1.0   INTRODUCTION

1.1   Purpose and Scope of this Guidance Document

This guidance document describes how to use *in vitro* cytotoxicity tests to estimate starting doses for acute oral lethality assays. Development of this document was recommended by participants in the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, held October 17-20, 2000, in Arlington, VA, U.S.A. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) convened the workshop to evaluate the validation status of available *in vitro* methods for assessing acute toxicity. A workshop breakout group reviewed the use of *in vitro* screening methods to estimate acute *in vivo* toxicity (i.e., LD_{50} values) and recommended the development of this guidance document which was written by three of its members.

This introduction summarizes background information about the correlation between *in vitro* cytotoxicity and acute lethality, explains the purpose of using *in vitro* cytotoxicity assays to predict starting doses for *in vivo* acute lethality assays, and describes a general approach for evaluating *in vitro* test performance. Chapter 2 describes the basic elements of *in vitro* assays for basal cytotoxicity and describes what investigators should consider before applying the results of these assays to their own situations. Chapter 3 describes the use of the Registry of Cytotoxicity (RC) prediction model to evaluate a candidate cytotoxicity assay. The RC prediction model is a regression analysis of LD_{50} values (the median lethal dose, i.e., the dose that produces lethality in 50% of the animals tested) and *in vitro* cytotoxicity IC_{50} values (i.e., concentration at which cell viability is inhibited by 50%) for 347 chemicals. Chapter 4 describes two candidate tests recommended for use with this method: neutral red uptake (NRU) assays using the mouse fibroblast cell line BALB/c 3T3 and normal human keratinocytes (NHK). Appendix A contains the RC data in spreadsheet format.

Appendix B contains a list of test protocols for basal cytotoxicity from the Scientific Information System (SIS) of the European Centre for the Validation of Alternative Methods (ECVAM). Appendices C-G provide detailed stand-alone protocols for BALB/c 3T3 and NHK NRU assays, additional guidance for implementing the protocols, and a standard template for data collection.

1.2   The Correlation between Basal Cytotoxicity and Acute Lethality

Acute oral toxicity testing is typically the first step in identifying and characterizing the hazards associated with a particular chemical. Information derived from acute toxicity tests in laboratory animals (mainly rodents) is used for several purposes, including: (a) hazard classification and labeling of chemicals in accordance with national and international regulations (e.g., 49 Code of Federal Regulations [CFR] 173; 16 CFR 1500; 29 CFR 1910; 40 CFR 156; Organisation for Economic Co-operation and Development [OECD], 1998a); (b) risk assessments pertaining to the acceptability of acute exposures in the workplace, at home, and upon accidental release; (c) clinical diagnosis, treatment and prognosis of acute human poisoning cases; and (d) design (e.g., dose-setting, identification of potential target organs) of longer-term (e.g., 28-day) toxicity studies. Historically, lethality estimated by the LD_{50} test has been a primary toxicological endpoint in acute toxicity tests, although more detailed toxicological information is sometimes collected. More recently, the conventional test procedure has been modified in various ways to refine and reduce animal use (OECD, 1992, 1996, 1998b). Aiding the acceptance of these alternative methods has been the recognition that the LD_{50} is not a biological constant, but is influenced by many factors (Klaassen and Eaton, 1991). For most purposes, the LD_{50} only needs to be characterized "within an order of magnitude range," according to Klaassen and Eaton (1991).

The use of cell cultures *in vitro* as alternatives to predict acute lethality *in vivo* has been under study for almost 50 years (Pomerat and Leake, 1954; Eagle and Foley, 1956; Smith et al., 1963).

Numerous demonstrations of strong correlations between cytotoxicity *in vitro* and animal lethality *in vivo* exist. (For reviews see Phillips et al., 1990, and Garle et al., 1994). Recently, several major international *in vitro* initiatives have been directed toward reducing the use of laboratory animals for acute toxicity testing (Curren et al., 1998; Ekwall et al., 2000; Ohno et al., 1998a, 1998b, 1998c; Seibert et al., 1996; Spielmann et al., 1999). The status of these initiatives was reviewed at the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, jointly sponsored by the National Institute of Environmental Health Sciences (NIEHS), the National Toxicology Program (NTP), and the U.S. Environmental Protection Agency (U.S. EPA). Conclusions and recommendations from the workshop are published in the *Report of the International Workshop on In Vitro Methods for Assessing Acute Systemic Toxicity* (NIEHS, 2001).

The RC has made a major contribution to the knowledge of the correlation between *in vitro* cytotoxicity and *in vivo* lethality (Halle and Spielmann, 1992; Halle, 1998). The most recent RC compilation (Halle, 1998) contains *in vitro* cytotoxicity information (1,912 single IC_{50} values averaged for each of 347 chemicals [i.e., one IC_{50x} value/chemical from multiple reports in the literature]) paired with 347 *in vivo* acute oral LD_{50} values (mmol/kg) for rats (282 values) or mice (65 values) from the National Institute for Occupational Safety and Health (NIOSH) Registry of Toxic Effects of Chemical Substances (RTECS). (See Appendix A for the RC data.) Criteria for data to be included in the RC database are fully described by Halle (1998) and briefly described by Spielmann et al. (1999). The combination of rat and mouse data was justified, since it yielded a regression that was not significantly different from those obtained with either rat data or mouse data alone. The RC data clearly demonstrate a strong relationship between *in vitro* cytotoxicity and acute lethality in rodents (Figure 1).
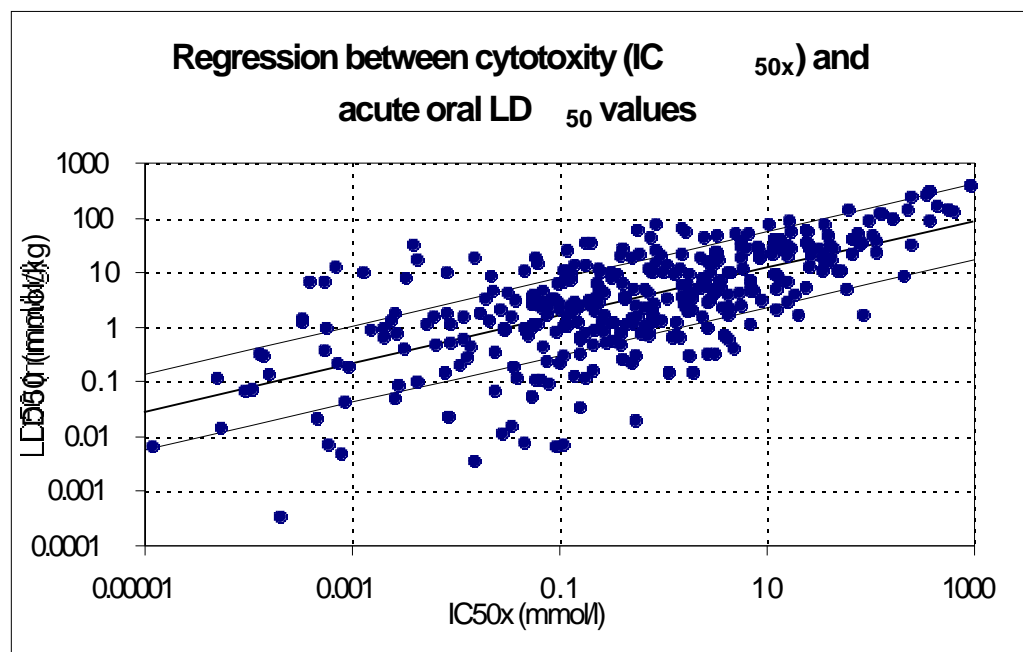
Figure 1. Registry of Cytotoxicity regression between cytotoxicity (IC_{50x}) and rodent acute oral LD_{50} values for 347 chemicals.
The heavy line shows the fit of the data to a linear regression model, log (LD_{50}) = 0.435 x log (IC_{50x}) + 0.625; r=0.67. The other lines show the empirical $F_G = \pm$ log 5 acceptance interval for the prediction model (Spielmann et al., 1999), which is based on the anticipated precision of LD_{50} values from rodent studies Halle (1998).

1.3 *In Vitro* Determination of Starting Dose for *In Vivo* Tests

Spielmann et al. (1999) have proposed – as an initial step – that the relationship found with the RC regression be used with *in vitro* data to predict starting doses for subsequent *in vivo* acute lethality assays. They suggest that before initiating any *in vivo* lethality assay for a chemical, an *in vitro* cytotoxicity assay should be conducted to estimate the LD_{50} for that chemical. The LD_{50} predicted from the RC regression equation should then be used to choose the most appropriate starting dose for the *in vivo* assay. The LD_{50} estimate from the RC regression is based on molar amounts of the chemical,

specifically a value in mmol/kg. This value must first be converted to a weight measurement expression, such as mg/kg, before using conventional LD_{50} dosing calculations. Using this estimate should make the conduct of *in vivo* assays much more efficient and result in savings both in the number of animals and in the amount of time required to obtain the final results. The workshop report (NIEHS, 2001) includes a discussion of the potential number of animals saved, based on several currently available *in vivo* protocols, e.g., protocols that use new sequential dosing methods such as the Acute Toxic Class method (ATC, OECD TG 423; OECD, 1996) and the Up-and-Down Procedure (UDP, OECD TG 425; OECD, 1998b). In these tests, using the

fewest animals possible depends upon the correct choice of starting dose since, on average, the number of consecutive dosing steps is minimal if the starting dose is close to the true toxicity class (ATC) or to the true LD_{50} (UDP).

1.4   Determination of *In Vitro* Test Performance Characteristics

Before the results obtained with any *in vitro* cytotoxicity test are used with the RC regression to generate an expected LD_{50} value, the performance characteristics of the new method should be determined and compared with those of the RC information as discussed in Section 3.1. Section 3.2 suggests a set of reference chemicals that should be tested with the candidate *in vitro* cytotoxicity method. The resultant regression line should then be compared with that of the current RC regression line. If the line falls within the $\pm$ log 5 boundaries indicated in Figure 1, then the regression parameters of the RC may be used to predict the LD_{50} starting dose.   Section 3.3 describes experimental trials, using two different cell types, performed after the workshop with the set of recommended reference chemicals. These experimental trials are included as examples of how to determine test performance for any *in vitro* test for basal cytotoxicity and to confirm the applicability of the test for use with the RC regression.