PLANNED FUTURE STUDIES/ACTIVITIES FOR *IN VITRO* CYTOTOXICITY TEST METHODS FOR ESTIMATING STARTING DOSES FOR ACUTE ORAL TOXICITY TESTING

1.0 BACKGROUND/INTRODUCTION

In January 2005, the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the European Centre for the Evaluation of Alternative Methods (ECVAM) completed a multi-laboratory validation study to evaluate animal reduction when using two mammalian cell types for *in vitro* basal cytotoxicity test methods with a neutral red uptake (NRU) cell viability endpoint to determine starting doses for acute oral systemic toxicity test methods. This validation study tested 72 reference chemicals to evaluate the *in vitro* NRU test methods and to determine the accuracy of the *in vitro* NRU test methods for estimating rat oral LD_{50} values (i.e., median lethal dose) across the five United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (UN 2005) categories of acute oral toxicity as well as unclassified toxicities (ICCVAM 2006a). While the use of these *in vitro* methods was shown to be helpful in identifying starting doses that would reduce animal use, the use of a single cytotoxicty test method was not found to be sufficiently predictive to replace the animal-based regulatory tests for hazard classification purposes. The accuracy of the *in vitro* test methods for predicting the correct acute toxicity regulatory hazard classification category (i.e., GHS category) was ~30%.

NICEATM, in conjunction with the Acute Toxicity Working Group (ATWG), prepared a draft background review document (BRD), which described the validation study results for the *in vitro* NRU test methods (ICCVAM 2006a). ICCVAM convened a Peer Review Panel (Panel) to peer review the BRD for errors and omissions, to assess the validation status of the methods, and to determine whether Draft ICCVAM Recommendations for Test Method Uses and Future Studies were supported by the BRD. The Panel agreed with these Draft ICCVAM Recommendations for Future Studies, which included the following:

- Additional efforts should be conducted to identify and study *in vitro* tests and other methods necessary to achieve accurate acute oral hazard classification.

- Standardized procedures to collect information pertinent to an understanding of the mechanisms of lethality should be included in future *in vivo* rat acute oral toxicity testing, and an expert group should be convened to identify the most appropriate *in vivo* endpoints for measurement.
- 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 systemic toxicity tests (e.g., widely available software that uses quantitative structure-activity relationships [QSAR]).
- Additional high quality comparative *in vitro* basal cytotoxicity data should be collected in tandem with *in vivo* rat acute oral toxicity data to supplement the high quality validation database started by this study.
- High quality reference values to expand the chemical database for acute oral toxicity should be collected.
- Additional data should be collected using the 3T3 NRU methods to evaluate its usefulness for predicting the *in vivo* acute oral toxicity of chemical mixtures.

The following sections provide additional details regarding a majority of these proposed studies/activities, with an overall description of each project and their associated primary objectives. Because the final activity listed above (i.e., additional data collection with chemical mixtures) entails a future validation study, it will be addressed in a separate document as an ICCVAM nomination for future study.

2.0 PROPOSED FUTURE STUDIES/ACTIVITIES TO BE CONDUCTED BY NICEATM

2.1 Identification of *In Vitro* Tests and Other Alternative Methods to Achieve Accurate Acute Oral Hazard Classification

2.1.1 Description of Project

The proposed activity is to identify *in vitro* tests and other methods necessary to achieve accurate acute oral hazard classification.

2.1.2 Objective

To identify *in vitro* tests and other alternative methods necessary to achieve accurate acute oral hazard classification and thus assist the development of testing schemes that reduce, refine[1], or replace the use of animals for *in vivo* acute toxicity testing.

2.1.3 Method/Proposed Activity

A concerted effort is underway in Europe known as the A-Cute-Tox Project. Under this project, ECVAM is investigating *in vitro* tests and other methods necessary to achieve accurate acute oral hazard classification. Studies will 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. At this stage, NICEATM and the ATWG will stay informed of ECVAM's progress to determine if any parallel and/or collaborative efforts might be beneficial to the objective outlined above.

[1] A reduction alternative is a new or modified test method that reduces the number of animals required. A refinement alternative is a new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal well-being (ICCVAM 2003).

2.1.4 Draft ICCVAM Recommended Priority: High

2.2 International Workshop to Identify Endpoints Appropriate for Elucidating the Mechanisms of Acute Oral Chemical Toxicity in Rats

2.2.1 Description of Project

The proposed activity is the organization of an international workshop to explore biomarkers (e.g., physiological, biochemical, etc.) that could be added to standardized rat acute oral toxicity tests either to predict lethality or to elucidate the mechanisms of acute chemical toxicity. Identifying the mechanisms of acute toxicity will guide the development of *in vitro* methods that can better predict acute oral toxicity, while identifying biomarkers that provide for earlier prediction of lethality can serve as earlier more humane endpoints for in vivo studies.

2.2.2 Objective

To reduce and potentially replace the use of animals for acute systemic toxicity testing by establishing the mechanisms of acute toxicity to guide the development of *in vitro* methods that can be used to make *in vitro* determinations of *in vivo* mechanisms of acute toxicity.

2.2.3 Method/Proposed Activity

NICEATM, in conjunction with the ATWG, ICCVAM, ECVAM, the Japanese Center for the Validation of Alternative Methods (JaCVAM), and various stakeholders will organize an international workshop to identify and standardize procedures for collecting information pertinent to an understanding of mechanisms of lethality in rats and identifying earlier more humane endpoints. The conclusions and recommendations of the workshop will be published in a workshop report, and the report made widely available for consideration by relevant stakeholders.

2.2.4 Draft ICCVAM Recommended Priority: High

2.3 Evaluation of the Utility of Other Non-Animal Approaches to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests

2.3.1 Description of Project

The proposed activity is to evaluate the utility of other non-animal approaches to estimate starting doses for acute oral systemic toxicity tests by using the established *in vivo* database constructed for the NICEATM/ECVAM validation study.

2.3.2 Objective

To evaluate available *in silico* methods to estimate LD_{50} values for use in predicting starting doses for acute oral toxicity testing thereby assisting the development of non-animal methods that reduce, refine[2], or replace the use of animals for *in vivo* acute toxicity testing.

2.3.3 Method/Proposed Activity

NICEATM will use available quantitative structure-activity relationship [QSAR] software (e.g., TOPKAT, DEREK, MCASE) and compare with the reference LD_{50} values determined for the validation study reference substances to estimate starting doses for acute oral toxicity testing. Results will be used in computer simulations to predict both animal use/reduction in the GHS acute oral toxicity classification system.

2.3.4 Draft ICCVAM Recommended Priority: Medium

2.4 Collection of High Quality Comparative *In Vitro* Basal Cytotoxicity Data in Tandem with *In Vivo* Rat Acute Oral Toxicity Testing

2.4.1 Description of Project

The proposed activity is to supplement the high quality validation database started by the NICEATM/ECVAM validation study by collecting additional high quality comparative *in vitro* basal cytotoxicity data in tandem with *in vivo* rat acute oral toxicity testing.

[2] A reduction alternative is a new or modified test method that reduces the number of animals required. A refinement alternative is a new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal well-being (ICCVAM 2003).

2.4.2 Objective

To supplement the *in vitro/in vivo* database to further characterize the usefulness and limitations of using *in vitro* basal cytotoxicity data as part of a weight-of-evidence approach to estimating doses for *in vivo* testing thereby assisting the development of *in vitro* methods that reduce, refine, or replace the use of animals for *in vivo* acute toxicity testing.

2.4.3 Method/Proposed Activity

NICEATM will solicit data from industry from regulatorily mandated *in vivo* rat acute oral toxicity studies that also include *in vitro* basal cytotoxicity data for the same test substances obtained in tandem with the *in vivo* test (i.e, the *in vitro* test was conducted to estimate the starting dose for the *in vivo* study). Literature searches and searches of publicly available toxicity databases and other secondary sources will also be conducted to identify similar information. *In vivo* testing will not be conducted solely to collect data to assess the usefulness of the *in vitro* basal cytotoxicity test methods. NICEATM will evaluate the data and compare the data to regression models developed in the validation study to further characterize the usefulness and limitations of basal cytotoxicity and other in vitro methods for estimating acute oral toxicity.

2.4.4 Draft ICCVAM Recommended Priority: High

2.5 Collection of High Quality Reference Values to Expand Chemical Database for Acute Oral Toxicity

2.5.1 Additional Background

The NICEATM/ECVAM validation study tested 58 chemicals from the Registry of Cytotoxicity (RC), a database that currently consists of *in vivo* acute toxicity data from rats and mice and *in vitro* cytotoxicity data from multiple cell lines/toxic endpoints for 347 substances (Halle 2003). A regression model constructed from these data was used in the validation study to determine starting doses for computer simulated acute oral systemic toxicity tests (Halle 2003; Spielmann et al. 1999). The validation study effort included the collection of LD_{50} data from literature searches and the development of high quality reference LD_{50} values (i.e., median lethal dose) for the reference substances tested. Rat oral LD_{50} values were identified for 55 of the 58 RC chemicals. During the Panel Meeting referenced in **Section**

1.0, the Panel agreed with the Draft ICCVAM Recommendation that an expanded list of reference substances with high quality rat LD_{50} data should be developed for use in future *in vitro* test method development and validation studies (ICCVAM 2006b).

2.5.2 Description of Project

The proposed activity is the collection and evaluation of rat acute oral lethality (i.e., LD_{50}) data for 442 chemicals included in the Registry of Cytotoxicity (RC).

2.5.3 Objective

To improve the correlation of *in vivo* and *in vitro* data by using high quality *in vivo* data thereby assisting the development of *in vitro* methods that reduce, refine[3], or replace the use of animals for *in vivo* acute toxicity testing.

2.5.4 Method/Proposed Activity

The RC database now has 500 chemicals. NICEATM will identify rat oral LD_{50} data for 442 RC chemicals (500 – 58 chemicals with reference LD_{50} values from the NICEATM/ECVAM validation study). Rat oral LD_{50} data will be identified through literature searches, searches of publicly available toxicity databases, and from secondary sources. NICEATM will evaluate the data retrieved to identify the appropriate LD_{50} values (those using adult laboratory rats and gavage administration in the absence of anesthesia) and calculate new reference values using a geometric mean of the acceptable LD_{50} values.

2.5.5 Draft ICCVAM Recommended Priority: Medium

3.0 REFERENCES

Halle W. 2003. The Registry of Cytotoxicity: Toxicity testing in cell cultures to predict acute toxicity (LD50) and to reduce testing in animals. Altern Lab Anim 31:89-198.

ICCVAM. 2006a. *In Vitro* Acute Toxicity Test Methods Draft Background Review Document. http://iccvam.niehs.nih.gov/methods/invidocs/brdvalstdy.htm. (accessed October 1, 2006)

[3] A reduction alternative is a new or modified test method that reduces the number of animals required. A refinement alternative is a new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal well-being (ICCVAM 2003).

ICCVAM. 2006b. PEER REVIEW PANEL REPORT: The Use of *In Vitro* Basal Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Testing. http://iccvam.niehs.nih.gov/methods/invidocs/panelrpt/ATpanelrpt.htm (accessed October 1, 2006)

Spielmann H, Genschow E, Liebsch M, Halle W. 1999. Determination of the starting dose for acute oral toxicity (LD_{50}) testing in the up and down procedure (UDP) from cytotoxicity data. Altern Lab Anim 27:957-966.