxicity data when rat acute oral toxicity testing is conducted. However, *in vivo* testing should not be conducted solely to collect data to assess the usefulness of the NRU test method. Conduct periodic evaluations of the expanded database to further characterize the usefulness and limitations of using *in vitro* cytotoxicity data as part of a weight-of-evidence approach to estimate starting doses.
3. Identify *in vitro* tests and other methods necessary to achieve accurate acute oral hazard classification; conduct studies to investigate the potential use of *in vitro* cell-based test methods that incorporate mechanisms of action and evaluations of absorption, distribution, metabolism, and excretion to provide improved estimates of acute toxicity hazard categories; develop methods to extrapolate from *in vitro* toxic concentrations to equivalent doses *in vivo*.
4. Employ the *in vivo* database of reference substances used in the NICEATM/ECVAM validation study to evaluate the utility of other non-animal approaches to estimate starting doses for acute oral toxicity tests (e.g., quantitative structure-activity relationship software).
5. Include standardized procedures to collect *in vivo* measurements and observations pertinent to an understanding of the mechanisms of lethality in future rat acute oral toxicity studies. Such information will support the further development of predictive mechanism-based *in vitro* methods.
6. Develop an expanded list of reference substances with rat acute oral LD_{50} values substantiated by high quality *in vivo* data (including proprietary and non-proprietary data currently held by industry) for use in future *in vitro* test method development and validation studies.

Conclusions

- ICCVAM recommends that, while the two standardized *in vitro* test methods (3T3 and NHK NRU test methods) are not sufficiently accurate to predict acute oral toxicity for the purposes of hazard classification, they can be used in a weight-of-evidence approach to determine the starting dose for the current acute oral *in vivo* toxicity protocols.
- ICCVAM recommends that these test methods be considered and used where determined appropriate before testing is conducted using animals. This approach should reduce the number of animals needed for acute oral toxicity testing studies, and for highly toxic substances, it should reduce the numbers of animals that die or need to be humanely killed.
- In accordance with the ICCVAM Authorization Act of 2000, the TMER will be made available to the public and provided to U.S. Federal agencies for consideration. Each federal agency then determines the regulatory acceptability of a method according to its statutory mandates. Agencies with applicable testing regulations, practices, guidelines and/or guidances are required by law to respond to ICCVAM within 180 days after receiving the recommendations. These responses will be made available to the public on the ICCVAM website (http://iccvam.niehs.nih.gov) in accordance with the ICCVAM Authorization Act requirements.

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