Reducing Animal Use for Acute Oral Toxicity Testing: ICCVAM Recommendations for the Use of *In Vitro* Cytotoxicity Test Methods

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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 (*In Vitro Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Tests;* ICCVAM 2006a) 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 (essential test method components, a list of minimum reference chemicals, and minimum performance characteristics)
- Future studies

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

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

Timeline for Development of the ICCVAM Test Method Evaluation Report

Oct 2000	ICCVAM 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*
Aug 2002	NICEATM[1]/ECVAM[2] *in vitro* basal cytotoxicity validation study initiated
Jan 2005	Validation study completed
Mar 2006	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
Jul 2006	Publication of Peer Review Panel Report
Aug 2006	SACATM and public comments on the Peer Review Panel Report
Oct 2006	ICCVAM approves final BRD and Test Method Evaluation Report
Nov 2006	Publication of *In Vitro* Acute Toxicity Test Methods BRD and ICCVAM Test Method Evaluation Report on ICCVAM/NICEATM website
2007	ICCVAM Recommendations forwarded to Federal agencies for consideration

[1] U.S. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

[2] European Centre for the Validation of Alternative Methods

International Workshop on In Vitro Methods for Assessing Acute Systemic Toxicity

- No *in vitro* methods had yet been validated for regulatory use
- Workshop and ICCVAM recommendations
  – Standardize and validate two *in vitro* basal cytotoxicity test methods: one using a human cell system and one using a rodent cell system
  – Support research to develop the additional components for a battery of *in vitro* tests necessary to accurately predict acute toxicity
- Published Workshop report (ICCVAM 2001a) and *Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses* (ICCVAM 2001b)

NIEHS National Institute of Environmental Health Sciences

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/

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) cytotoxicity 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)
  – 72 reference substances tested to determine accuracy and reproducibility for prediction of Globally Harmonized System of Classification and Labelling of Chemicals (GHS; UN 2005) acute oral hazard category
  – Estimated potential animal savings for the UDP and ATC using computer simulations of animal testing for the 72 chemicals
- Provides high quality database to determine the additional *in vitro* methods that will be necessary to accurately predict acute oral toxicity hazards without animals
- Results and analyses of accuracy and reliability provided in a draft BRD prepared by NICEATM in consultation with ICCVAM and the ICCVAM Acute Toxicity Working Group

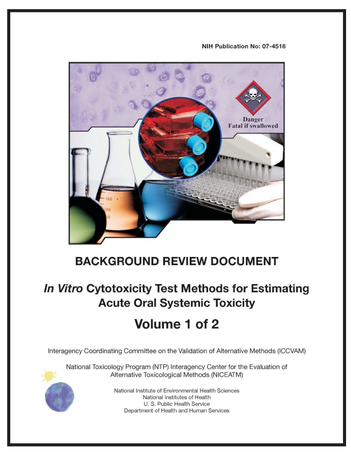
Conclusions from an Independent Scientific Peer Review Panel Meeting

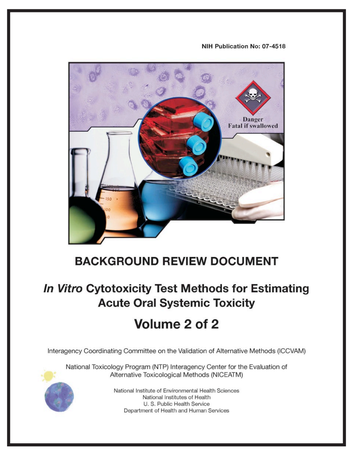
The Panel agreed that the applicable validation criteria had been adequately addressed for using these *in vitro* test methods in a weight-of-evidence approach to determine the starting dose for acute oral systemic toxicity tests. The Panel agreed also that the validation study showed that the two NRU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

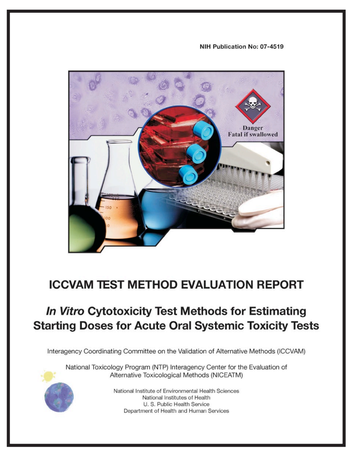
Available at http://iccvam.niehs.nih.gov/methods/acutetox/invidocs/panelrpt/ATpanelrpt.htm.

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

- SACATM commented on the *In Vitro* Acute Toxicity Test Methods BRD and ICCVAM Test Method Evaluation Report and agreed with the future studies recommendations (SACATM 2006)







- The BRD provides data and analyses from the NICEATM/ECVAM validation study for using *in vitro* basal cytotoxicity NRU test methods for estimating acute oral rat toxicity and for estimating starting doses for acute oral toxicity tests with rats (ICCVAM 2006b)
- The Test Method Evaluation Report provides test method recommendations, including usefulness and limitations, protocols, performance standards, and recommendations for future studies (ICCVAM 2006a)

ICCVAM Recommendations: Test Method Uses

1. The 3T3 and NHK NRU cytotoxicity test methods are not sufficiently accurate to predict acute oral toxicity for the purpose of regulatory hazard classification.
2. The 3T3 and NHK NRU cytotoxicity 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. The revised RC millimole regression line based on substances with rat LD_{50} values in mmol/kg and IC_{50} values in mmol/L should be used to determine starting doses for test substances with known molecular weights and high purity. The revised RC regression line based on substances with rat LD_{50} values in mg/kg and IC_{50} values in µg/mL should be used 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.

ICCVAM Recommendations: Test Method Protocols

- ICCVAM recommends using the standardized 3T3 and NHK *in vitro* NRU protocols available at http://iccvam.niehs.nih.gov/.

ICCVAM Recommendations: Test Method Performance Standards

Performance standards communicate the basis by which validated new test methods have been determined to have sufficient accuracy and reliability for specific purposes. Performance standards can then be used to evaluate the accuracy and reliability of other test methods that are based on similar scientific principles and that measure or predict the same biological or toxic effect. The three elements of performance standards are:

- Essential test method components (i.e., unique characteristics of the test method, critical procedural details, and quality control measures)
- Minimum list of 30 reference substances for assessing the accuracy and reliability of the proposed test method
- Accuracy and reliability values that should be achieved by the proposed test method using the 30 reference substances

ICCVAM Recommendations: 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. 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. Additional high quality comparative *in vitro* basal cytotoxicity data should be collected 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 *in vitro* NRU test method. Periodic evaluations of the expanded database should be conducted 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. *In vitro* tests and other methods necessary to achieve accurate acute oral hazard classification should be identified; 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 should be conducted 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*.
4. The *in vivo* database of reference substances used in the NICEATM/ECVAM 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., quantitative structure-activity relationship software).
5. Standardized procedures to collect *in vivo* measurements/observations pertinent to understanding the mechanisms of lethality in future rat acute oral toxicity studies should be used. Such data will support further development of predictive mechanism-based *in vitro* methods.
6. 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 held by industry) should be developed for use in future *in vitro* test method development and validation studies.

Conclusions

- The two standardized *in vitro* cytotoxicity test methods (3T3 and NHK NRU test methods) are not sufficiently accurate to predict acute oral toxicity for the purposes of hazard classification; however, they can be used in a weight-of-evidence approach to determine the starting dose for the current rodent acute oral toxicity protocols.
- These *in vitro* test methods should be considered and used where determined appropriate before acute oral toxicity 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, ICCVAM recommendations 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.

References

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- Institute for *In Vitro* Sciences, Gaithersburg, MD

Recommended neutral red uptake protocols for 3T3 and NHK cells are available at: http://iccvam.niehs.nih.gov/methods/acutetox/invitrocyto/invcytoval.htm