APPENDIX D

Background Document for Workshop Participants

This document was provided in the Background Materials and Supplemental Information Notebook for the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity [Section I].

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

October 17-20, 2000
Hyatt Regency Crystal City
Arlington, VA, U.S.A.

Background Document

National Toxicology Program (NTP)
Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)
National Institute of Environmental Health Sciences
Research Triangle Park, NC 27709

September 2000

TABLE OF CONTENTS

LIST OF TABLES

LIST OF APPENDICES ***[Note: TABs refer to the Background Materials Document binder provided for the Workshop]***

LIST OF ABBREVIATIONS AND ACRONYMS

ATC	Acute Toxic Class Method
ATP	Adenosine triphosphate
CFN	Swedish National Board for Laboratory Animals
CPSC	Consumer Product Safety Commission
CTLU	Cytotoxicology Laboratory, Uppsala
DOT	Department of Transportation
ECVAM	European Center for the Validation of Alternative Methods
ECITTS	ERGATT/CFN Integrated Toxicity Testing Scheme
EDIT	Evaluation-Guided Development of *In Vitro* Tests
EPA	Environmental Protection Agency
ERGATT	European Research Group for Alternatives in Toxicity Testing
FDP	Fixed Dose Procedure
IC_{50}	Inhibitory Concentration - the concentration of a material estimated to reduce the biological endpoint (e.g., cell growth, ATP levels) being evaluated as a measure of toxicity by 50%.
$IC_{50}\ \bar{x}$	Mean of two or more IC_{50} values
ID_{50}	Model body doses that affect the endpoint in question by 50%.
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
i.p.	Intraperitoneal
LD_{50}	Dose producing lethality in 50% of the animals
LDH	Lactate dehydrogenase
MEIC	Multicenter Evaluation of *In Vitro* Cytotoxicity
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NLM	National Library of Medicine
NRU	Neutral Red Uptake
NTP	National Toxicology Program
OECD	Organization for Economic Cooperation and Development
OSHA	Occupational Safety and Health Administration
PBBK	Physiologically Based Biokinetic
PBPK	Physiologically Based Pharmacokinetic
QSAR	Quantitative Structure Activity Relationship
TG	Test Guideline
UDP	Up-and-Down Procedure
U.S.	United States
ZEBET	The German Center for Documentation and Evaluation of Alternative Methods to Animal Experiments

1.0 Preface

[Note: This document has been modified for inclusion in this *In Vitro* Workshop Report.]

This document provides background information to facilitate discussion at the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, to be held on October 17-20, 2000, at the Hyatt Regency Crystal City in Arlington, VA, U.S. Undoubtedly, other information on this topic exists. Participants are encouraged to bring relevant information to the attention of NICEATM for consideration at the workshop. The Introduction (**Section 2**) provides information on acute toxicity, the uses of acute toxicity testing data by regulatory authorities and clinicians, and the U.S. and OECD *in vivo* test methods currently used for assessing acute toxicity. **Section 3** discusses general strategies for using *in vitro* test methods to assess *in vivo* toxicity, including the use of quantitative structure activity relationships (QSAR). Sections 4 - 7 provide information relevant to each of the four Workshop Breakout Groups: Breakout Group 1: *In Vitro* Screening Methods for Assessing Acute Toxicity; Breakout Group 2: *In Vitro* Methods for Assessing Acute Toxicity –Toxicokinetic Determinations; Breakout Group 3: *In Vitro* Methods for Assessing Acute Toxicity - Specific Organ Toxicity and Mechanisms; and Breakout Group 4: Chemical Data Sets for Validation of *In Vitro* Toxicity Tests, including lists of relevant publications. Information on potentially useful general databases is provided in **Section 8**, a complete list of references cited is provided in **Section 9**, and a Glossary in **Section 10.**

2.0 Introduction

Acute toxicity testing in animals is typically the initial step in the assessment and evaluation of the health effects characteristics of a test substance, and its primary purpose is to provide information on potential health hazards that may result from a short-term exposure (OECD, 1987). This information is used to properly classify and label materials as to their toxicity in accordance with national and international regulations and guidelines. An internationally harmonized system has also been proposed (OECD, 1998a). Another purpose of such studies is to help guide the design of longer-term health effects studies. Acute oral toxicity is defined as the adverse effects occurring within a short time (i.e., up to a few weeks) of oral administration of a single dose of a substance or multiple doses given within 24 hours (OECD, 1987). It is typically presented as an LD_{50} value, which is a statistically derived estimate of the single dose of a substance that can be expected to cause death in 50 percent of the treated animals. LD_{50} data are expressed in terms of amount of the test substance per unit body weight of the animal (e.g., g or mg/kg). Potential target organ toxicity, toxicokinetic parameters, and dose-response relationships may also be evaluated in acute toxicity studies. While animals are currently used to evaluate acute toxicity, recent studies suggest that *in vitro* methods might be helpful in predicting acute toxicity and in estimating *in vivo* toxic chemical concentrations.

Studies by Spielmann et al. (1999) suggest that *in vitro* cytotoxicity data may be useful in identifying an appropriate starting dose for *in vivo* studies, and thus may potentially reduce the number of animals necessary for such determinations. Other studies (e.g., Ekwall et al., 2000) have indicated an association between chemical concentrations leading to *in vitro* cytotoxicity and human lethal blood concentrations. A program to estimate toxicokinetic parameters and target organ toxicity utilizing *in vitro* methods has been proposed that may provide enhanced predictions of toxicity, and potentially reduce or replace animal use for some tests (Ekwall et. al., 1999). However, many of the necessary *in vitro* methods for this program have not yet been developed. Other methods have not been evaluated for reliability and relevance, and their usefulness and limitations for generating information to meet regulatory requirements for acute toxicity testing have not been assessed.

The International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity will examine the status of available *in vitro* methods for assessing acute toxicity. The methods to be addressed will include screening methods for acute toxicity, such as methods that might be used

to predict the starting dose for *in vivo* animal studies, and methods for generating information on toxicokinetics, target organ toxicity, and mechanisms of toxicity. The Workshop will develop recommendations for validation efforts necessary to characterize the usefulness and limitations of these methods. Recommendations will also be developed for future mechanism-based research and development efforts that might further improve *in vitro* assessments of acute systemic lethal and non-lethal toxicity.

The objectives of the Workshop are to:

- Review the status of *in vitro* methods for assessing acute systemic toxicity:
  a. Review the validation status of available *in vitro* screening methods for their usefulness in estimating *in vivo* acute toxicity;
  b. Review *in vitro* methods for predicting toxicokinetic parameters important to acute toxicity (i.e., absorption, distribution, metabolism, elimination);
  c. Review *in vitro* methods for predicting specific target organ toxicity;
- Recommend candidate methods for further evaluation in prevalidation and validation studies;
- Recommend validation study designs that can be used to adequately characterize the usefulness and limitations of proposed *in vitro* methods;
- Identify reference chemicals that can be used for development and validation of *in vitro* methods for assessing *in vivo* acute toxicity;
- Identify priority research efforts necessary to support the development of mechanism-based *in vitro* methods to assess acute systemic toxicity. Such efforts might include incorporation and evaluation of new technologies, such as gene microarrays, and development of methods necessary to generate dose response information.
-

2.1 Uses of Acute Toxicity Testing Data by Regulatory Authorities

Internationally, the most common use of acute systemic toxicity data is to provide a basis for hazard classification and the labeling of chemicals for their manufacture, transport, and use (**Table 1, OECD, 1998a**). Other, potential uses for acute toxicity testing data include:

- Establish dosing levels for repeated-dose toxicity studies;
- Generate information on the specific organs affected;
- Provide information related to the mode of toxic action;
- Aid in the diagnosis and treatment of toxic reactions;
- Provide information for comparison of toxicity and dose response among substances in a specific chemical or product class;
- Aid in the standardization of biological products;
- Aid in judging the consequences of exposures in the workplace, home, or from accidental release, and
- Serve as a standard for evaluating alternatives to animal tests.

.

Table 1. OECD Harmonized Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances—Oral Toxicity (OECD, 1998a)

	Class 1	Class 2	Class 3	Class 4	Class 5
Oral (mg/kg)	5	50	300	2000	5000

2.2 Uses of Acute Toxicity Testing Data by Clinicians

In an effort to obtain information on the uses of acute toxicity data by clinicians, NICEATM contacted Ms. Kathy Kirkland, the Director of the Association of Occupational and Environmental Clinics. Ms. Kirkland queried the clinicians within the Association for such information. The following outlines the responses from two physicians.

In a clinic that deals primarily with cases of heavy metal and pesticides exposures, LD_{50} values are used to assess the dose and likelihood of toxic effects in a patient. However, many of the cases deal with mixed or unknown exposures, and LD_{50} values are not available for these materials. *In vitro* cytotoxicity data is utilized in a body of evidence approach to the extent that it is available.

In another clinical practice that treats mainly chronic toxicity cases (e.g., pneumonoconiosis, malignancy, solvent neurotoxicity), the clinicians tend to rely on historical human toxicity data, such as published reports of previous industrial toxicity, for which there is much literature. It was felt that animal toxicity data alone is not very useful in the absence of a clinical database, but that animal studies are helpful in supporting human epidemiological literature for occupational cancer. No specific response was provided on the use of *in vitro* cytotoxicity test data.

2.3 Current *In Vivo* Methods for Assessing Acute Toxicity

The first of the methods described in this section (the conventional LD_{50} test) is the approach used historically to provide acute toxicity data (LD_{50} value, slope of the dose-response curve, confidence interval), and information regarding toxic signs. Compared to other, more recently developed alternative *in vivo* methods for evaluating acute toxicity, the conventional LD_{50} test requires the use of more animals. For this reason, there are considerable international efforts through the OECD to delete the test guideline for this method (Test Guideline [TG] 401). These efforts have prompted a re-assessment of all of the OECD *in vivo* test guidelines for acute toxicity to ensure that regulatory needs are met while minimizing animal usage and maximizing data quality. Each of the OECD *in vivo* test methods is described in this section.

In these *in vivo* test methods, rats are the preferred species, although other rodent species may be used. Oral gavage is the primary route for administration of solid and liquid test substance. Doses that are known to cause marked pain and distress due to corrosive or severely irritant actions are not used. In the draft alternative *in vivo* test method guidelines, animals of a single sex are considered sufficient. Females are given preference because literature surveys of test results using the OECD TG 401 method have shown that although there is little difference in sensitivity between the sexes, in those cases where significant differences were observed, females were more frequently the more sensitive sex.

2.3.1 *The Conventional LD_{50} Test (OECD TG 401)*

OECD TG 401 (OECD, 1987) outlines the conventional LD_{50} test to assess acute oral toxicity. The use of five animals (of the same sex) using at least three dose levels in the toxic/lethal range is recommended. The test often

uses five or more dose levels. When testing is completed in one sex, at least one group of five animals of the other sex is dosed to establish that animals of this sex do not have markedly different sensitivity to the test substance. When testing substances for which no relevant toxicity information is available, a range-finding or sighting study that uses up to five animals must be conducted. Thus, a minimum of 20 to 25 animals would be used in each study. Generally, the test substance is administered to all animals within a study on the same day to eliminate potential differences in preparing the test substance solutions on different days. The goal of the test is to produce at least two dose groups in which at least one, but not all, of the animals is killed by the test substance with 14 days. If this occurs, the LD_{50}, its confidence interval, and the slope of the dose-response curve can be calculated using probit analysis, and a hazard classification determined.

When it is suspected that the test substance may have little or no toxicity, a limit test may be conducted. TG 401 specifies testing five animals of each sex at 2000 mg/kg. If test substance-related mortality is produced, a full study may need to be conducted. If no mortality occurs, the substance is classified as having an LD50 of >2000 mg/kg

2.3.2 *Fixed Dose Procedure (FDP) (Draft OECD TG 420)*

The draft OECD TG 420 (OECD, 1999a) describes the FDP for acute toxicity testing. The method is designed so that only moderately toxic doses are administered (i.e., doses that are expected to be lethal are avoided). The method allows test substances to be ranked and classified according to a globally harmonized system for the classification of chemicals that cause acute toxicity (**Table 1**) (OECD, 1998a).

Specifically, groups of animals of a single sex are dosed in a step-wise procedure using fixed doses of 5, 50, 300, and 2000 mg/kg (exceptionally, an additional fixed dose of 5000 mg/kg may be considered, if required for a specific regulatory purpose). The initial dose for the main study is selected on the basis of a sighting study as the dose expected to produce some signs of toxicity without causing severe toxic effects or mortality. The initial fixed dose selected for the sighting study is one expected to produce evident toxicity based, when possible on evidence from structurally related chemicals. In the absence of such information, the sighting fixed dose is 300 mg/kg; the test substance is administered to a single animal per dose group in a sequential manner, with at least 24 hours allowed between the dosing of each animal. Subsequent animals are dosed at higher or lower fixed doses depending on the absence or presence of toxic signs or mortality, respectively. The procedure continues until the dose causing evident toxicity, or not more than one death, is identified, or when no effects are observed at the limit dose, or when deaths occur at the lowest dose.

In the main test, five animals per dose level are usually used. The animals tested during the sighting study are included in that total. Thus, if an animal had been tested at a specific dose level in the sighting study, only four more animals would be tested at that same dose level, if it were selected as an appropriate dose to test further.

In vivo and modeling studies have shown the FDP to be reproducible (OECD, 1999a). The method is considered advantageous because it:

- Uses fewer animals than OECD TG 401,
- Causes less suffering than tests that primarily use lethality and morbidity as the endpoint, and
- Is able to rank test substances in a similar manner to other *in vivo* alternative acute toxicity test methods (e.g., the Acute Toxic Class Method [ATC]).

The FDP is not intended to allow for the calculation of the LD_{50} value or of a dose-response slope.

2.3.3 *Acute Toxic Class Method (ATC) (Draft OECD TG 423)*

The ATC is a step-wise procedure that uses three animals of a single sex per step (OECD, 1999b). Testing is conducted at defined doses of 5, 50, 300, and 2000 mg/kg (exceptionally, an additional

fixed dose of 5000 mg/kg may be considered, if required for a specific regulatory purpose) that allow a test substance to be ranked and classified according to a globally harmonized system for the classification of chemicals that cause acute toxicity (**Table 1**) (OECD, 1998a). The dose level to be used as the starting dose is selected from one of the four fixed dose levels based on an expectation that mortality would be induced in at least some of the dosed animals. When available information suggests that mortality is unlikely at the limit dose, then a limit test should be conducted. A limit test involves testing three animals of the same sex at the limit dose. When there is no information on a substance to be tested, it is recommended for animal welfare concerns that the starting dose be 300 mg/kg. Depending on the mortality and/or moribund status of the animals, an average of two to four steps may be necessary to allow judgement of the acute toxicity potential of the test substance. The time interval between treatment groups is determined by the onset, duration, and severity of toxic signs. Treatment of animals at the next higher dose should be delayed until one is confident of survival of the previously dosed animals. The number of animals used per test is generally in the range of six to 12. The method is based on biometric evaluations, and has been validated internationally (OECD, 1999b).

The ATC is not intended to allow for the calculation of the LD_{50}, but does allow for the determination of defined exposure ranges where lethality is expected, since death of a proportion of animals is a major endpoint of the test. An LD_{50} can be calculated only when at least two doses result in mortality in some, but not all, animals. The main advantage of this method is that it requires fewer animals than OECD TG 401. In theory, the method also should increase laboratory-to-laboratory reproducibility because the provisions for dose selection and interpretation are specifically set.

2.3.4 *Up-and-Down Procedure (UDP) (U.S. EPA Draft OECD TG 425)*

The U.S. EPA draft of OECD TG 425 (OECD, 1998b) specifies the approach for conducting the UDP. In this procedure, animals are dosed sequentially at 48-hour intervals. The first animal receives a dose at the best estimate of the LD_{50}; when no information is available, an initial dose of 175 mg/kg is recommended. Depending on the outcome for the previous animal, the dose for the next animal is adjusted upwards or downwards by a dose-spacing factor of 3.2 (half-log). If an animal survives, the dose for the next animal is higher; if the animal dies or is moribund, the dose for the next animal is lowered. Dosing continues depending on the fixed-time interval outcomes of all the animals up to that time. The testing stops when (1) three consecutive animals survive at the limit dose (or three consecutive animals die at a predetermined lower limit dose, or (2) five reversals occur in 6 animals started, or (3) at least 4 animals have followed the first reversal and the criteria of the stopping rules based on likelihood-ratios are met (OECD, 1998b). A reversal is a situation where nonresponse is observed at some dose, and a response is observed at the next dose tested. Calculations are made with each dose, following the fourth animal after the first reversal. For a wide variety of combinations of LD50 and slopes as low as 2.5, the stopping rule (i.e., the criteria for terminating the study) will be satisfied with four to six animals after the first reversal. However, for chemicals with a shallow dose-response slope, more animals (but not more than 15) may be needed. When the stopping criteria have been attained after the initial reversal, the estimated LD50 should be calculated from the animal outcomes at test termination using the statistical method described in the Guideline (OECD, 1998b). The LD_{50} is calculated using the method of maximum likelihood.

When weak toxicity is suspected, a limit test may be used. A single animal is tested at the limit dose of 2000 or 5000 mg/kg. Which limit dose is used depends on the regulatory requirement being fulfilled. If the animal survives, then two additional animals receive the same dose. If one or more of these two animals die, a fourth and perhaps a fifth animal is placed on test at the same dose. At 5000 mg/kg, the test is terminated whenever a total of three animals have survived or have died. At 200 mg/kg, all 5 animals must be tested. If three animals survive, the LD50 is above the limit dose; if three animals die, the LD50 is below the limit dose. In situations where

the first animal dies, the UDP main test is conducted. Also, if three animals have died and an LD50 value is required, the UDP main test is conducted.

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center on the Validation of Alternative Toxicological Methods (NICEATM) recently coordinated a peer review of U.S. EPA draft TG 425; the peer review report for that meeting will be available soon.

3.0 *In Vitro* Test Methods for Predicting *In Vivo* Toxicity—General Strategies

Cytotoxicity is defined as the adverse effects resulting from interference with structures and/or processes essential for cell survival, proliferation, and/or function. These effects may involve the integrity of membranes and the cytoskeleton, cellular metabolism, the synthesis and degradation or release of cellular constituents or products, ion regulation, and cell division. Generally, three principal mechanisms for toxicity have been identified. These include general (also known as basal) toxicity, selective toxicity, and cell-specific function toxicity. General cytotoxicity involves one or more structures or processes that would be expected to be intrinsic to all cell types (e.g., mitochondrial function, membrane integrity). Selective cytotoxicity occurs when some types of differentiated cells are more sensitive to the effects of a particular toxicant than others, potentially as a result of, for example, binding to specific receptors, or uptake by a cell-type specific mechanism. Cell-specific function cytotoxicity occurs when the toxicant affects structures or processes that may not be critical for the affected cells themselves, but which are critical for the organism as a whole. For example, such toxicity can involve effects on cell-to-cell communication, via the synthesis, release, binding and degradation of cytokines, hormones and transmitters.

Numerous assays have been developed for assessing cytotoxicity *in vitro (*see **Table 2**). However, until recently, there has been little emphasis on to how to apply the resulting data to predicting *in vivo* toxicity and to the regulatory decision-making process. Several large scale, international multi-laboratory studies have attempted to address the issue of using *in vitro* toxicity information to predict *in vivo* test substance-induced toxic effects (Fentem et al., 1993; Garle et al., 1994); some of these studies will be discussed in subsequent sections. The goals of these studies have ranged from a complete replacement of *in vivo* acute toxicity tests by *in vitro* tests (e.g., see **Section 4.1**) to reducing animal usage by using *in vitro* cytotoxicity data to identify the optimal starting dose for an *in vivo* acute toxicity test (e.g., see **Section 4.3**), or to determine whether a limit test should be conducted first.

Several work groups have proposed the potential use of *in vitro* cytotoxicity test methods in a tiered testing scheme. For the sake of brevity, only two examples are provided here although other, generally similar approaches have been presented in different forums (e.g., see **Section 6.1**).

In 1996, Seibert et al. reported on an international evaluation of selected *in vitro* toxicity test systems for predicting acute systemic toxicity (see also Fentem et al., 1993). The goal of the evaluation was to identify strategies for using data obtained from *in vitro* tests as a basis for classifying and labelling new chemicals, thereby reducing (and possibly replacing) the need for acute toxicity tests in animals. A diverse group of 42 chemicals were evaluated; the chemicals had been tested in a range of *in vitro* systems (bovine spermatozoa, BALB/c 3T3 cells, rat hepatocytes, rat skeletal muscle cells, hepatocyte/3T3 co-cultures, V79 cells, 3T3-L1 cells, and V79/hepatocyte co-cultures), employing various exposure periods and endpoint measurements. *In vitro* effective concentration values were compared with *in vivo* rodent LD50 values. Based on the recommendations of the participants, the following tiered testing scheme for assessing acute toxicity was proposed.

In Stage 1, basal cytotoxicity is determined using cell proliferation inhibition as the endpoint. In Stage 2, a test is conducted to determine hepatocyte-specific cytotoxicity and to define the role of metabolism in the cytotoxic effects of the

test chemical. Finally, in Stage 3, additional testing is conducted that would provide information on selective cytotoxicity (other than hepatocyte-specific cytotoxicity) as well as an indication of any interference with important specific, but non-vital, cell functions. Many test systems may be appropriate for this level of testing, including the use of cells from the nervous system, heart, or kidney.

More recently, and based also on discussion at a meeting that focused on validation and acute toxicity testing, Curren et al. (1998) also suggested the use of *in vitro* cytotoxicity and other information tests in a tiered testing approach. Step one would be the collection and integration of information on the physical/chemical properties of a compound, including literature reviews and analysis of the structure-activity relationships (when possible). Step two would be the determination of general cytotoxicity using an *in vitro* model system. This Step would include gathering information (via *in vitro* models) on gastrointestinal uptake, the penetration of the blood-brain barrier, and biotransformation. In Step three, general cytotoxicity information could be reinforced and supplemented with computer-based modeling of biokinetic data.

Curren et al. (1998) concluded that these steps might provide sufficient information to estimate the hazard classification for some compounds. In cases where additional information is needed, tests using a limited number of animals might be conducted to supplement the data obtained from literature review, *in vitro* testing, and computer modeling. Curren et al. (1998) recognized also that the use of this tiered testing strategy is currently limited because there is insufficient information on structure-activity relationships with respect to acute systemic toxicity, most likely because of the large number of mechanisms involved in the expression of this type of toxicity. Thus, substantial additional investigation into the cause of chemically induced lethality is needed. Curren concluded that the *in vitro* models used to determine gastrointestinal uptake, blood-brain barrier passage, and biotransformation have not been formally validated.

A variety of *in vitro* tests have been developed to evaluate the types of cytotoxicity (general or basal, selective, cell-specific function) that have the potential to result in acute systemic toxicity, with the greater effort focused on general toxicity. Any strategy used to extrapolate *in vitro* toxicity test results to an *in vivo* toxicity response must consider all of these possibilities, as well as toxicokinetics. To provide some indication of the range of biological endpoints used to assess cytotoxicity *in vitro*, **Table 2** summarizes the *in vitro* toxicity endpoints/test systems used in three large studies. Information on the reliability (intra-laboratory repeatability and inter-laboratory reproducibility) of any *in vitro* toxicity test method was not located. The studies considered for this document evaluated the correlation between *in vitro* test method results and animal LD50 or human lethal blood concentrations; test method reliability was not addressed.

3.1 Quantitative Structure Activity Relationship (QSAR) Methods

The potential uses of QSAR as part of an *in vitro* strategy will need to be evaluated during the Workshop. QSAR methods are models that relate the biological activities of a series of similar compounds to one or more physicochemical or structural properties of the compounds (Barratt et al., 1995). 'Similar' includes compounds that exhibit the same mechanism of action in addition to those that have related chemical structures. However, it is often difficult to determine mechanism of action, whereas it is less difficult to establish chemical similarity. Therefore, QSAR models are usually developed for sets of chemically similar compounds on the assumption that they will have the same mechanism of action. Any compounds that do not act by the same mechanism are likely to poorly fit the correlation, and would thus not be accurately modeled or predicted.

Table 2. Various *In Vitro* Cytotoxicity Endpoints Evaluated in MEIC and Spielmann et al. (1999)

Endpoint	Measured as	Cell Line(s)	Study
Cell viability	ATP content or leakage	ELD cells (mouse); erythrocytes (mouse); LS-L929 cells (mouse); hepatocytes (rat); spermatozoa (bovine); HL-60 cells (human)	MEIC
	Cell morphology	C9 cells (rat); hepatocytes (rat); L2 cells (rat); MDBK cells (bovine); Chang liver cells (human); HeLa cells (human); McCoy cells (human); WI-1003/Hep-G2 cells (human)	MEIC
	Chromium release	LS-L929 cells (mouse)	MEIC
	Creatine kinase activity	Muscle cells (rat)	MEIC
	Hemolysis	Erythrocytes (human)	MEIC
	Killing index (*sic*)	SQ-5 cells (human)	MEIC
	LDH release	3T3 Cells (mouse); hepatocytes (rat, human); Hep-2 cells (human); Hep-G2 cells (human); lymphocytes (human); SQ-5 cells (human)	MEIC
	Neutral Red Uptake	3T3 cells (mouse); L929 cells (mouse); NB41-A3 cells (mouse); BHK cells (hamster); hepatocytes (rat, human); HeLa cells (human); Hep-2 cells (human); keratinocytes (human)	MEIC; Spielmann et al. (1999)
	Plating efficiency	HeLa cells (human)	MEIC
	^{86}Rb leakage	Not designated	MEIC
	Viable cell count	LS-L929 cells (mouse); polymorphonuclear leukocytes (human)	MEIC
Cell growth	Cell cycle distribution	Daudi cells (human), RERF-LC-AI cells (human)	MEIC
	Glucose consumption	Muscle cells (rat)	MEIC
	Macromolecule content	HTC cells (rat); Hep-G2 cells (human)	MEIC
	MTT metabolism	3T3 cells (mouse); L929 cells (mouse); NG108-15 cells (mouse, rat); V79 cells (hamster); hepatocytes (rat, human); Detroit 155, DET dermal fibroblasts (human); FaO cells (human); Hep-G2 cells (human); HFL1 cells (human); 3D Skin^2, Dermal Model ZK1100 keratinocytes (human); lymphocytes (human); RERF-LC-AI cells (human); WS1 cells (human)	MEIC
	pH change	L2 cells (rat); Chang liver cells (human); HeLa cells (human); WI-1003/Hep-G2 cells (human)	MEIC

Endpoint	Measured as	Cell Line(s)	Study
Specialized function effects	Protein content	3T3 or 3T3-L1 cells (mouse); Hepa-1c1c7 (mouse); L929 cells (mouse); V79 cells (hamster); hepatocytes (rat); PC12h cells (rat); LLC-PK1 cells (pig); HeLa cells (human); Hep-2 cells (human); Hep-G2 cells (human); MRC-5 cells (human); NB-1 cells (human); Chinese hamster V79 cells	MEIC; Spielmann et al. (1999); Fry et al., 1990
	Tritiated-proline uptake	L2 cells (rat)	MEIC
	Tritiated-thymidine incorporation	Peripheral lymphocytes (human)	MEIC, Spielmann et al. (1999)
	Cell resting membrane potential	NG108-15 (mouse, rat)	MEIC
	Chemotaxis/locomotion stimulated by chemotactic peptide	Polymorphonuclear leukocytes (human)	MEIC
	EOD activity	Hepatocytes (rat)	MEIC
	Inhibition of NK cell-mediated cytotoxicity activity	Natural killer cells, including over 90% CD16+ or CD56+ cells (human)	MEIC
	Intracellular glycogen content	Hepatocytes (rat)	MEIC
	Motility or velocity	Spermatozoa (bovine)	MEIC
	Spontaneous contractility	Muscle cells (rat)	MEIC

Abbreviations: ATP = Adenosine triphosphate; CR = calorimetric respirometric ratio; EOD = 7-ethoxycoumarin *O*-deethylase; LDH = Lactate dehydrogenase; MTT = 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide; MEIC = Multicenter Evaluation of *In Vitro* Cytotoxicity (see summary in **Appendix 6 [Appendix E *of the In Vitro Workshop Report*]**).

In a review of QSAR studies, Phillips et al. (1990) concluded that QSAR methods have shown some success in relating LD_{50} values to certain physicochemical properties of a compound (especially lipophilicity). However, QSAR appears to be less successful in correlating electronic properties of molecules (related to reactivity) or structural variables with LD_{50} values.

Of the numerous QSAR studies intended to rationalize and predict the *in vivo* mammalian toxicity of chemicals based on properties related to structure, one popular approach is the linear free-energy, extra-thermodynamic method developed by Hansch and colleagues (Phillips et al., 1990). The basic assumption of this approach is that the effect of the substituents on the magnitude of a compound's interaction with biological receptors or other molecules is an additive combination of the substituents' interactions in simpler systems.

A second common approach was developed by Free and Wilson in 1964 (Phillips et al., 1990). It is based on the assumption that, for congeneric

series of compounds with multiple sites of substitutions, the observed activity can be expressed in terms of the mutually independent contributions from the various substituents of the molecule.

Requirements/caveats for the successful development and use of QSAR methods include the following:

- There should be a well-defined mechanism of action for the compound(s) used to derive the QSAR model (Phillips et al., 1990; Barratt et al., 1995);
- The compounds should form part of a congeneric group (Phillips et al., 1990) and should be pure (i.e., not mixtures) (Barratt et al., 1995);
- There should be a common site of action for the biological effect (Phillips et al., 1990);
- As for any comparative purpose, concentrations or doses should be presented in molar (not weight) units (Barratt et al., 1995);
- Each QSAR model should be validated by investigating its predictive ability using a different set of compounds from its learning set, which should cover the same ranges of parameter space as the original test chemicals (Barratt et al., 1995); and
- The QSAR should not be applied outside of its domain of validity (i.e., outside the parameter space covered by the training set) (Barratt et al., 1995).

3.1.1 *Publications Containing Further Information*

Free, S.M., And J.W. Wilson. 1964. A Mathematical Contribution To Structure-Activity Studies. J. Med. Chem. 7: 395-399.

Hansch, C., and T. Fujita. 1964. ρ, σ, π Analysis. A method for the correlation of biological activity and chemical structure. J. Am. Chem. Soc. 86: 1616-1626.

4.0 *In Vitro* Screening Methods for Assessing Acute Toxicity (Breakout Group 1)

This Breakout Group will evaluate the validation status of available *in vitro* methods for estimating *in vivo* acute toxicity. The Group will identify the most promising methods and recommend appropriate validation studies that might be completed within the next one to two years. The potential uses of QSAR as part of an *in vitro* strategy will also be evaluated (see **Section 3.1**). Most of the *in vitro* test method development for assessing cytotoxicity has focused on general (or basal) cytotoxicity. General cytotoxicity is independent of cell type and involves one or more adverse effects that interfere with structures and/or processes essential for cell survival, proliferation, and/or function. These effects may include adverse effects on the integrity of membranes (including the cytoskeleton), general metabolism, ion regulation, and cell division. Studies conducted to evaluate the suitability of *in vitro* general cytotoxicity methods for predicting *in vivo* toxicity are described briefly; more detailed information can be obtained as indicated.

4.1 The Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC)

Additional details of the MEIC study are reported in the MEIC Summary prepared by NICEATM (**Appendix A [Appendix E of the *In Vitro* Workshop Report]**) and in the list of MEIC-related publications provided in **Section 4.1.4**.

4.1.1 *General Study Description*

The MEIC program was organized by the Scandinavian Society for Cell Toxicology in 1989. The intent of the program was to investigate the relevance of *in vitro* test results for predicting the acute toxic action of chemicals in humans. Given that such relevance was identified, the next goal was to establish batteries of existing *in vitro* toxicity tests that have the potential to serve as replacements for acute toxicity tests using laboratory mammals.

MEIC was a voluntary effort involving 96 international laboratories that evaluated the

effectiveness of *in vitro* cytotoxicity tests originally developed as alternatives to (or supplements for) laboratory mammal tests for acute and/or chronic systemic toxicity, organ toxicity, skin irritancy, or other forms of general toxicity. Minimal methodological directives were provided in order to maximize protocol diversity among the laboratories. The collection of test method data was completed in 1996; to date, 24 publications originating from these studies have been published.

By the end of the project, 39 laboratories had tested the first 30 reference chemicals in 82 *in vitro* assays, while the last 20 chemicals were tested in 67 *in vitro* assays. The primary 82 assays included 20 human cell line assays; seven human primary culture assays utilizing hepatocytes, keratinocytes, and polymorphonuclear leukocytes; 19 animal cell line assays, 18 animal primary culture assays, and 18 ecotoxicological tests utilizing bacteria, rotifer, crustacea, plant, and fish cells. Thirty-eight of these assays were based on viability, 29 on growth, and the remaining assays involved more specific endpoints, such as locomotion, contractility, motility, velocity, bioluminescence, and immobilization. The endpoints assessed were based on exposure durations ranging from five minutes to six weeks. The analyses conducted by the MEIC management team were based on *in vitro* toxicity data presented as IC50 values (i.e., the dose estimated to affect the endpoint in question by 50%). The types of comparative data used to evaluate the predictive accuracy of the *in vitro* IC50 toxicity data for *in vivo* acute toxicity included oral rat and mouse LD50 values, acute oral lethal doses in humans, clinically measured acute lethal serum concentrations in humans, acute lethal blood concentrations in humans measured post-mortem, human pharmacokinetics following single doses, peaks from curves of an ~50% lethal blood/serum concentration over time after ingestion.

4.1.2 *List of Chemicals Tested and Selection Rationale*

The chemical set (50 chemicals) used in the MEIC studies is provided in the MEIC Summary (**Appendix A [Appendix E of the *In Vitro* Workshop Report]**)). These chemicals were selected because of the availability of human data on acute toxicity (e.g., lethal blood concentrations).

4.1.3 *Summary Conclusions*

Based on the results obtained, a battery of four endpoints/two exposure times (protein content/24 hours; ATP content/24 hours; inhibition of elongation of cells/24 hours; pH change/7 days) in three human cell line tests was found to be highly predictive of the peak human lethal blood concentrations (LC50) of chemicals when incorporated into an algorithm developed by the MEIC management team. The MEIC management team concluded that the battery could be used directly as a surrogate for a LD50 test. However, since the battery predicts lethal blood concentrations, not lethal oral dosages, it is not a direct counterpart of the animal LD50 test. Thus, the battery must be supplemented with data on gut absorption as well as the distribution volumes of chemicals. Furthermore, in this study, there was no assessment of test method reliability, either within or between laboratories.

4.1.4 *Publications Containing Additional Study Information*

Balls, M., B.J. Blaauboer, J.H. Fentem, L. Bruner, R.D. Combes, B. Ekwall, R.J. Fielder, A. Guillouzo, R.W. Lewis, D.P. Lovell, C.A. Reinhardt, G. Repetto, D. Sladowski, H. Spielmann, and F. Zucco. 1995. Practical Aspects of the Validation of Toxicity Test Procedures –The Report and Recommendations of ECVAM Workshop 5. ATLA 23: 129-147.

Bernson, V., I. Bondesson, B. Ekwall, K. Stenberg, and E. Walum. 1987. A Multicentre Evaluation Study of *In Vitro* Cytotoxicity. ATLA 14: 144-145.

Bondesson, I., B. Ekwall, K. Stenberg, L. Romert, and E. Walum. 1988. Instruction for Participants in the Multicentre Evaluation Study of *In Vitro* Cytotoxicity (MEIC). ATLA 15: 191-193.

Bondesson, I., B. Ekwall, S. Hellberg, L. Romert, K. Stenberg, and E. Walum. 1989. MEIC - A

New International Multicenter Project to Evaluate the Relevance to Human Toxicity of *In Vitro* Cytotoxicity Tests. Cell Biol. Toxicol. 5: 331-347.

Clemedson, C., and B. Ekwall. 1999. Overview of the Final MEIC Results: I. The *In Vitro-In Vivo* Evaluation. Toxicol. *In Vitro* 13: 1-7.

Clemedson, C, E. McFarlane-Abdulla, M. Andersson, F.A. Barile, M.C. Calleja, C. Chesné, R. Clothier, M. Cottin, R. Curren, E. Daniel-Szolgay, P. Dierickx, M. Ferro, G. Fiskesjö, L. Garza-Ocanas, M.J. Gómez-Lechón, M. Gülden, B. Isomaa, J. Janus, P. Judge, A. Kahru, R.B. Kemp, G. Kerszman, U. Kristen, M. Kunimoto, S. Kärenlampi, K. Lavrijsen, L. Lewan, H. Lilius, T. Ohno, G. Persoone, R. Roguet, L. Romert, T. Sawyer, H. Seibert, R. Shrivastava, A. Stammati, N. Tanaka, O. Torres Alanis, J.-U. Voss, S. Wakuri, E. Walum, X. Wang, F. Zucco, and B. Ekwall. 1996. MEIC Evaluation of Acute Systemic Toxicity. Part I. Methodology of 68 *in vitro* toxicity assays used to test the first 30 reference chemicals. ATLA 24 (Suppl. 1): 249-272.

Clemedson, C, E. McFarlane-Abdulla, M. Andersson, F.A. Barile, M.C. Calleja, C. Chesné, R. Clothier, M. Cottin, R. Curren, P. Dierickx, M. Ferro, G. Fiskesjö, L. Garza-Ocanas, M.J. Gómez-Lechón, M. Gülden, B. Isomaa, J. Janus, P. Judge, A. Kahru, R.B. Kemp, G. Kerszman, U. Kristen, M. Kunimoto, S. Kärenlampi, K. Lavrijsen, L. Lewan, H. Lilius, A. Malmsten, T. Ohno, G. Persoone, R. Pettersson, R. Roguet, L. Romert, M. Sandberg, T. Sawyer, H. Seibert, R. Shrivastava, M. Sjöström, A. Stammati, N. Tanaka, O. Torres Alanis, J.-U. Voss, S. Wakuri, E. Walum, X. Wang, F. Zucco, and B. Ekwall. 1996. MEIC Evaluation of Acute Systemic Toxicity. Part II. *In vitro* results from 68 toxicity assays used to test the first 30 reference chemicals and a comparative cytotoxicity analysis. ATLA 24 (Suppl. 1): 273-311.

Clemedson, C., F.A. Barile, B. Ekwall, M.J. Gómez-Lechón, T. Hall, K. Imai, A. Kahru, P. Logemann, F. Monaco, T. Ohno, H. Segner, M. Sjöström, M. Valentino, E. Walum, X. Wang, and B. Ekwall. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part III. *In vitro* results from 16 additional methods used to test the first 30 reference chemicals and a comparative cytotoxicity analysis. ATLA 26 (Suppl. 1): 91-129.

Clemedson, C., Y. Aoki, M. Andersson, F.A. Barile, A.M. Bassi, M.C. Calleja, A. Castano, R.H. Clothier, P. Dierickx, B. Ekwall, M. Ferro, G. Fiskesjö, L. Garza-Ocanas, M.J. Gómez-Lechón, M. Gülden, T. Hall, K. Imai, B. Isomaa, A. Kahru, G. Kerszman, P. Kjellstrand, U. Kristen, M. Kunimoto, S. Kärenlampi, L. Lewan, H. Lilius, A. Loukianov, F. Monaco, T. Ohno, G. Persoone, L. Romert, T.W. Sawyer, R. Shrivastava, H. Segner, H. Seibert, M. Sjöström, A. Stammati, N. Tanaka, A. Thuvander, O. Torres-Alanis, M. Valentino, S. Wakuri, E. Walum, A. Wieslander, X. Wang, F. Zucco, and B. Ekwall. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part IV. *In vitro* results from 67 toxicity assays used to test reference chemicals 31-50 and a comparative cytotoxicity analysis. ATLA 26 (Suppl. 1): 131-183.

Clemedson, C., F.A. Barile, C. Chesné, M. Cottin, R. Curren, Ba. Ekwall, M. Ferro, M.J. Gomez-Lechon, K. Imai, J. Janus, R.B. Kemp, G. Kerszman, P. Kjellstrand, K. Lavrijsen, P. Logemann, E. McFarlane-Abdulla, R. Roguet, H. Segner, H. Seibert, A. Thuvander, E. Walum, and Bj. Ekwall. 2000. MEIC Evaluation of Acute Systemic Toxicity. Part VII. Prediction of human toxicity by results from testing of the first 30 reference chemicals with 27 further *in vitro* assays. ATLA 28 (Suppl. 1): 161-200.

Ekwall, B. 1989. Expected Effects of the MEIC-Study. In: Report from the MEIC *In Vitro* Toxicology Meeting, Stockholm 9/3/1989. (Jansson, T., and L. Romert, eds). Swedish National Board for Technical Development, pp. 6-8.

Ekwall, B. 1995. The Basal Cytotoxicity Concept. In Proceedings of the World Congress on Alternatives and Animal Use in the Life Sciences: Education, Research, Testing. Alternative Methods in Toxicology and the Life Sciences 11: 721-725. Mary Ann Liebert, New York, 1995.

Ekwall, B. 1999. Overview of the Final MEIC Results: II. The *in vitro/in vivo* evaluation, including the selection of a practical battery of cell tests for prediction of acute lethal blood concentrations in humans. Toxicol. *In Vitro* 13(4-5): 665-673.

Ekwall, B., M.J. Gómez-Lechón, S. Hellberg, L. Bondsson, J.V. Castell, R. Jover, J. Högberg, X. Ponsoda, K. Stenberg, and E. Walum. 1990. Preliminary Results from the Scandinavian Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC). Toxicol. *In Vitro* 4: 688-691.

Ekwall, B., E. Abdulla, F. Barile, I. Bondesson, C. Clemedson, R. Clothier, R. Curren, P. Dierickx, G. Fiskesjö, L. Garza-Ocanas, M.J. Gómez-Lechón, M. Gülden, K. Imai, J. Janus, U. Kristen, M. Kunimoto, S. Kärenlampi, K. Lavrijsen, L. Lewan, A. Malmsten, T. Miura, M. Nakamura, T. Ohno, H. Ono, G. Persoone, R. Rouget, L. Romert, M. Sandberg, T. Sawyer, H. Seibert, R. Shrivastava, A. Stammati, N. Tanaka, E. Walum, X. Wang, and F. Zucco. 1992. Acute Lethal Toxicity in Man Predicted by Cytotoxicity in 55 Cellular Assays and by Oral LD50 Tests in Rodents for the First 30 MEIC Chemicals. In: Proceedings of the Japanese Society for Alternatives to Animal Experiments, 6th annual meeting in Tokyo, Dec 17-18, 1992. (S. Sato, ed). pp. 114-115.

Ekwall, B., E. Abdulla, F. Barile, C. Chesne, R.H. Clothier, M. Cottin, R. Curren, E. Daniel-Szolgay, P. Dierickx, M. Ferro, G. Fiskesjö, L. Garza-Ocanas, M.J. Gómez-Lechón, M. Gülden, B. Isomaa, A. Kahru, R.B. Kemp, G. Kerszman, U. Kristen, M. Kunimoto, S. Kärenlampi, K. Lavrijsen, L. Lewan, T. Ohno, G. Persoone, R. Pettersson, R. Rouget, L. Romert, T. Sawyer, H. Seibert, R. Shrivastava, M. Sjöström, N. Tanaka, F. Zucco, E. Walum, and C. Clemedson. 1994. A Comparative Cytotoxicity Analysis of the Results from Tests of the First 30 MEIC Reference Chemicals in 68 Different *In Vitro* Toxicity Systems. In Alternatives Research - Proceedings of the 8th Annual Meeting of the Japanese Society for Alternatives to Animal Experiments in Tokyo, Nov. 28-29, 1994, pp. 117-118.

Ekwall, B., C. Clemedson, B. Crafoord, Ba. Ekwall, S. Hallander, M. Sjöström, and E. Walum. 1997. Correlation Between *In Vivo* and *In Vitro* Acute Toxicity Tests: Results of the MEIC project. In: Development of Ecotoxicity and Toxicity Testing of Chemicals - Proceeding of the 2nd Network Meeting, TemaNord 1997: 524, Nordic Council of Ministers, Copenhagen. pp. 82-83.

Ekwall, B., C. Clemedson, B. Crafoord, Ba. Ekwall, S. Hallander, E. Walum, and I. Bondesson. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part V. Rodent and human toxicity data for the 50 reference chemicals. ATLA 26 (Suppl. 2): 569-615.

Ekwall, B., F.A. Barile., A. Castano, C. Clemedson, R.H. Clothier, P. Dierickx, Ba. Ekwall, M. Ferro, G. Fiskesjö, L. Garza-Ocanas, M.J. Gómez-Lechón, M. Gülden, T. Hall, B. Isomaa, A. Kahru, G. Kerszman, U. Kristen, M. Kunimoto, S. Kärenlampi, L. Lewan, A. Loukianov, T. Ohno, G. Persoone, L. Romert, T.W. Sawyer, H. Segner, R. Shrivastava, A. Stammati, N. Tanaka, M. Valentino, E. Walum, and F. Zucco. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part VI. Prediction of human toxicity by rodent LD50 values and results from 61 *in vitro* tests. ATLA 26 (Suppl. 2): 617-658.

Hellberg, S., I. Bondesson, B. Ekwall, M.J. Gómez-Lechón, R. Jover, J. Högberg, X. Ponsoda, L. Romert, K. Stenberg, and E. Walum. 1990. Multivariate Validation of Cell Toxicity Data: The first ten MEIC chemicals. ATLA 17: 237-238.

Hellberg, S., L. Eriksson, J. Jonsson, F. Lindgren, M. Sjöström, S. Wold, B. Ekwall, M.J. Gómez-Lechón, R. Clothier, N.J. Accomando, G. Gimes, F.A. Barile, M. Nordin, C.A. Tyson, P. Dierickx, R.S. Shrivastava, M. Tingsleff-Skaanild, L. Garza-Ocanas, and G. Fiskesjö. 1990. Analogy Models for Prediction of Human Toxicity. ATLA 18: 103-116.

Shrivastava, R., C. Delomenie, A. Chevalier, G. John, B. Ekwall, E. Walum, and R. Massingham. 1992. Comparison of *In Vivo* Acute Lethal

Potency and *In Vitro* Cytotoxicity of 48 Chemicals. Cell Biol. Toxicol. 8(2): 157-170.

Walum, E. 1998. Acute Oral Toxicity. Environ. Hlth Perspect. 106 (Suppl. 2): 497-504.

Walum, E., M. Nilsson, C. Clemedson, and B. Ekwall. 1995. The MEIC Program and its Implications for the Prediction of Acute Human Systemic Toxicity. In: Proceedings of the World Congress on Alternatives and Animal Use in the Life Sciences: Education, Research, Testing. Alternative Methods in Toxicology and the Life Sciences 11: 275-282. Mary Ann Liebert, New York.

4.2 Correlation of acute lethal potency with *in vitro* cytotoxicity. (Fry et al., 1990)

Fry et al. (1990) evaluated the *in vitro* cytotoxicity of 27 compounds believed to act by interference with cell basal functions/structures. The cytotoxic endpoint assessed was growth inhibition in Chinese hamster V79 cells. ID_{50} values were calculated and compared to either oral or intraperitoneal (i.p.) LD_{50} values from mice or rats. Although significant positive correlations were found when either log i.p. or log oral LD50 values were compared to log ID_{50} values, the correlation was 'better' when log i.p. LD_{50} values were used. A further improvement was obtained when data from three compounds (>10%) were excluded for which metabolism is a major determinant of toxicity *in vivo*. Close correlations of log i.p. LD_{50}/log ID_{50} values were obtained with groups of six anti-metabolites and six alkylating agents, although the locations of the regression lines for these two groups were significantly different. Based on these results, the authors concluded that the *in vitro* cytotoxicity of compounds that exert their toxicity by interference with cell basal functions/structures is correlated with their intrinsic lethal potency. However, information on absorption, metabolism, and disposition is required before *in vitro* cytotoxicity data can be used to assess *in vivo* potency. The data also indicated that the precise relation of LD_{50} to ID_{50} values was determined by the mode of toxicity. In this study, there was no assessment of test method reliability, either within or between laboratories.

4.3 Determination of the starting dose for acute oral toxicity (LD50) testing in the up and down procedure (UDP) from cytotoxicity data. (Spielmann et al., 1999)

Additional details of this study are reported in Spielmann et al. (1999), while related information are provided in **Appendix B [Section 7.0 of the *In Vitro* Workshop Report]**.

4.3.1 *General Study Description*

The Spielmann et al. (1999) study was conducted to investigate the feasibility of using the standard regression between mean IC_{50} ($IC_{50}\,\bar{x}$) and acute oral LD_{50} values reported for rats and mice in the Register of Cytotoxicity (Halle and Goeres, 1988) to determine the starting dose for *in vivo* acute toxicity testing. The linear regression line determined using 347 chemicals was used to predict the LD_{50} values for nine chemicals that had been investigated in an evaluation study of the UDP (Lipnick et al., 1995).

4.3.2 *List of Chemicals Tested and Selection Rationale*

Since the focus of the study was to determine if the linear regression extrapolation method could be used to adequately predict starting doses for the UDP, chemicals evaluated in a study considered to be the official evaluation for OECD acceptance of the UDP (Lipnick et al., 1995) were used. Lipnick et al. (1995) investigated 35 materials. Nine of those were excluded from the Spielmann et al. (1999) study because they were mixtures or formulations (e.g., laundry detergent). Of the remaining 26 chemicals, nine (acetonitrile, *p*-aminophenol, caffeine, coumarin, dimethylformamide, mercury (II) chloride, nicotine, phenylthiourea, and resorcinol) were also reported in the Register of Cytotoxicity, and thus were selected for evaluation.

4.3.3 *Summary Conclusions*

The predicted LD_{50} values for seven of the nine chemicals were the same as those calculated from *in vivo* testing. For the two remaining chemicals,

the dose-range differed from *in vivo* test results by one order of magnitude. The authors concluded that this method of predicting starting doses seemed promising, given the results from the limited data set, and that the use of this technique, coupled with the use of the UDP in place of the conventional LD_{50} test, would reduce animal use. However, the use of the IC_{50}/LD_{50} linear regression to estimate *in vivo* acute toxicity from cytotoxicity data assumes that a linear relationship exists between the IC_{50} and the LD_{50} values. This linear relationship could only be expected if all of the reference chemicals were found to be mechanistically similar and if all of the reference chemicals demonstrated similar toxicokinetics.

4.3.4 *Publications Containing Additional Study Information*

Seibert, H., M. Gülden, And J.-U. Voss. 1994b. An *In Vitro* Toxicity Testing Strategy For The Classification And Labelling Of Chemicals According To Their Potential Acute Lethal Potency. Toxicol. *In Vitro* 8: 847-850.

5.0 *In Vitro* Methods for Assessing Acute Toxicity –Toxicokinetic Determinations (Breakout Group 2)

This Breakout Group will evaluate the capabilities of *in vitro* methods for providing toxicokinetic information (absorption, distribution, metabolism, and elimination) that can be used to estimate target organs and dosimetry for acute toxicity testing and to provide recommendations for future research needs to accomplish this goal. The role of QSAR in toxicokinetic determinations will also be explored.

The toxicity of a substance *in vivo* is strongly influenced by the time-dependent processes of intake, uptake (absorption), distribution, biotransformation (metabolism), and elimination (excretion). As a consequence, such information is essential for the accurate prediction of *in vivo* toxicity from *in vitro* cytotoxicity test results. This need has been recognized by a number of investigators (see also **Sections 3** and **6.1**).

One method for estimating toxicokinetic parameters is through physiologically based biokinetic (PBBK) [or physiologically based pharmacokinetic modeling (PBPK)] or modeling. However, the method is complex and requires a great deal of knowledge about *in vivo* target organs and about various *in vivo* toxicokinetic parameters for the chemical under investigation. Whether PBBK modeling can be considered to be a suitable method for assessing a large number of chemicals remains to be determined.

Another approach would be to use a few, carefully selected *in vivo* toxicokinetic parameters, such as the fraction absorbed from the intestine and the apparent volume of distribution in combination with other information (e.g., lipid solubility, pKa) to estimate body doses from *in vitro* concentrations and to estimate organ concentrations from body doses. If such *in vivo* data is not available, the fraction absorbed from the intestine could be estimated from knowledge about the general relationships between physicochemical properties of chemicals and their absorption in the gastrointestinal tract, or from *in vitro* experimental data. One *in vitro* approach is the use of two-compartment systems comprising epithelia-like monolayers of human colon carcinoma cells (e.g., Caco-2 or HT-29 cells).

Additionally, *in vitro* data on specific chemicals and parameters defining the composition/compartmentalization of the *in vivo* model can be used as the basis for converting *in vitro* effective concentrations into equivalent body doses. This requires the following information/tools at a minimum:

- Various physicochemical characteristics of the chemical (e.g., pKa, lipophilicity, or volatility);
- Quantitative estimates of protein binding;
- Basis characteristics of the *in vitro* system (e.g., cell concentration, cell protein concentration, ratio of cell-medium volumes, and medium albumin concentration); and
- A mathematical model that permits the calculation of equivalent body doses, such as one described by Gülden et al. (1994), who derived a formula that allows for the conversion of calculated EC_{50} values to

ED_{50} values, which can then be compared to known LD_{50} values.

5.1 Tests for Metabolic Effects

Because the liver is the primary organ involved in xenobiotic metabolism, liver-derived *in vitro* systems have been used to estimate metabolic activation and the production of toxic metabolites. Test systems commonly used include whole liver homogenates, subcellular fractions (e.g., microsomes), liver slices, freshly isolated hepatocytes in suspension, primary monolayer hepatocyte cultures, metabolically competent hepatocyte or hepatoma cell lines, and cell lines transfected with human or rodent cytochromes. Studies of metabolism require the use of preparations that maintain appropriate and sufficient metabolic competence. Noted limitations of these *in vitro* tests include a lack of Phase II enzymes that are not membrane bound in some tests using liver homogenates and subcellular fractions, and variable stability in the expression of both Phase I and II enzyme activities in tests using freshly isolated hepatocytes or primary hepatocyte cultures. Co-culturing metabolically active hepatocytes with targets cells is one promising approach for assessing the role of metabolism in *in vivo* toxicity. An alternative (but less attractive) approach would be to expose the hepatocytes to the test substance, and then culture the target cells in the resulting conditioned culture medium. The advantages of the former method are that it enables the detection of hepatocyte-specific cytotoxicity, interference with specific functions of hepatocytes, and metabolism-mediated effects on target cells.

5.1.1 *Publications Containing Further Information*

Blaauboer, B.J., A.R. Boobis, J.V. Castell, S. Coecke, G.MM. Groothuis, A. Guillouzo, T.J. Hall, G.M. Hawksworth, G. Lorenzen, H.G. Miltenburger, V. Rogiers, P. Skett, P. Villa, and F.J Wiebel. 1994. The Practical Applicability of Hepatocyte Cultures in Routine Testing. The Report and Recommendations of ECVAM Workshop 1. ATLA 22: 231-241.

Ericsson, A.C., and E. Walum. 1988. Differential Effects of Allyl Alcohol on Hepatocytes and Fibroblasts Demonstrated in Roller Chamber Co-Cultures. ATLA 15: 208-213.

Paillard, F., F. Finot, I. Mouche, A. Prenez, and J. A. Vericat. 1999. Use of Primary Cultures of Rat Hepatocytes to Predict Toxicity in the Early Development of New Chemical Entities. Toxicol. *In Vitro* 13: 693-700.

Voss, J.-U., and H. Seibert. 1992. Toxicity of Glycols and Allyl Alcohol Evaluated by Means of Co-Cultures of Microcarrier-Attached Rat Hepatocytes and Balb/c 3T3 Mouse Fibroblasts. ATLA 20: 266-270.

Voss, J.-U., and H. Seibert. 1991. Microcarrier-Attached Rat Hepatocytes as a Xenobiotic-Metabolizing System in Cocultures. Cell Biol. Toxicol. 7(4): 387-397.

6.0 *In Vitro* Methods for Assessing Acute Toxicity - Specific Organ Toxicity and Mechanisms (Breakout Group 3)

This Breakout Group will review *in vitro* methods that can be used to predict specific organ toxicity or toxicity associated with alteration of specific cellular or organ functions, and will develop recommendations for priority research efforts necessary to support the development of methods that can accurately assess target organ toxicity.

While the focus of most *in vitro* cytotoxicity research for predicting *in vivo* acute toxicity has been on an assessment of general cytotoxicity, the accurate prediction of *in vivo* acute toxicity for many substances absolutely requires critical information on the potential for organ-specific toxicity. Selective toxicity occurs when some types of differentiated cells are more sensitive to the effects of a particular toxicant than others, potentially as a result of, for example, biotransformation, binding to specific receptors, or uptake by a cell-type specific mechanism. A number of specific cell type assays (e.g., liver, nervous system, heart, kidney) have been developed for assessing selective toxicity. In the absence of appropriate information on target organ specificity for structurally-related

substances, detection of selective cell toxicity requires the evaluation of toxicity of the same test substance in multiple cell types.

Not specifically considered, but potentially relevant to specific organ toxicity is so-called specific function cytotoxicity. This type of toxicity occurs when the toxicant affects structures or processes that may not be critical for the affected cells themselves, but which are critical for the organism as a whole. For example, such toxicity can involve effects on cell-to-cell communication, via the synthesis, release, binding and degradation of cytokines, hormones and transmitters. No specific studies evaluating this type of toxicity were located.

Studies conducted to evaluate the suitability of *in vitro* organ-specific toxicity methods for predicting *in vivo* toxicity are described briefly; more detailed information can be obtained as indicated.

6.1 Evaluation-Guided Development of *In Vitro* Tests (EDIT)

In recognition that additional *in vitro* tests were needed to enhance the accuracy of the proposed MEIC *in vitro* battery for predicting human acute toxicity, a second multicenter program was initiated by the Cytotoxicology Laboratory, Uppsala (CTLU). The CTLU designed a blueprint for an extended battery and invited interested laboratories to develop the "missing" tests of this battery (i.e., extracellular receptor toxicity, excitatory toxicity, passage across blood-brain barrier, absorption in the gut, blood protein binding, distribution volumes, metabolic activation to more toxic metabolites) within the framework of the EDIT program. More information is available on the Internet (www.ctlu.se). The aim of EDIT is to provide a full replacement of the animal acute toxicity tests. Among the needed developments are assays for the accumulation of chemicals in cells, passage across the intestinal and blood-brain barriers, and biotransformation to more toxic metabolites. Purported advantages of the project are as follows. First, the evaluation-guided test development in EDIT is rational since tests are designed according to specific needs and as tests of single processes that can be integrated into sequential testing models. This is the potential strength of the *in vitro* toxicity testing strategy. Second, the direct testing of chemicals in newly developed *in vitro* assays will lead to a rapid evaluation of the potential value of each assay. Further information is provided in the MEIC Summary prepared by NICEATM (**Appendix A [Appendix E of the *In Vitro* Workshop Report]**)).

6.1.1 *Publications Containing Further Information*

Ekwall, B., C. Clemedson, Ba. Ekwall, P. Ring, And L. Romert. 1999. Edit: A New International Multicentre Programme To Develop And Evaluate Batteries Of *In Vitro* Tests For Acute And Chronic Systemic Toxicity. Atla 27: 339-349.

6.2 European Research Group for Alternatives in Toxicity Testing (ERGATT)/ Swedish National Board for Laboratory Animals (CFN) Integrated Toxicity Testing Scheme (ECITTS)

6.2.1 *General Study Description*

The ECITTS approach was to develop integrated testing schemes by combining sets of test batteries for predicting local and systemic toxicity in ways that would be more efficient than animal-based methods (Seibert et al., 1996). Evaluation of basal cytotoxicity and biokinetic parameters were considered to be essential to the investigation, although further testing would be adapted based on the test chemical; such testing may involve evaluation of developmental toxicity, immunotoxicity, nephrotoxicity, or neurotoxicity, as deemed appropriate. The basal cytotoxicity data were specifically used to interpret specific effects on potential target cells and tissues, while protein binding and biotransformation data were used to evaluate biokinetics.

In an initial pilot study reported by Blaauboer et al. (1994), the neurotoxic properties of five chemicals (acrylamide, lindane, methyl mercury (II) chloride, trethyltin chloride, and *n*-hexane) were studied in combination with biokinetic

analysis, in which blood and brain concentrations were predicted from biokinetic modeling. A follow-up study was conducted by Forsby et al. (1995), in which four of these chemicals (acrylamide, lindane, methyl mercury (II) chloride, and trethyltin chloride) were evaluated for general cytotoxicity and neurite degeneration in human epithelial and neuronal cells.

6.2.2 *Publications Containing Further Study Information*

Forsby, A., F. Pilli, V. Bianchi, And E. Walum. 1995. Determination Of Critical Cellular Neurotoxic Concentrations In Human Neuroblastoma (Sh-Sy5y) Cell Cultures. Atla 23: 800-811.

Walum, E., M. Balls, B. Bianchi, B. Blaauboer, G. Bolcsfoldi, A. Guillouzo, G.A. Moor, L. Odland, C.A. Reinhardt, and H. Spielmann. 1992. ECITTS: An Integrated Approach for the Application of *In Vitro* Test Systems for the Hazard Assessment of Chemicals. ATLA 20: 406-428.

6.3 Institute of Toxicology, University of Kiel

6.3.1 *General Study Description*

The study used a continuous cell line (Balb/c 3T3 cells) and differentiated mammalian cells (primary cultures of rat hepatocytes, rat skeletal muscle cells, and bovine spermatozoa) to assess acute systemic toxicity (Seibert et al., 1996). The importance of comparative cell toxicology and physicochemical data were emphasized. Comparative cell toxicology was investigated using tests with different endpoints, tissues, and species, while tests for effects such as lipophilicity were used to assess physicochemical interactions.

Chemicals evaluated in Seibert et al. (1994a) included 2,4-dinitrophenol, cyclophosphamide, and lidocaine. The paper demonstrated a comparative cell toxicological approach that enabled the detection of various toxic potencies and provided a limited interpretation of the mechanisms behind the toxic actions. Such information could serve as the basis for the assessment of the toxicological characteristics of a new chemical by providing information on which to base decisions on appropriate further testing.

Gülden et al. (1994) used the first 30 chemicals tested in the MEIC battery to evaluate the relevance of *in vitro* test systems for acute toxicity assessment. In order to make an appropriate comparison, the calculated EC_{50} values for inhibition of spontaneous contractility of primary cultured rat skeletal muscle cells were converted to ED_{50} values (i.e., effective model body doses) that were then compared directly to the known LD_{50} values for these chemicals. Although the extrapolation model was based on oversimplifications, the investigators concluded that the approach shows promise and that more complex models should be investigated.

6.3.2 *Publications Containing Further Study Information*

Gülden, M., H. Seibert, and J.-U. Voss. 1994. Inclusion of Physicochemical Data in Quantitative Comparisons of *In Vitro* and *In Vivo* Toxic Potencies. ATLA 22: 185-192.

Gülden, M., H. Seibert, and J.-U. Voss. 1994. The Use of Cultured Skeletal Muscle Cells in Testing for Acute Systemic Toxicity. Toxicol. *In Vitro* 8: 779-782.

Halle, W., and H. Spielmann. 1992. Two Procedures for the Prediction of Acute Toxicity (LD50) from Cytotoxicity Data. ATLA 20: 40-49.

Seibert, H., M. Gülden, And J.-U. Voss. 1994b. An *In Vitro* Toxicity Testing Strategy For The Classification And Labelling Of Chemicals According To Their Potential Acute Lethal Potency. Toxicol. *In Vitro* 8: 847-850.

7.0 Chemical Data Sets for Validation of *In Vitro* Toxicity Tests (Workshop Group 4)

This Breakout Group has the responsibility of defining what chemical data sets are required for validation studies, identifying existing resources,

and recommending approaches for using existing data sets and/or compiling or developing new data sets. Developing a single listing of chemicals that will address all test validation needs is not feasible. Instead, a library of useful chemicals should be developed that could be used when designing test development or validation efforts. Using this library, chemicals can be selected according to the purpose of the test and of the validation study. Developing appropriate criteria for chemical selection is a critical aspect of this process. Examples of selection criteria to be considered include:

- Chemicals that cover a wide range of acute LD50's, corresponding to the dose ranges used in the OECD classification (**Table 1**).
- Different chemical classes (structure; use; activity).
- Chemicals that are directly active and those that require metabolic activation (by internal organs; gut flora).
- General toxins and specific organ toxins.
- Chemicals active by different mechanisms.
- Chemicals that are commercially available in high purity, and relatively inexpensive.
- Gases; insolubles; immiscible liquids; unstable substances; dangerous substances should be avoided.
- Controlled substances (e.g., requiring a license) or those with shipping and handling restrictions should be avoided.

The most important components of the database will be the chemical name, CASRN, Smiles (or other structure-search) code, and biological endpoints. These endpoints could include acute toxicity data (e.g., LD50); organ/tissue specificity (e.g., hepatotoxin; neurotoxin; etc.); and ADME-related information (e.g., metabolism; peak blood levels; organ distribution; membrane permeability; excretion route). At a second level, the database should also include physico-chemical parameters (e.g., pH, volatility, and solubility), and product and use classes.

This database will enable users to pick the endpoint of interest (e.g., LD50; hepatotoxicity) and select the chemicals that can be used to validate the *in vitro* test. The candidate chemicals selected for use in the validation test can then be further grouped by class (e.g., chemical; product; use). If the chemical structure data are appropriately entered, the chemical classes that best correspond to the chemicals showing a specific endpoint can be defined by the database user.

Chemicals selected should be backed with adequate animal data showing acute toxicity, organ specificity, general mechanism of action, metabolic and toxicokinetic requirements, etc.

Where possible, structurally related chemicals with differing toxicities should be used to determine if the *in vitro* system could distinguish among them. It would be helpful to find homologous series of chemicals with differing toxicities.

Databases specific to *in vitro* cytotoxicity tests for use in assessing acute toxicity include the following:

- The Register of Cytotoxicity is a collection of acute oral LD_{50} values from rats and mice, as listed in the NIOSH Registry of Toxic Effects of Chemical Substances (RTECS), and mean cytotoxicity data (IC_{50} *x*) on chemicals and drugs (Halle and Goeres, 1988).
- The MEIC i*n vitro* database contains both the methods used in testing (Part I, http://www.cctoxconsulting.a.se/Web/Met/default.htm) and the results (Part II, http://www.cctoxconsulting.a.se/Web/Res/default.htm) for the 50 chemicals tested in the MEIC study. The associated MEMO database (http://www.cctoxconsulting.a.se/meicinvivo.htm) contains the human lethal blood concentration data used for comparison against the *in vitro* test results.

An *in vivo* acute toxicity database that may be useful is provided in **Appendix C [Appendix F of the *In Vitro* Workshop Report]**). In the United States, regulations regarding packaging, labeling,

and transport of acutely toxic liquids or solids are provided under 49 CFR 173. Materials with oral LD_{50} values less than or equal to 200 mg/kg (for solids) or 500 mg/kg (for liquids), dermal LD_{50} values less than or equal to 1000 mg/kg, or inhalation LC_{50} values less than or equal to 10 mg/L are considered to be poisonous and to pose a hazard to human health during transport. These materials, listed in the regulation as Division 6.1 materials, are further categorized into packing groups based on the level of hazard. Information on packing group designations, materials reported in the DOT regulation as Division 6.1 (49 CFR 172.101) hazardous materials and their packing group designations are provided in **Appendix C [Appendix F of the *In Vitro* Workshop Report]**), along with their packing group designation.

A list of 375 substances tested *in vitro* with comparative *in vivo* data, as reported in five studies (MEIC, Fry et al., 1990; Gülden et al., 1994; Lipnick et al., 1995; Spielmann et al. 1999), as well as in the Register of Cytotoxicity database developed under the direction of W. Halle, has been compiled for this Workshop (**Appendix B [Section 7.0 of the *In Vitro* Workshop Report]**). Detailed information on the cell system/endpoint used to assess cytotoxicity and the IC50 and/or ID50 values, the oral corresponding LD50 for rat and/or mouse, and the average or acute human lethal dose, can be obtained in the appropriate citations.

8.0 Relevant General Databases

Relevant general databases that may include pertinent information for this Workshop include:

- INVITTOX is a searchable database of protocols for *in vitro* toxicity test methods. Its aim is to provide precise and up-to-date technical information on the performance of the *in vitro* techniques currently in use and under development, their applications, advantages, and disadvantages. Sixty-two protocols, as well as information on the number of chemicals tested using the protocols and relevant publications, are available at http://embryo.ib.amwaw.edu.pl/invittox/invittox.htm.
- The German Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET) searchable database contains information on 300 alternatives in biomedicine fields and contains about 4,000 bibliographical references. It is available at http://gripsdb.dimdi.de/engl/guieng.html.
- The National Library of Medicine (NLM) maintains a bibliography of publications on alternatives to animal testing. This bibliography is available at http://www.sis.nlm.nih.gov/altanimal.cfm.
- The Akademie für Tierschutz, which is part of the German Animal Welfare Federation, has established a bibliographical database on alternatives. It contains 15,000 references and is available on floppy disk. Requests may be directed to akademie.fuer.tierschutz@muenchen.org.
- The Galileo Databank contains toxicology data from alternative studies, mostly related to cosmetics testing. The databank contains data on over 800 ingredients, over 300 cosmetic formulations, 50 methods, 26 animal models, and over 100 biosystems, with a total of nearly 21,000 individual results. The databank is not currently available online, but printouts may be requested by contacting Gregorio Loprieno, Technical Services SAS, Via Vecchia Lucchese 59, I-56123, Pisa, Italy, 39-50-555-685 (phone), 39-50-555-687 (fax).
- VetBase is a database of literature references to over 12,000 doses for 800 veterinary drugs in 130 species, including farm and laboratory animals, zoo species, fish, birds, amphibians and reptiles. The database is a custom-made MS Windows application, and is available by contacting J.D.Kuiper@cc.ruu.nl.

9.0 References

Barratt, M.D., J.V. Castell, M. Chamberlain, R.D. Combes, J.C. Dearden, J.H. Fentem, I. Gerner, A. Giuliani, T.J.B. Gray, D.J. Livingstone, W.

Mclean Provan, F.A.J.J.L. Rutten, H.J.M. Verhaar, And P. Zbinden. 1995. The Integrated Use Of Alternative Approaches For Predicting Toxic Hazard: The Report And Recommendations Of ECVAM Workshop 8. ATLA 23: 410-429.

Blaauboer, B.J., And J. Dejongh. 1998. An Integrated Approach To The Prediction Of Systemic Toxicity Using Computer-Based Biokinetic Models And Biological In Vitro Test Methods. Report For The Dutch Platform Alternatives To Animal Testing (Pad), The Hague, The Netherlands. 34 Pp.

Curren, R., L. Bruner, A. Goldberg, And E. Walum. 1998. Validation And Acute Toxicity Testing. Environ. Hlth Perspect. 106 (Suppl. 2): 419-426.

Ekwall, B., C. Clemedson, Ba. Ekwall, P. Ring, And L. Romert. 1999. EDIT: A New International Multicentre Programme To Develop And Evaluate Batteries Of *In Vitro* Tests For Acute And Chronic Systemic Toxicity. ATLA 27: 339-349.

Ekwall, B., Ekwall, B., And Sjorstrom, M. 2000. MEIC Evaluation Of Acute Systemic Toxicity: Part VIII. Multivariate Partial Least Squares Evaluation, Including The Selection Of A Battery Of Cell Line Tests With A Good Prediction Of Human Acute Lethal Peak Blood Concentrations For 50 Chemicals. ATLA 28 (Suppl. 1): 201-234.

Fentem, J., J. Fry, M. Garle, M. Gülden, H. Seibert, J-U. Voss, O. Wassermann, M. Perchermeier, And F.Wiebel. 1993. An International Evaluation Of Selected *In Vitro* Toxicity Test Systems For Predicting Acute Systemic Toxicity. A Report Prepared For DGXI, CEC; Contract Numbers B92/B4-3063/14086 & B92/B4-3040/14087. FRAME, Nottingham.

Forsby, A., F. Pilli, V. Bianchi, And E. Walum. 1995. Determination Of Critical Cellular Neurotoxic Concentrations In Human Neuroblastoma (Sh-Sy5y) Cell Cultures. Atla 23: 800-811.

Fry, J.R., M.J. Garle, A.H. Hammond, And A. Hatfield. 1990. Correlation Of Acute Lethal Potency With *In Vitro* Cytotoxicity. Toxicol. *In Vitro* 4(3): 175-178.

Garle, M.J., J.H. Fentem, And J.R. Fry. 1994. *In Vitro* Cytotoxicity Tests For The Prediction Of Acute Toxicity *In Vivo*. Toxicol. *In Vitro* 8: 1303-1312.

Gülden, M., H. Seibert, And J.-U. Voss. 1994. Inclusion Of Physicochemical Data In Quantitative Comparisons Of *In Vitro* And *In Vivo* Toxic Potencies. ATLA 22: 185-192.

Halle, W., H. Spielmann, And M. Liebsch. 2000. Prediction Of Human Lethal Concentrations By Cytotoxicity Data From 50 MEIC Chemicals. ALTEX 17: 75-79.

Halle, W., And E. Goeres. 1988. Register Der Zytotoxizität (IC50) In Der Zellkultur Und Möglichkeiten Zur Abschätzung Der Akuten Toxizität (LD50). In: Beiträge Zur Wirkstoffforschung, Institute Für Wirkstoffforschung. (Oehme, P., H. Loewe, And E. Goeres, Eds). Berlin, Germany.

Lipnick, R.L., J.A. Cotruvo, R.N. Hill, R.D. Bruce, K.A. Stitzel, A.P. Walker, I. Chu, M. Goddard, L. Segal, J.A. Springer, And R.C. Myers. 1995. Comparison Of The Up-And-Down, Conventional LD50, And Fixed-Dose Acute Toxicity Procedures. Food Chem. Toxicol. 33: 223-231.

OECD (Organisation For Economic Co-Operation And Development). 1987. OECD Guideline For Testing Of Chemicals 401: Acute Oral Toxicity. OECD, Paris.

OECD. 1998a. Harmonized Integrated Hazard Classification System For Human Health And Environmental Effects Of Chemical Substances As Endorsed By The 28th Joint Meeting Of The Chemicals Committee And The Working Party On Chemicals In November 1998, Part 2, P. 11. Available On The Internet At Http://Www.Oecd.Org/Ehs/Class/HCL6.Htm.

OECD. 1998b. OECD Guideline For Testing Of Chemicals 425: Acute Oral Toxicity—Up-And-Down Procedure. OECD, Paris.

OECD. 1999a. OECD Guideline For Testing Of Chemicals, Revised Draft Guideline 420: Acute Oral Toxicity—Fixed Dose Procedure. OECD, Paris.

OECD. 1999b. OECD Guideline For Testing Of Chemicals, Revised Draft Guideline 423: Acute Oral Toxicity—Acute Toxic Class Method. OECD, Paris.

Phillips, J.C., W.B. Gibson, J. Yam, C.L. Alden, And G.C. Hard. 1990. Survey Of The QSAR And *In Vitro* Approaches For Developing Non-Animal Methods To Supersede The *In Vivo* LD50 Test. Food Chem. Toxicol 28(5): 375-394.

Seibert, H., M. Gülden, M. Kolossa, And G. Schepers. 1992. Evaluation Of The Relevance Of Selected *In Vitro* Toxicity Test Systems For Acute Systemic Toxicity. Atla 20: 240-245.

Seibert, H., M. Gülden, And J.-U. Voss. 1994. Comparative Cell Toxicology: The Basis For *In Vitro* Toxicity Testing. ATLA 22: 168-174.

Spielmann, H., E. Genschow, M. Leibsch, And W. Halle. 1999. Determination Of The Starting Dose For Acute Oral Toxicity (LD50) Testing In The Up-And-Down Procedure (UDP) From Cytotoxicity Data. ATLA 27(6): 957-966.

10.0 Glossary

[See Section 6.0 of the In Vitro Workshop Report]