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**DRAFT**

**Validation Study to Evaluate the Usefulness of Two Cytotoxicity  
Methods for Estimating Acute Systemic Toxicity**

**Study Design**

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**National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative  
Toxicological Methods (NICEATM)**

**National Institute of Environmental Health Sciences (NIEHS)  
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## 1. INTRODUCTION

Acute oral toxicity testing is typically the initial step in identifying and characterizing the hazards associated with a particular chemical. Historically, lethality estimated by the LD<sub>50</sub> test has been a primary toxicological endpoint in acute toxicity tests, although more detailed toxicological information is sometimes collected. Information derived from acute oral toxicity tests, which use laboratory animals, is employed for several purposes, including:

- Hazard classification and labeling of chemicals in accordance with national and international regulations (e.g., 49 CFR 173; 16 CFR 1500; 29 CFR 1910; 40 CFR 156; OECD, 2001);
- Risk assessments pertaining to the acceptability of acute exposures in the workplace, at home, and upon accidental release;
- Clinical diagnosis, treatment and prognosis of acute human poisoning cases; and
- Design of longer-term (e.g., 28-day) toxicity studies (dose-setting, identifying potential target organs).

### 1.1 Reduction, Replacement, and Refinement

The conventional LD<sub>50</sub> test procedure has been modified in various ways to refine and reduce animal use (OECD, 1992, 1996, and 1998). Other initiatives directed toward reducing and replacing the use of laboratory animals for acute toxicity testing include the use of *in vitro* cytotoxicity assays to predict the results of acute *in vivo* lethality tests. As reviewed by Phillips et al. (1990) and Garle et al. (1994), a number of strong correlations between cytotoxicity *in vitro* and animal lethality *in vivo* have been demonstrated. The status of several major international *in vitro* initiatives directed toward reducing the use of laboratory animals for acute toxicity testing was reviewed in October 2000, at the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity (ICCVAM, 2001a). The workshop participants concluded that none of the proposed *in vitro* models had been evaluated in any formal studies for reliability and relevance, and that their usefulness and limitations for generating information to meet regulatory requirements for acute toxicity testing had not been assessed.

## **1.2 Cytotoxicity Assays**

One of the workshop recommendations for reducing and refining the use of animals for lethality assays in the near-term was the publication of guidance for using *in vitro* cytotoxicity assays to estimate starting doses for acute oral lethality assays. A *Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses for Acute Toxicity* (ICCVAM, 2001b), which provides details and examples on how to implement such an approach, has been published. The *Guidance Document* explains how to use IC<sub>50</sub> values (i.e., the concentration estimated to affect the endpoint in question by 50%) from cytotoxicity studies to calculate *in vivo* starting doses for acute oral lethality assays (Spielmann et al., 1999). The approach uses a regression formula derived from mean IC<sub>50</sub> values (i.e., IC<sub>50x</sub>) and acute oral LD<sub>50</sub> data for 347 chemicals from the Registry of Cytotoxicity (RC), which was compiled by ZEBET (German Centre for the Documentation and Validation of Alternative Methods) (Halle 1998). The RC is a database of acute oral LD<sub>50</sub> data from rats and mice (taken from the NIOSH Registry of Toxic Effects of Chemical Substances [RTECS]) and IC<sub>50x</sub> values (i.e., mean IC<sub>50</sub> of several published values) of chemicals and drugs from *in vitro* cytotoxicity assays (Halle, 1998; Halle and Spielmann, 1992). The RC linear regression analysis between rodent LD<sub>50</sub> values and IC<sub>50x</sub> values demonstrates a strong relationship between *in vitro* cytotoxicity and acute lethality in rodents ( $r = 0.67$ ). [NOTE: See Section 2 of ICCVAM (2001a) for more information on the RC].

## **2. STUDY OBJECTIVES**

The proposed validation study will further evaluate the usefulness of *in vitro* basal cytotoxicity assays for predicting *in vivo* acute systemic toxicity. The major objectives are:

- To further standardize and optimize two *in vitro* cytotoxicity protocols in order to maximize intra- and inter-laboratory reproducibility.
- To assess the accuracy of two standardized *in vitro* cytotoxicity assays for estimating rodent oral LD<sub>50</sub> values and human lethal concentrations across the five Globally Harmonised System (GHS; OECD 2001) categories of acute oral toxicity as well as unclassified toxicities.

- To estimate the reduction and refinement (i.e., reduced deaths) in animal use that would result from using *in vitro* cytotoxicity assays to estimate starting doses for *in vivo* acute toxicity testing.
- To generate a high quality *in vitro* database that can be used to support investigation of other methods necessary to improve the accuracy of *in vitro* assessments of acute systemic toxicity.

### 3. PROPOSED STUDY DESIGN

#### 3.1 Prediction Models

The RC prediction model will be used to assess the relevance of two standardized *in vitro* cytotoxicity assays for estimating rodent oral LD<sub>50</sub> values. A linear regression analysis of the IC<sub>50</sub> data generated in Phases I and II for each cell type and the LD<sub>50</sub> data from RTECS (as used in the RC) will be performed. If the regression formula is not significantly different from the RC prediction model, then the RC prediction model will be used to predict starting doses for LD<sub>50</sub> assays:

$$\log(\text{LD}_{50} [\text{mmol/kg}]) = 0.435 \times \log(\text{IC}_{50} [\text{mmol/l}]) + 0.625 \quad (r = 0.67)$$

The prediction model will then be refined by the use of high quality rodent LD<sub>50</sub> values to again determine whether the regression: (a) is significantly different from the RC prediction model, and (b) significantly improves in correlation. The high quality rodent LD<sub>50</sub> data will be selected prior to chemical testing.

To date, a human prediction model based on a single *in vitro* endpoint has not been reported. The feasibility of developing such a model with either 3T3 fibroblast or normal human keratinocytes (NHK) data will be evaluated by using the *in vitro* results for the 12 chemicals tested in Phases I and II and corresponding human sublethal and lethal blood concentrations (MEMO database; Ekwall et al. 1998). If it is possible to develop a preliminary human prediction model on the basis of the data obtained, *in vitro* data for Phase III chemicals will then be used to assess its predictive capacity.

### 3.2 Selection of Cytotoxicity Methods

The proposed *in vitro* cytotoxicity assays are the neutral red uptake (NRU) assay using mouse fibroblast (BALB/c) 3T3 cells and the NRU assay using normal human keratinocytes (NHK). These assays are suggested in the *Guidance Document* for the purpose of obtaining cytotoxicity information to predict starting doses for LD<sub>50</sub> assays. The BALB/c 3T3 NRU (link to <http://iccvam.niehs.nih.gov/methods/invidocs/phIIIprot/3t3phIII.pdf>) has been highly reproducible in several validation studies. NHK NRU (link to <http://iccvam.niehs.nih.gov/methods/invidocs/phIIIprot/nhkphIII.pdf>) has been less frequently used, but has also yielded good results in validation studies. In addition, both cell types are easily obtainable from commercial sources. More information on the repeatability and reliability of the 3T3 and NHK NRU assays is provided in Section 4 of the *Guidance Document* (ICCVAM, 2001b).

Clemenson et al. (1996) have shown that mammalian cell types produce similar, but not exactly the same, basal cytotoxicity results as human cell lines ( $R^2 = 0.77$  for 30 chemicals and  $R^2 = 0.93$  after the exclusion of one chemical). Thus, a rodent cell line, such as BALB/c 3T3, is expected to predict rodent lethality somewhat better than a human cell line. A human cell line is chosen for this study so as to develop an historical database of human cytotoxicity on which to build future efforts for predicting acute toxicity in humans. [NOTE: See Section 2 of the *Guidance Document* (ICCVAM, 2001b) for information on the preferred elements of a standard test for basal cytotoxicity.]

### 3.3 Chemical Selection

The chemicals proposed for the validation study were selected to represent a wide range of toxicity from highly toxic (LD<sub>50</sub> ≤ 5 mg/kg) to nonclassified toxicity (LD<sub>50</sub> > 5000 mg/kg) (OECD, 2001). The existence of human exposure and toxicity data, or at least the potential for human exposure to occur, was a major criterion for chemical selection.

Seventy-two chemicals were selected for testing, with 12 chemicals from each of the six acute oral toxicity classification groups of the GHS (OECD, 2001):

<u>Class</u>	<u>LD<sub>50</sub></u>
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Class 1	$\leq 5$ mg/kg
Class 2	$> 5 - \leq 50$ mg/kg
Class 3	$> 50 - \leq 300$ mg/kg
Class 4	$> 300 - \leq 2000$ mg/kg
Class 5	$> 2000 - \leq 5000$ mg/kg
Unclassified	$> 5000$ mg/kg

### **3.3.1 Candidate Chemical Database**

Chemicals were selected from a candidate database of 116 chemicals (see file SelectedAlternateChemicals.xls) that was compiled in the following manner:

- Started with the 50 Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) chemicals since these chemicals have good human exposure and toxicity data that have been collected and analyzed by Ekwall et al.
- Added MEIC chemicals 51-65 at the request of ECVAM. These included carbamazepine, chloral hydrate, diquat, meprobamate, pentobarbital, strychnine, valproic acid, glutethimide, maprotiline, colchicine, disopyramide, diphenhydramine, haloperidol, methadone, and procainamide.
- Added 13 chemicals nominated by U.S. EPA Office of Pesticide Programs: fenpropathrin, endosulfan, bromoxynil (phenol), fipronil, carbaryl, rotenone, metaldehyde, molinate, 1,3-dichloropropene, dichlorvos (DDVP), chlorpyrifos, sodium arsenite, triphenyltin hydroxide, and boric acid. Cycloheximide, acrolein, and pentachlorophenol were also nominated, but were already on the candidate list.
- Added chemicals with the highest frequency of human toxic exposures from Poison Control Centers (Litovitz et al., 2000). The top five chemicals for total human toxic exposures were hypochlorite, acetaminophen, ethanol, diphenhydramine, and isopropanol. The top five chemicals for childhood exposures were the same except that oxalate replaced ethanol. Only hypochlorite and diphenhydramine were added to the list since the other chemicals were already on the MEIC list.
- Added 11 chemicals from the *Guidance Document* (ICCVAM, 2001b) that are recommended for qualifying cytotoxicity assays and that have been tested using the new recommended protocols for BALB/c 3T3 NRU and the NHK NRU assays. These added chemicals are in

every toxicity class except for  $LD_{50} \leq 5$  mg/kg, and included: sodium dichromate dihydrate, cadmium chloride, p-phenylenediamine, DL-propranolol HCl, trichlorfon, ibuprofen, nalidixic acid, salicylic acid, antipyrine, dimethylformamide, and glycerol.

- Added chemicals of current interest to the National Toxicology Program (NTP), which also signifies significant human exposure potential. Four hepatotoxins to be tested by the NTP Center for Toxicogenomics were added: furfural, methyl eugenol, and methylphenidate. Acetaminophen is also to be tested by the Center, but was already included in the candidate list because it was used in the MEIC study. Chromium (VI) is currently of interest to the NTP due to potential drinking water exposures and is represented by sodium dichromate dihydrate, which had already been added to the candidate list.
- Several chemicals with  $LD_{50} > 5000$  mg/kg that have been evaluated by the NTP were added to increase the number of chemicals in that toxicity range. These chemicals include: dibutyl phthalate, 5-aminosalicylic acid, propylparaben, gibberellic acid, and diethyl phthalate. It is important to include chemicals of low toxicity since Spielmann et al. (1999) indicate that 75% of 1115 industrial chemicals submitted to the Federal Institute for Health Protection of Consumers and Veterinary Medicine in Germany since 1982 had  $LD_{50}$ s over 2000 mg/kg.
- Ten of the 11 very toxic (i.e.,  $LD_{50} \leq 5$  mg/kg) chemicals from the RC were added to increase the number of chemicals in that category. These chemicals are: triethylenemelamine, busulphan, cycloheximide, disulfoton, parathion, aminopterin, phenylthiourea, epinephrine bitartrate, and aflatoxin b1.
- Lactic acid, citric acid, and acetonitrile from the NTP database and HPV list, and trichloroacetic acid from NTP database were added to increase the number of chemicals in the  $LD_{50} > 2000 - \leq 5000$  mg/kg category.
- Tert-butylamine, 2,4-dinitrophenol, and acrolein from both the NTP database and HPV list were added to increase the number of chemicals in the  $LD_{50} > 5 - \leq 50$  mg/kg category. Sodium selenate, from the NTP database, was added to increase the number of chemicals in the  $LD_{50} \leq 5$  mg/kg category to 12.

### **3.3.2 Criteria for Selecting Chemicals**

The criteria for selecting chemicals to test from the list of 116 candidate chemicals are:

- Twelve chemicals to represent each of the five toxicity categories in the GHS classification of oral toxicity and unclassified chemicals (OECD, 2001);
- MEIC chemicals;
- Nonvolatile;
- Not restricted by the U.S. Drug Enforcement Agency (DEA).
- Corrosives are given a lower testing priority than noncorrosives since regulatory guidelines state that corrosive chemicals should not be tested in animals for acute toxicity. U.S. Department of Transportation (DOT) Packing Group (PG) designations were used to determine relative Corrosivity (although toxicity is also considered in assigning PGs to chemicals). Chemicals in DOT PG I are lowest in testing priority. Chemicals in DOT PG II and III are higher priority.

If more than one chemical in a GHS category meets the above criteria, chemicals were chosen so as to represent the range of toxicity in each GHS category, and/or so that the entire set of chemicals has no more chemicals that were more than half log (i.e., 0.699) from the RC regression (proportionally) than the entire RC database (referred to as “RC outliers” by the authors of the RC database).

### ***3.3.3 Selection Process for GHS Categories***

The selected and candidate chemicals are listed in file SelectedAlternateChemicals.xls. The selection process for each GHS category is summarized below.

**Class 1 ( $LD_{50} \leq 5$  mg/kg).** Ten of the 12 candidate chemicals are in the RC and two of the 11 chemicals are MEIC chemicals. Since there were only 12 candidate chemicals in this class, all 12 were intended for testing. However, since the cost of aflatoxin B1 was prohibitive, it was moved to the alternate list. Physostigmine was then added to the database as one of the chemicals to be tested.

**Class 2 ( $LD_{50} > 5 - < 50$  mg/kg).** Fifteen candidate chemicals were proposed in this class. Three chemicals were excluded. Acrolein was excluded because of its volatility. t-Butylamine and 2,4-dinitrophenol were excluded because they were not MEIC chemicals and because they

were in the most corrosive packing groups. Of the 12 chemicals included, six are MEIC chemicals and five of these are Evaluation-Guided Development on *In Vitro* Tests (EDIT) chemicals (Ekwall et al. 1999). EDIT chemicals, which should have a rich cytotoxicity and human toxicity database, were chosen to evaluate batteries of *in vitro* tests to predict acute human toxicity. No proposed MEIC chemicals in this class were excluded.

**Class 3 ( $LD_{50} > 50 - < 300$  mg/kg).** Twenty-six chemicals were proposed for this class.

Seventeen were MEIC chemicals. Three DEA Schedule II controlled substances, which were also MEIC chemicals, were excluded: amphetamine sulfate, pentobarbital, and methadone.

Eleven of the 12 chemicals selected were MEIC chemicals. One non-MEIC chemical, cadmium chloride, was selected in preference to three remaining MEIC chemicals because it has good rodent  $LD_{50}$  data as evidenced by its use in an acute toxic class methodology validation (Schlede et al. 1995). In addition, two of the three remaining MEIC chemicals were not selected because they were “RC outliers”.

**Class 4 ( $LD_{50} > 300 - < 2000$  mg/kg).** Thirty-eight chemicals were proposed for this class.

Twenty-eight are MEIC chemicals. The 12 chemicals selected are all MEIC chemicals in the less corrosive packing group, PG III, and were chosen to represent, as much as possible, the entire range of toxicity. Three of these chemicals are also EDIT chemicals.

Several MEIC chemicals were excluded. Warfarin and malathion were excluded because they are in the most corrosive packing group (PG I). Diazepam was excluded because it is a DEA Schedule II controlled substance and dichloromethane and chloroform were excluded because they are relatively volatile. Other chemicals designated as somewhat corrosive by PG II or PG III were viewed as low priority.

**Class 5 ( $LD_{50} > 2000 < 5000$  mg/kg).** Twelve chemicals were proposed for this class, so all 12 will be tested. Six are MEIC chemicals and two of these are also EDIT chemicals.

**Unclassified ( $LD_{50} > 5000$  mg/kg).** Twelve chemicals were proposed for this class, so all 12 are selected for testing. Five are MEIC chemicals and two of these are also EDIT chemicals.

### 3.3.4 MEIC and EDIT Chemical GHS Classifications

The candidate and selected MEIC and EDIT chemicals are distributed according to GHS class as described in the following table. The table shows that 42 of the 72 selected chemicals are MEIC chemicals and 17 are EDIT chemicals.

**Table 1. MEIC<sup>1</sup> and EDIT<sup>2</sup> Chemical Distribution by GHS<sup>3</sup> Oral Toxicity Category**

<b>GHS Category</b>	<b>Selected Chemicals/ Candidate Chemicals</b>	<b>Selected MEIC/ MEIC Candidates</b>	<b>Selected EDIT/ EDIT Candidates</b>
Category 1	12/13	2/2	1/1
Category 2	12/15	6/6	5/5
Category 3	12/26	11/17	4/5
Category 4	12/38	12/29	3/5
Category 5	12/12	6/6	2/2
Unclassified	12/12	5/5	2/2
<b>Total</b>	<b>72/116</b>	<b>42/65</b>	<b>17/20</b>

<sup>1</sup>MEIC: Multicentre Evaluation of *In Vitro* Cytotoxicity (Ekwall et al., 1998)

<sup>2</sup>EDIT: Evaluation-guided Development of New In Vitro Cytotoxicity Tests (Ekwall et al., 1999)

<sup>3</sup>GHS: Globally Harmonised System of acute oral toxicity hazard classification (OECD, 2001).

Table 2 summarizes the number of RC chemicals in each GHS oral toxicity category, the number of RC chemicals considered as candidates for this study, the number of RC chemicals selected for testing, the number of “outliers” in the RC, and the number of RC “outliers” selected for testing. Although the percentage of “outliers” for the selected chemicals in most GHS categories is similar to the RC, the total percentage of RC “outliers” identified in the set of selected chemicals (i.e., 38%) is greater than the total percentage of outliers in the RC (i.e., 27%).

**Table 2. Distribution of Registry of Cytotoxicity (RC) Chemicals and “Outliers”<sup>1</sup> by Chemical Class**

Registry of Cytotoxicity		Candidate and Selected Chemicals		
GHS <sup>2</sup> Category	“Outliers”/Total Chemicals	Candidate Chemicals	Selected RC Chemicals/ RC Candidates	Selected RC “Outliers”/Selected RC Chemicals
Category 1	9/11 (82%)	13	9/10	8/9 (89%)
Category 2	15/26 (58%)	15	8/10	4/8 (50%)
Category 3	24/70 (34%)	26	10/17	4/10 (40%)
Category 4	14/139 (10%)	38	8/28	0/8 (0%)
Category 5	12/57 (21%)	12	10/10	0/10 (0%)
Unclassified	20/44 (45%)	12	11/11	5/11 (45%)
Total	94/347 (27%)	116	56/86	21/56 (38%)

<sup>1</sup>Chemicals falling outside the empirical  $F_G = \pm \log 5$  acceptance interval for the RC prediction model (Halle, 1998).

<sup>2</sup>GHS: Globally Harmonised System of acute oral toxicity hazard classification (OECD, 2001)

### 3.4 Phase I, II, and III Activities

The laboratory work will proceed in three phases. All work will be in accordance with Good Laboratory Practice (GLP) Standards (U.S. Food and Drug Administration, Title 21 CFR Part 58; Environmental Protection Agency, Title 40 CFR Part 160). If a non-GLP lab is to be used, the lab must adhere to the GLP principles put forth in the test method protocols and statement of work. Documentation and accountability must be equivalent to GLP requirements. Aspects of noncompliance should be documented prior to conduct of the study. A lead lab will be designated for each cytotoxicity method to assist the Management Team with troubleshooting laboratory challenges.

#### 3.4.1 Phase I

Phase I is the laboratory training phase. Based on the test method protocols and suggested statement of work provided by NTP, the laboratories will develop SOPs for the two NRU cytotoxicity assays. During Phase Ia, an historical database for the positive control chemical,

sodium laurel sulfate (CASN 151-21-3) will be established by performing 10 concentration-response assays (two assays/day) so as to determine acceptable response limits. Acceptable response limits for the assays will be the mean  $IC_{50S}$  +/- two standard deviations. The Management Team will evaluate the repeatability and reproducibility of the positive control data and if there is excessive variation within or among labs, the lead lab for each method will assist in determining the cause and appropriate actions necessary to reduce the variation. The Management Team will determine when testing should proceed to Phase Ib.

During Phases Ib and II the laboratories will test a total of 12 chemicals of varying cytotoxicities. There will be two chemicals to represent each of the six toxicity categories. The chemicals will be coded so that the test labs will not know the identities.

The criteria for choosing Phase I and II chemicals from the list of proposed chemicals are:

- Log  $LD_{50}$  (mmol/kg) must be within 0.699\* of the RC prediction (i.e., NOT an “RC outlier”) as recommended by the *Guidance Document* (ICCVAM, 2001b) for evaluating a cytotoxicity test for use with the RC prediction model;
- Two chemicals to represent each of the six toxicity categories in GHS classification of oral toxicity (OECD, 2001);
- Preferably be MEIC chemicals;
- Preferably not corrosive, volatile, or controlled by the U.S. DEA.

If more than one chemical in a GHS category meets the above criteria, chemicals are chosen so as to be closest to the RC prediction and/or to represent the range of toxicity in each GHS category.

Limited testing is proposed in Phase Ib so that any laboratory and/or protocol refinements that need to be made are incurred at a minimum expense. Only three coded chemicals will be tested so as to limit the expense of repeat testing, if required. For Phase Ib, one chemical each in GHS Classes 2, 4 and “unclassified” will be tested. Eight concentrations of each chemical will be tested using six replicates per concentration. This concentration-response experiment for each

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\* This percentage factor characterizes the dosage range of  $LD_{50}$  deviating from the regression line by the minimum and maximum residuals  $\leq \pm 0.699$ .

chemical shall be performed three times on three different days. These chemicals are the training set to assure that the laboratories obtain sufficiently similar results. The Management Team will evaluate the repeatability and reproducibility of the data and if there is excessive variation within or among labs, the lead lab for each method will assist in determining the cause and appropriate actions necessary to reduce the variation. The correspondence of the results with RC data will also be evaluated. The Management Team will determine when to proceed to Phase II.

### **3.4.2 Phase II**

The second phase of the laboratory study is the laboratory qualification phase. The remaining nine chemicals selected for Phases I and II will be tested in Phase II to assure that any corrective actions taken in Phase I have achieved the desired results. Chemicals will be coded and tested in both *in vitro* cytotoxicity assays in a concentration-response fashion with three replicate assays. The chemicals to be tested include representatives from all six GHS oral toxicity classes.

The Management Team will analyze the results, and if there is excessive variation within or among labs, the lead lab for each method will assist in determining the cause and appropriate actions necessary to reduce the variation. Additional details may be added to the test method protocol as necessary, and testing repeated until acceptable proficiency is achieved. The Management Team will decide when to proceed to Phase III.

### **3.4.3 Phase III**

The third phase requires testing 60 of the selected chemicals in the same manner as in Phase II (i.e., coded and in two *in vitro* cytotoxicity assays in a concentration-response fashion with three replicate assays).

## **3.5 Chemical Distribution**

Certificates of analysis provided by chemical suppliers will verify the identity and purity of the chemicals to be tested. Chemical samples will be packaged for distribution so as to minimize damage during transit and will be shipped to each laboratory according to proper regulatory procedures. With the exception of the positive control chemical, all chemicals will be shipped

coded so as to conceal their identities. Chemicals will be accompanied by data sheets giving a minimum of essential information, including color, odor, physical state, weight or volume of sample, and storage requirements. Participating laboratories shall be instructed to treat all blind chemicals as very hazardous and potentially carcinogenic. Health and safety information such as first-aid and spill procedures shall be sealed in a separate envelope addressed to the laboratory safety officer. The safety officer will retain the sealed health and safety information package and pass the samples to an experimenter. If the laboratory must open the health and safety package, the safety officer shall notify the Management Team. At the end of the study, the safety officer shall return the unopened health and safety package to the management team. Chemical codes will be broken after the management team receives all blind chemical test results.

### **3.6 Data Analyses**

Labs will calculate IC<sub>20</sub>s, IC<sub>50</sub>s, and IC<sub>80</sub>s in µg/ml and submit to the study Management Team. The Management Team will evaluate intra- and interlaboratory reproducibility for Phases I and II by ANOVA and may recommend revision of the SOPs so as to improve repeatability and reproducibility. IC<sub>50</sub> data will also be compared to published IC<sub>50</sub> values as a check for consistency. The Management Team will evaluate intra- and interlaboratory reproducibility for Phase III and convert IC<sub>50</sub>s to mmol/l to use the values for the prediction of starting doses for LD<sub>50</sub> assays using the RC prediction model:

$$\log (\text{LD}_{50} [\text{mmol/kg}]) = 0.435 \times \log (\text{IC}_{50} [\text{mmol/l}]) + 0.625$$

For Phases I and II chemicals, a regression will be calculated for each lab using the average of the three IC<sub>50</sub>s for each chemical and then all lab results will be combined for a regression analysis. These regressions will be compared to the RC prediction model. If they are similar to the RC prediction model, the model will be used to predict starting doses for all chemicals tested (if the protocols are not changed between phases).

Using simulation modeling, the Management Team will calculate the reduction in the use of animals using the predicted starting dose vs a standard starting dose. A simulation model for each chemical for each lab will calculate how many animals are used and killed to compare

results using cytotoxicity data to determine starting dose vs the default method of determining starting dose.

To determine the best cell type for the prediction of starting dose, the reduction in animal use for each cell type will be compared. Another way to determine which cell type is best is to compare the predicted LD<sub>50</sub> from the NHK and 3T3 cell types for each chemical to the actual LD<sub>50</sub> and use a rank test to determine whether one cell type is significantly more predictive than the other. In addition, the Management Team will evaluate the quality of existing LD<sub>50</sub> data in an effort to refine the prediction model with the best quality data available. Then the same analyses to determine the reduction in animal use and the best cell type for the prediction will be performed.

### **3.6 Scheduling**

The final study design and award of contracts were completed in June 2002. Testing began in July 2002 and is expected to be completed by June 2004. Management Team data analysis and report writing will be done June-December 2004. See StudyTimeline.doc for a more detailed schedule.

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