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

Results of an International Workshop Organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Toxicology Program
P.O. Box 12233
Research Triangle Park, NC 27709

August 2001
NIH Publication No. 01-4499

National Institute of Environmental Health Sciences
National Institutes of Health
US Public Health Service
Department of Health and Human Services

Table of Contents

List of Tables

List of Figures

List of Tables

List of Figures

List of Acronyms/Abbreviations

A549	Human alveola Type II epithelia – lung carcinoma
ADAPT	A commercially available system for the evaluation of LD50s and MTDs
ADME	Absorption, distribution, metabolism, elimination
ANOVA	Analysis of Variance
ATC	Acute Toxic Class
ATP	Adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry/DHHS
BALB/c	Inbred strain of mouse
BBB	Blood-Brain Barrier
BEAS-2B	Human Bronchial-tracheal epithelia/transformed
BgVV	Federal Institute for Health Protection of Consumers and Veterinary Medicine (Germany)
BG1	Breakout Group 1: *In Vitro* Screening Methods for Assessing Acute Toxicity
BG2	Breakout Group 2: *In Vitro* Methods for Assessing Acute Toxicity Biokinetic Determinations
BG3	Breakout Group 3: *In Vitro* Methods for Organ-Specific Toxicity
BG4	Breakout Group 4: Chemical Data Sets for Validation of *In Vitro* Toxicity Tests
BFU-E	Burst-forming unit -- erythrocytes
BMC	Bone marrow cell
BTS	British Transplantation Society
b.w.	Body weight
Caco-2	Human acute leukemia cell line
CASE	QSAR Software
CAS	Chemical Abstract Service
CBC	Cord blood cell
CBER	Center for Biologics Evaluation and Research/FDA
CCL-30	Human nasal septum cells – squamous cell carcinoma
CDC	Centers for Disease Control and Prevention/DHHS
CDER	Center for Drug Evaluation and Research/FDA
CFN	The National Board for Laboratory Animals, Stockholm, Sweden
CFR	Code of Federal Regulations
CFU-GM	Colony-forming unit – granulocyte/macrophage
CFU-MK	Colony-forming unit – megakaryocytes
CFSAN	Center for Food Safety and Nutrition/FDA
CNN	Computational Neural Network
CNS	Central Nervous System
CPH 100	Human neuroblastoma cell line -- differentiated
CPSC	Consumer Product Safety Commission
CTLU	Cytotoxicology Laboratory, Uppsala
DEREK	Deduction of Risk from Existing Knowledge (a commercially available knowledge-based expert system - QSAR);
DHHS	Department of Health and Human Services
DIMDI	The German Institute for Medical Documentation and Information

DIV-BBB	Dynamic *in vitro* blood-brain barrier model
DOD	Department of Defense
DOE	Department of Energy
DOT	Department of Transportation
EC50	Effective concentration of compound that causes 50% of the maximum response
ECITTS	ERGATT/CFN Integrated Toxicity Testing Scheme
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
EC/HO	European Commission/British Home Office
ECVAM	European Centre for the Validation of Alternative Methods
EDIT	Evaluation-Guided Development on *In Vitro* Tests
ELISA	Enzyme-Linked Immunosorbent Assay
ERGATT	European Research Group for Alternatives in Toxicity Testing
EPA	Environmental Protection Agency
EU	European Union
EUCLID	Electronically Useful Chemistry Laboratory Instructional Database
FACS	Fluorescence activated cell sorting
FDA	Food and Drug Administration/DHHS
FDP	Fixed-Dose Procedure
FOIA	Freedom of Information Act
FRAME	Fund for the Replacement of Animals in Medical Experiments
$GABA_A$	gamma-aminobutyric acid; type A receptor is a ligand-gated ion channel complex
Galileo	Publicly available database of chemicals tested for toxicity
GFAP	Glial Fibrillary Acidic Protein
GHS	Globally Harmonized System
GLP	Good Laboratory Practice
H441	Human pulmonary adenocarcinoma cell line
Hb/g	Blood-air partition
HeLa	Human cervical adenocarcinoma cell line
HepG2	Human hepatocellular carcinoma cell line
HESI	Health and Environmental Science Institute
HL-60	Human acute leukemia cell line
HPV	High Production Volume
IC50	Inhibitory concentration estimated to affect endpoint in question by 50%
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use;
ILSI	International Life Sciences Institute
IMR32	Human neuroblastoma cell line -- differentiated
INVITTOX	ERGATT FRAME ECVAM Data Bank of In Vitro Techniques in Toxicology (on-line)
IUPAC	The International Union of Pure and Applied Chemistry
JSAAE	Japanese Society for Alternatives to Animal Experiments
Km	Constant that reflects affinity of the enzyme for its substrate

Ko/w	Octanol-water partition; lipophilicity
LC	Lethal blood (or serum) Concentration
LD50	Dose producing lethality in 50% of the animals (median lethal dose)
LDH	Lactate Dehydrogenase
LLC-PK_1	Porcine kidney cell line
LOAEL	Lowest Observable Adverse Effect Level
LR	Likelihood-Ratio
MCASE	A QSAR system for the evaluation of LD50s and MTDs
MDCK	Madin Darby Canine Kidney cells
MEIC	Multicentre Evaluation of *In Vitro* Cytotoxicity
MTD	Maximum Tolerated Dose
MTS	3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MEMO	MEIC Monographs (monographs for 50 MEIC chemicals available from CTLU)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NCI	National Cancer Institute/NIH
NHK	Normal human keratinocyte
NHNP	Human brain neural progenitor cell line
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences/NIH
NIH	National Institutes of Health/DHHS
NIOSH	National Institute for Occupational Safety and Health
NLM	National Library of Medicine/NIH
NMDA	N-methyl-D-aspartate; receptor for neurotransmitter glutamate
NOAEL	No Observed Adverse Effect Level
NRU	Neutral Red Uptake
NT2	Human brain neural progenitor cell line; from teratocarcinoma
NTE	Neuropathy Target Esterase
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OPP	Office of Pesticide Programs/EPA
OPPT	Office of Pollution Prevention and Toxics/EPA
OPPTS	Office of Prevention, Pesticides, and Toxic Substances/EPA
PBBK	Physiologically-Based Biokinetics
PCA	Principal Component Analysis
PCC	Poison Control Center
PCNA	Proliferating cell nuclear antigen
PLS	Partial Least Square Analysis
QSAR	Quantitative Structure-Activity Relationship
QSPR	Quantitative Structure-Property Relationship
QPPR	Quantitative Property-Property Relationship
RC	Registry of Cytotoxicity/ZEBET
RITOX	Research Institute of Toxicology – Utrecht University, the Netherlands

ROS	Reactive Oxygen Species
RTECS	Registry of Toxic Effects of Chemical Substances/NIOSH
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SAR	Structure Activity Relationship
SAS	Statistical Analysis System – (SAS Institute, Inc., Cary, NC, USA)
SGOMSEC	Scientific Group on Methodologies for the Safety Evaluation of Chemicals
SH-SY5Y	Human neuroblastoma cell line -- differentiated
SMILES	Simplified Molecular Input Line Entry Specification (chemical nomenclature)
SR-4897	Murine stromal cells
SOP	Standard Operating Procedures
Sw	Water solubility
TD10	Toxic Dose for 10% of the individuals
TG 401	Test Guideline 401 (Acute Oral Toxicity) [OECD]
TG 420	Test Guideline 420(Acute Oral Toxicity - Fixed Dose Method) [OECD]
TG 423	Test Guideline 423 (Acute Oral toxicity - Acute Toxic Class Method) [OECD]
TG 425	Test Guideline 425 (Acute Oral Toxicity: Up-and-Down Procedure) [OECD]
TOPKAT	QSAR Software for the evaluation of LD50s and MTDs
UDP	Up-and-Down Procedure
Vd	Volume of distribution
Vmax	Maximum initial rate of reaction
WEHI-3B	Murine leukemia (myelomonocytic) cells
XTT	sodium 3,3-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate
ZEBET	German Centre for the Documentation and Validation of Alternative Methods (at BgVV)
3Rs	Refinement, Reduction, and Replacement (of Animal Use)
3T3	BALB/c mouse fibroblast cells
9L	Rat glioma cells

Workshop Breakout Groups

The following scientists were invited to serve on the Breakout Group Panels for the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, October 17-20, 2000.

Breakout Group 1
In Vitro Screening Methods for Assessing Acute Toxicity

Julia Fentem, Ph.D. (Co-Chair)
Unilever Research
Shambrook Bedforshire, United Kingdom

Charles Tyson, Ph.D. (**Co-Chair)**
SRI International
Menlo Park, CA

Robert Combes, **Ph.D.**
Fund for the Replacement of Animals in Medical Experiments (FRAME)
Nottingham, United Kingdom

Rodger Curren, **Ph.D.**
Institute for In Vitro Sciences, Inc.
Gaithersburg, MD

Elke Genschow, Ph.D.
German Centre for the Documentation and Validation of Alternative Methods (at BgVV) (ZEBET)
Berlin, Germany

Alan Goldberg, Ph.D.
Johns Hopkins University Center for Alternatives to Animal Testing
Baltimore, MD

A. Wallace Hayes, **Ph.D., D.A.B.T., D.A.T.S.**
The Gillette Company
Boston, MA

Manfred Liebsch, **Ph.D.**
German Centre for the Documentation and Validation of Alternative Methods (at BgVV) (ZEBET)
Berlin, Germany

Lennart Romert, **Ph.D.**
Swedish National Chemicals Inspectorate
Solna, Sweden

Noriho Tanaka, Ph.D.
Hatano Research Institute
Kanagawa, Japan

ICCVAM Agency Participants

Kailash Gupta, **D.V.M., Ph.D.**
U.S. CPSC, Bethesda, MD

Kenneth Hastings, **Ph.D.**
U.S. FDA/CDER, Rockville, MD

Richard N. Hill, M.D., Ph.D.
U.S. EPA/OPPT, Washington, D.C.

Abdullah Khasawinah, Ph.D.
U.S. EPA/OPP/HED, Arlington, VA

Elizabeth Margosches, **Ph.D.**
U.S. EPA/OPPT/OPPTS, Washington, D.C.

Philip Sayre, **Ph.D.**
U.S. EPA/OPPT, Washington, D.C.

Leonard Schechtman, **Ph.D.**
U.S. FDA/CDER, Rockville, MD

Suhair Shallal, Ph.D.
U.S. EPA/OPP/HED, Arlington, VA

William S. Stokes, **D.V.M., D.A.C.L.A.M.**
NIEHS, Research Triangle Park, NC

Breakout Group 2
In Vitro Methods for Toxicokinetics (ADME)

Bas Blaauboer, **Ph.D. (Co-Chair)**
Utrecht University
Utrecht, The Netherlands

Harvey Clewell, **Ph.D. (Co-Chair)**
KS Crump Group/ICF Kaiser
Ruston, LA

Richard Clothier, Ph.D.
University of Nottingham
Nottingham, United Kingdom

Charles Crespi, **Ph.D.**
Gentest Corp
Woburn, MA

Benjamin Gerson, **M.D.**
BIEX Clinical Laboratory
Philadelphia, PA

Gabrielle Hawksworth, **Ph.D.**
University of Aberdeen
Aberdeen, United Kingdom

Gregory Kedderis, **Ph.D.**
Chemical Industry Institute of Toxicology
Research Triangle Park, NC

Karl Rozman, **Ph.D., D.A.B.T.**
Kansas University Medical Center
Kansas, KS

Calvin Willhite, **Ph.D.**
State of California, Department of Toxic Substances
Berkeley, CA

ICCVAM Agency Participants

John Frazier, **Ph.D.**
U.S. Air Force, Wright Patterson AFB, OH

Stephen Hundley, **Ph.D.**
U.S. FDA/CDER, Rockville, MD

Michael Cunningham, **Ph.D., D.A.B.T.**
NIEHS, Research Triangle Park, NC

Harry Salem, Ph.D.
U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD

Breakout Group 3
In Vitro Methods for Organ-Specific Toxicity

Katherine Stitzel, **D.V.M., (Co-Chair)**
Proctor & Gamble
Cincinnati, OH

Eric Walum, **Ph.D. (Co-Chair)**
Pharmacia & Upjohn AB
Stockholm, Sweden

Daniel Bagley, **Ph.D., D.A.B.T.**
Colgate-Palmolive
Piscataway, NJ

Michael Balls, **Ph.D.**
European Centre for the Validation of
Alternative Methods (ECVAM)
Ispra, Italy

Eugene Elmore, **Ph.D.**
University of California, Irvine
Irvine, CA

Anna Forsby, **Ph.D.**
Stockholm University
Stockholm, Sweden

Sidney Green, **Ph.D.**
Howard University
Washington, D.C.

ICCVAM Agency Participants

James Antonini, Ph.D.
NIOSH, Morgantown, WV

Rajendra S. Chhabra, Ph.D., D.A.B.T
NIEHS, Research Triangle Park, NC

Karen Hamernik, **Ph.D.**
U.S. EPA/OPP, Washington, D.C.

Jerrold Heindel, **Ph.D.**
NIEHS, Research Triangle Park, NC

David Lester, Ph.D.
U.S. FDA/CDER, Laurel, MD

Breakout Group 4
Reference Chemicals for Validation of *In Vitro* Acute Toxicity Test Methods

Angela Auletta, **Ph.D. (Co-Chair)**
U.S. EPA/OPPTS
Washington, D.C.

Leon Bruner, **D.V.M., Ph.D**. **(Co-Chair)**
The Gillette Company
Needham, MA

Martin Barratt
Martin Consultancy
Bedford, United Kingdom

James Cone, **M.D., M.P.H.**
California Department of Health Services
Oakland, CA

John Harbell, **Ph.D.**
Institute of In Vitro Sciences
Gaithersburg, MD

Peter Jurs, Ph.D.
Pennsylvania State University
University Park, PA

Gilles Klopman, **Ph.D.**
Case Western Reserve University
Cleveland, OH

ICCVAM Agency Participants

Thomas Collins, **Ph.D.**
U.S. FDA/CFSAN, Laurel, MD

George Cushmac, Ph.D.
U.S. DOT, Washington, D.C.

Leonard Keiffer, **Ph.D.**
U.S. EPA/OPPT, Washington, D.C.

Alberto Protzel, **Ph.D.**
U.S. EPA/OPP, Washington, D.C.

Errol Zeiger, **Ph.D.,**
NIEHS, Research Triangle Park, NC

Acknowledgements

The following individuals are acknowledged for their contributions to the workshop.

ICCVAM Organizing Committee for the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity

Consumer Product Safety Commission (CPSC)
Kailash Gupta, D.V.M., Ph.D.

Department of Defense (DOD)
John Frazier, Ph.D., (Organizing Committee co-chair)

Department of Transportation (DOT)
George Cushmac, Ph.D.

Environmental Protection Agency (EPA)
Richard Hill, M.D., Ph.D. (ICCVAM Co-Chair)
Angela Auletta, Ph.D.
Elizabeth Margosches, Ph.D.
Karen Hamernik, Ph.D.
Philip Sayre, Ph.D. (Organizing Committee co-chair)
Maurice Zeeman, Ph.D.

Food and Drug Administration (FDA)
Leonard Schechtman, Ph.D.
Melvin Stratmeyer, Ph.D.
Ronald Brown, Ph.D.
Thomas Collins, Ph.D.
Peter Goering, Ph.D.
Stephen Hundley, Ph.D.

National Institute of Environmental Health Sciences (NIEHS)
Jerrold Heindel, Ph.D.
William Stokes, D.V.M. (ICCVAM Co-Chair)
Errol Zeiger, Ph.D., J.D.

National Institute for Occupational Safety and Health (NIOSH)
Kenneth Weber, Ph.D.

National Library of Medicine (NLM)
Vera Hudson, M.S.L.S.

Occupational Safety and Health Administration (OSHA)
Surender Ahir, Ph.D.

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

Integrated Laboratory Systems (ILS), Inc.
Bradley Blackard, M.S.P.H.
Sue Brenzel
Ashlee Duncan, M.S.
Thomas Goldworthy, Ph.D.
Christina Inhof, M.S.P.H.
Linda Litchfield
Barry Margolin, Ph.D.
Judy Strickland, Ph.D., D.A.B.T.
Michael Paris
Barbara Shane, Ph.D., D.A.B.T.
Raymond Tice, Ph.D.

National Institute of Environmental Health Sciences (NIEHS)
Loretta Frye
Debbie McCarley
William S. Stokes, D.V.M., D.A.C.L.A.M. (Director)

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

Agency for Toxic Substances and Disease Registry (ATSDR)
*William Cibulas, Ph.D.

Consumer Product Safety Commission (CPSC)
*Marilyn L. Wind, Ph.D.
Susan Aitken, Ph.D.
Kailash C. Gupta, D.V.M., Ph.D.

Department of Defense (DOD)
*Harry Salem, Ph.D., U.S. Army Edgewood Chemical Biological Center
John M. Frazier, Ph.D., U.S. Air Force, Wright-Patterson AFB

Department of Energy (DOE)
*Marvin Frazier, Ph.D.

Department of Interior (DOI)
*Barnett A. Rattner, Ph.D.

Department of Transportation (DOT)
*James K. O'Steen
George Cushmac, Ph.D.

Environmental Protection Agency (EPA)
*Richard N. Hill, M.D., Ph.D. (Co-Chair)
Office of Pollution Prevention and Toxics
+Angela Auletta, Ph.D
Philip Sayre, Ph.D.
Office of Pesticides Programs
+Karen Hamernik, Ph.D.
Amy Rispin, Ph.D.
Office of Research and Development
+Harold Zenick, Ph.D.
Suzanne McMaster
OECD Test Guidelines Program
Maurice Zeeman, Ph.D.

Food and Drug Administration (FDA)
*Leonard M. Schechtman, Ph.D.
Suzanne C. Fitzpatrick, Ph.D., D.A.B.T.

Center for Drug Evaluation and Research
+Joseph DeGeorge, Ph.D.
Joseph F. Contrera, Ph.D.
Abby C. Jacobs, Ph.D.
Center for Devices and Radiological Health
+Mel E. Stratmeyer, Ph.D.
Raju G. Kammula, D.V.M., Ph.D., D.A.B.T.
Center for Biologics Evaluation and Research
+Patrick G. Swann
Center for Food Safety and Nutrition
+David G. Hattan, Ph.D.
Victor P. Frattali
Center for Veterinary Medicine
+Devaraya Jagannath, Ph.D.
Louis T. Mulligan, D.V.M.
National Center for Toxicological Research
+William T. Allaben, Ph.D.
Office of Regulatory Affairs
+Atin Datta, Ph.D.

National Cancer Institute (NCI)
*David Longfellow, Ph.D.
Yung-Pin Liu, Ph.D.

National Institute of Environmental Health Sciences (NIEHS)
*William S. Stokes, D.V.M., D.A.C.L.A.M. (Co-Chair)
John R. Bucher, Ph.D., D.A.B.T.
Rajendra S. Chhabra, Ph.D., D.A.B.T

National Institute for Occupational Safety and Health (NIOSH)
*Doug Sharpnack, D.V.M., D.A.C.V.P.
+Paul Nicolaysen, V.M.D.

National Institutes of Health (NIH)
*Margaret D. Snyder, Ph.D.

National Library of Medicine (NLM)
*Vera Hudson, M.S.L.S.

Occupational Safety and Health Administration (OSHA)
*Surender Ahir, Ph.D.

* Principal Agency Representatives
\+ Principal Program Representatives 6/01

Special Acknowledgement

Björn Ekwall, 1940 – 2000

Dr. Björn Ekwall is acknowledged for his extraordinary contributions to alternative test method development and his dedication to the implementation of alternative tests for regulatory purposes. Results from his work in the area of *in vitro* cytotoxicity served as a major impetus for organization of the International Workshop on *In Vitro* Methods for Assessing Acute Toxicity.

Born in Uppsala in 1940, he attended Uppsala University Medical School and obtained his MD in 1969. After a short time as a General Practitioner, he became a lecturer at the Department of Anatomy, Uppsala University, where he earned his Ph.D. in toxicology. He was a postdoctoral fellow for 6 months at Materials Science Toxicology Laboratories, Memphis, TN, 1981-1982, and a Consultant at the Toxicology Laboratory of the Swedish Food Administration, 1982-1983. Between 1989 and 1996 he was an Associate Professor at the Division of Toxicology, Department of Pharmaceutical Biosciences, Uppsala University.

Dr. Ekwall introduced the concept of testing compounds in systems such as cell cultures and extrapolating the results to human toxicity. He felt that one could break down toxicity in complex biological systems to basic elements that could then be analyzed by using *in vitro* methods. To advance these ideas, he founded a small non-profit research institute, the Cytotoxicology Laboratory, Uppsala (CTLU) in 1983. He also established the Scandinavian Society for Cell Toxicology whose mission is to gather scientists for meetings and show that chemical effects on cells should translate to *in vivo* effects. These organizations coordinated a large international evaluation study, the Multicenter Evaluation of In Vitro Cytotoxicity Tests (MEIC), that began in 1989 to scrutinize the relevance of *in vitro* cytotoxicity tests for human acute toxicity of chemicals, and to select batteries of *in vitro* assays for practical testing of chemicals as alternatives to animal acute toxicity tests. Sixty-five different test methods were employed for testing 50 chemicals. Dr. Ekwall continued to work with the MEIC project until his untimely death on August 19, 2000.

Dr. Ekwall published 69 articles/book chapters on *in vitro* toxicology, plus 30 abstracts published in journals and another 30 abstracts published in conference proceedings. He received many international awards and was a member of many scientific societies and associations.

Preface

Acute systemic toxicity testing is conducted to determine the relative health hazard of chemicals and various products. Substances found to cause lethality in animals at or below prescribed doses are labeled to identify their hazard potential. While acute toxicity testing is currently conducted using animals, studies published in recent years have shown a correlation between *in vitro* and *in vivo* acute toxicity. These studies suggest that *in vitro* methods may be helpful in predicting *in vivo* acute toxicity.

An extensive evaluation of *in vitro* methods for acute toxicity, known as the Multicenter Evaluation of *In Vitro* Toxicity (MEIC) Program, was initiated by the Scandinavian Society for Cell Toxicology in 1989 under the direction of Dr. Bjorn Ekwall, Director of the Cytotoxicity Laboratory at the University of Uppsula. Fifty reference chemicals were selected for which there was acute oral toxicity data from animal testing and blood concentrations from fatal human poisonings. Ninety-six laboratories evaluated 30 of the chemicals in 82 different *in vitro* cytotoxicity assays, and all 50 chemicals were evaluated in 61 assays. Detailed analysis of the results identified a battery of three human cell line basal cytotoxicity assays that were highly correlative with peak human lethal blood concentrations.

In 1998, Dr. Willi Halle from Germany published a Register of Cytotoxicity consisting of *in vivo* acute toxicity data and *in vitro* cytotoxicity data for 347 chemicals. These data were used to construct a regression model that could be used to predict estimated LD50 values based on cytotoxicity data. Dr. Horst Spielmann and his colleagues at the German Centre for the Documentation and Evaluation of Alternatives to Testing in Animals subsequently proposed that cytotoxicity methods could be useful for predicting starting doses for *in vivo* acute oral toxicity studies, thereby reducing the number of animals necessary for such determinations.

In 1999, amidst growing awareness of the MEIC and other studies, the National Institute of Environmental Health Sciences (NIEHS) received over 800 letters requesting that the MEIC program results be evaluated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Also in 1999, the U.S. Environmental Protection Agency (EPA) Office of Pesticides, Prevention, and Toxic Substances asked ICCVAM to review the validation status of the MEIC proposals.

ICCVAM discussed these requests at its August 1999 meeting and asked the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to prepare a technical summary of the extensive publications resulting from the MEIC studies. ICCVAM reviewed the MEIC results at its October 1999 meeting and recommended that an expert workshop should be convened to: a) evaluate the current validation status of the proposed MEIC test battery and other available *in vitro* tests that might be useful for predicting acute toxicity; and b) identify research, development, and validation efforts that might further enhance the use of *in vitro* methods to assess acute systemic toxicity.

Names of appropriate scientists to serve on an ICCVAM Workshop Organizing Committee were requested from participating ICCVAM Agencies. The Committee was charged with working with NICEATM to develop the Workshop objectives and program and to identify appropriate expert scientists to participate. The Committee held its first of several meetings in February 2000. Dr. Philip Sayre of the EPA and Dr. John Frazier of the U.S. Air Force co-chaired the Organizing Committee and guided the development of the scope and breadth of the Workshop.

In June of 2000, the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity was announced in a *Federal Register* notice. Relevant data and nominations of

scientists that should be invited to participate in the Workshop were also requested in the notice. The Organizing Committee invited 33 expert scientists from academia, industry, and Federal agencies to participate in the Workshop. NICEATM assembled relevant background materials for distribution to the invited expert scientists, other workshop participants, and the public. The Organizing Committee also identified knowledgeable agency scientists to participate in the workshop, and developed a series of questions for four breakout groups to address during the three and a half-day meeting. In September 2000, a second *Federal Register* notice announced the availability of the Workshop agenda and background materials, and requested public comments.

Invited scientific experts and ICCVAM agency scientists were assigned to one of the following four Breakout Groups:

- *In Vitro* Screening Methods for Assessing Acute Toxicity;
- *In Vitro* Methods for Toxicokinetic Determinations;
- *In Vitro* Methods for Predicting Organ Specific Toxicity; and
- Chemical Data Sets for Validation of *In Vitro* Acute Toxicity Test Methods.

The Workshop was convened in Arlington, VA on October 17-20, 2000. The NTP, the NIEHS and the EPA sponsored the Workshop, and NICEATM provided logistical, technical, and administrative support. The Workshop was open to the public and was attended by 110 participants from nine countries. In the opening plenary session, speakers provided an overview of *in vitro* acute toxicity methods and described the regulatory use of acute toxicity data. Breakout Groups were then charged with their assigned objectives and asked to develop responses to questions provided by the Organizing Committee.

The Groups reported on their progress each morning of the second and third days and gave a final report on the last day of the meeting. Opportunity for public comment was provided in all plenary and breakout sessions. Following the Workshop, each of the Breakout Groups prepared reports that represented the consensus of the invited scientists assigned to that Group.

The NICEATM subsequently assembled the Breakout Group reports and other relevant information into this Workshop Report. A separate *Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses for Acute Toxicity*, based on contributions from Drs. Rodger Curren, Julia Fentem, and Manfred Liebsch, was also prepared after the workshop. The Organizing Committee and ICCVAM reviewed the report and guidance document, and developed test recommendations to forward with these publications to Federal agencies for their consideration in accordance with Public Law 106-545. The ICCVAM recommendations are included in this report as Appendix I. Both publications are available on the Internet at the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov), and copies may be requested from NICEATM through email at: NICEATM@niehs.nih.gov.

On behalf of the ICCVAM, we gratefully acknowledge the unselfish contributions of all of the Workshop participants. We extend a special thanks to the Breakout Group co-chairs who worked diligently to ensure the timely completion and accuracy of their Group reports. The efforts of the Organizing Committee members and especially the co-chairs, Drs. John Frazier and Philip Sayre, were instrumental in assuring a productive and useful Workshop. The efforts of the NICEATM staff in coordinating local arrangements, providing timely distribution of information, and preparing the final report are acknowledged and appreciated. We especially acknowledge Dr. Ray Tice for preparation of the comprehensive background materials, Brad Blackard for coordinating communications and logistics throughout the entire project, and Michael Paris and Judy Strickland for their efforts in compiling the final workshop report.

William S. Stokes, D.V.M.
Co-Chair, ICCVAM, NIEHS

Richard N. Hill, M.D., Ph.D.
Co-Chair, ICCVAM, U. S. EPA

Executive Summary

Toxicity testing is conducted to determine the potential human health hazards of chemicals and products. Acute systemic toxicity testing is used to properly classify and appropriately label materials with regard to their lethality potential in accordance with established regulatory requirements (49 CFR 173; 16 CFR 1500; 29 CFR 1910; 40 CFR 156). Non-lethal parameters may also be evaluated in acute systemic toxicity studies to identify potential target organ toxicity, toxicokinetic parameters, and dose-response relationships. While animals are currently used to evaluate acute toxicity, recent studies suggest that *in vitro* methods may also be helpful in predicting acute toxicity.

To evaluate the validation status and current potential uses of *in vitro* methods as predictors of acute *in vivo* toxicity, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) held a four-day workshop—the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, October 17-20, 2000, in Arlington, VA, U.S.A. The Workshop provided a public venue for invited experts and ICCVAM agency participants to review the validation status of available *in vitro* methods for assessing acute systemic toxicity and to develop recommendations for validation efforts necessary to further characterize the usefulness and limitations of these methods. Workshop participants also developed recommendations for future mechanism-based research and development efforts to improve *in vitro* assessments of acute systemic lethal and non-lethal toxicity.

Specific objectives of the Workshop were to:

- Review the status of *in vitro* methods for assessing acute systemic toxicity:
  - — Review the validation status of available *in vitro* screening methods for their usefulness in estimating *in vivo* acute systemic toxicity;
  - — Review *in vitro* methods for predicting toxicokinetic parameters important to acute toxicity (i.e., absorption, distribution, metabolism, and elimination);
  - — 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 to develop and validate *in vitro* methods for assessing *in vivo* acute toxicity.

Four Breakout Groups were assigned specific objectives and asked to develop responses to questions grouped into general areas of (a) identifying needs, (b) current status, and (c) future directions. Breakout Group 1 (BG1) addressed the use of *in vitro* screening methods to estimate acute *in vivo* toxicity (i.e., median lethal dose [LD50 values]). Breakout Group 2 (BG2) discussed the role of *in vitro* methods for estimating toxicokinetic parameters needed to assess acute *in vivo* toxicity. Breakout Group 3 (BG3) examined *in vitro* methods for assessing target organ toxicity and mechanisms, and Breakout Group 4 (BG4) addressed chemical data sets for validation of acute *in vitro* toxicity tests.

In Vitro **Screening Methods for Assessing Acute Toxicity**

BG1 was asked to evaluate the validation status of available *in vitro* methods for estimating *in vivo* acute toxicity. The Group identified methods and

appropriate validation studies that might be completed within the next one to two years. The potential uses of quantitative structure-activity relationships (QSAR) as part of an *in vitro* strategy were also considered.

In identifying needs, BG1 noted that the ultimate goal is to be able to predict acute toxicity in humans. To that end, the long-term goal is to develop a battery of *in vitro* tests employing human cells and to integrate the resulting information with that derived from other sources on key physico-chemical parameters (e.g., kinetics, metabolism, and dynamics) to predict human acute toxicity. The Group also recommended investigating ways to reduce and replace animal use in acute oral toxicity tests as detailed and described in the Organisation for Economic Co-operation and Development (OECD) test guidelines 401, 420, 423, and 425. The Group recognized that the use of QSAR (e.g., Barratt et al., 1998) can provide key information in a number of areas, including the selection of test chemicals for validation studies, the interpretation of outliers, and the grouping of chemicals by structure and biological mechanisms of toxicity.

To characterize the current status of the use of *in vitro* cytotoxicity assays to predict acute *in vivo* lethality, BG1 reviewed a number of approaches but focused on the Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) and the German Center for the Documentation and Validation of Alternative Methods (ZEBET) approaches. The MEIC program investigated the relevance of *in vitro* test results for predicting acute toxicity in humans by coordinating the generation of *in vitro* cytotoxicity data for 50 chemicals by 96 laboratories using different *in vitro* methods. The MEIC management team correlated the *in vitro* findings with data compiled from human poisoning reports. The ZEBET approach involved using data from the Registry of Cytotoxicity (RC), which contains a regression analysis of *in vitro* cytotoxicity IC50 values and rodent LD50 values for 347 chemicals, to determine starting doses for LD50 tests. BG1 concluded that none of the available *in vitro* methods or proposed testing strategies had been evaluated adequately to replace the use of animals for acute systemic toxicity testing.

In the future, to reduce the use of animals in acute lethality assays, BG1 recommended using *in vitro* cytotoxicity data to predict starting doses for *in vivo* lethality studies as proposed by ZEBET (Spielmann et al., 1999). Data were presented indicating that this approach would reduce and refine animal use for acute toxicity testing. BG1 recommended that test laboratories evaluate and compare the performance of several *in vitro* cytotoxicity tests with the existing RC data. An appropriate *in vitro* cytotoxicity assay for this purpose would be a protocol employing the BALB/c 3T3 mouse fibroblast cell line, a 24-hour exposure time, and neutral red uptake as the measurement endpoint (of cytotoxicity). Other cell lines and cell viability assays could serve the same purpose equally well.

The Group also recommended that to further the goal of replacing the use of animals in acute lethality assays a prevalidation study should be initiated as soon as possible to evaluate various cell types, exposure periods, and endpoint measurements as predictors of acute toxicity. The assay, or battery of assays, determined to be the best predictor of *in vivo* lethality could be optimized further to identify, standardize, and validate simple predictive systems for gut absorption, blood-brain barrier (BBB) passage, kinetics, and metabolism. Such information has been identified as necessary to improve the ability of *in vitro* cytotoxicity data to predict *in vivo* LD50 values (Curren et al., 1998; Seibert et al., 1996; Ekwall et al., 1999). Additionally, other concepts such as TestSmart (CAAT, 1999, 2001), an approach to determine whether "one can measure cellular changes that will predict acute system failure" (A. Goldberg, personal communication) could be incorporated into *in vitro* strategies for predicting acute toxicity *in vivo.*

In the longer-term, preferably as a parallel activity, BG1 recommended focusing on the development and validation of human *in vitro* test systems for predicting human acute toxicity, integrating the approaches suggested by Breakout Groups 2 and 3. BG1 recommended that future

studies identify and evaluate mechanism-based endpoints. The Group also recognized the potential impact of genomics and proteomics in many areas of toxicology, but noted that acute toxicity testing is not currently an area of high priority for the application of these new technologies.

BG1 made the following recommendations for the prevalidation, validation, and future development of *in vitro* assays for acute lethal toxicity:

- To further reduce the use of animals in acute lethality assays, a guidance document on the application of *in vitro* cytotoxicity data for predicting *in vivo* starting doses, including details of current test protocols and their application should be prepared.
- To support a testing strategy that might eventually replace the use of animals in acute lethality assays, a working group of scientific experts should be established to identify and/or define specific *in vitro* cytotoxicity test protocols for inclusion in a prevalidation study of their use for predicting LD50 values. The working group should design and plan the study in detail and take into account the suggestions made by BG1 (Section 2.7) regarding cell type, exposure period, and endpoint measurement.
- It is anticipated that the use of simple systems that predict gut absorption, BBB passage, key kinetic parameters, and metabolism will improve the ability of *in vitro* cytotoxicity assays to predict rodent LD50 values, or any *in vivo* toxic effects. Continued development and optimization of such systems for this application is encouraged and should receive regulatory support.
- In principle, QSAR approaches, including expert systems and neural networks, could be developed and validated for predicting acute systemic toxicity. Initially, an up-to-date review of current QSAR systems for predicting rodent oral LD50 values should be undertaken. In addition, QSARs for predicting gut absorption, metabolism, and BBB passage should be developed and evaluated and initiatives to increase data sharing should be established.
- The development of simple predictive models for human acute toxicity should be a major focus.
- The evaluation and ultimate acceptance of *in vitro* assays for human acute toxicity will need a larger reference database than is presently available for validation purposes. The MEIC human database should be peer-reviewed, modified if needed, and expanded as soon as possible so that data will be available for future validation studies.

In Vitro **Methods for Assessing Acute Toxicity: Biokinetic Determinations**

The second Breakout Group, BG2, was charged with 1) evaluating the capabilities of *in vitro* methods for providing toxicokinetic information (i.e., absorption, distribution, metabolism, and elimination) that can be used to estimate target organ dosimetry for acute toxicity testing, and 2) providing recommendations for future research to accomplish this goal. BG2 also explored the role of QSAR in toxicokinetic determinations.

In identifying needs, BG2 focused on a short-term goal of improving the prediction of acute lethal effects in rodents and a long-term goal of using *in vitro* techniques to evaluate chemical kinetics and ultimately to predict sublethal acute toxic effects in humans. Needs include the ability to use *in vitro* determinations of metabolic rate and passage of a chemical across membrane barriers to improve kinetic modeling. Such information may be useful for estimating LD50 values from basal cytotoxicity data. BG2 identified the following techniques that need further development to advance *in vitro* determinations of biokinetic parameters:

- *In vitro* determination of partition coefficients, metabolism, protein binding, and stability;
- Characterization of biotransformation enzymology;

- Structural knowledge and its translation into "chemical functionalities," estimation of partition coefficients, metabolism, etc. (i.e., "*in silico*" methods such as QSAR/quantitative structure-property relationships [QSPR]);
- Biokinetic modeling, including the integration of toxicodynamic and biokinetic modeling in predicting systemic toxicity.

Evaluation of the current status of the use of *in vitro* methods to obtain biokinetic information involved a survey of *in vitro* systems for estimating metabolism and passage of membrane barriers. Biotransformation information can currently be obtained using human or animal liver preparations; however, conditions for the preparation and incubation need to be standardized. Several *in vitro* systems for measuring intestinal absorption are also available, but some cell lines lack transporters that are present *in vivo*. Glomerular filtration and reabsorption in the proximal tubule determine the renal excretion of most compounds and can be predicted from a compound's physico-chemical properties and plasma protein binding. Many of the available renal cell lines or primary cultures lack specific transporters implicated in the accumulation of several nephrotoxic compounds.

Future directions for research outlined by BG2 include using a conceptual structure to integrate kinetic information into the estimation of acute oral toxicity. Available *in vitro* data on the absorption, tissue partitioning, metabolism, and excretion of a test material could be used to parameterize a chemical-specific biokinetic model (Clewell, 1993). The model could then be used to relate the concentration at which *in vitro* toxicity occurs to the equivalent dose that would be expected to produce *in vivo* toxicity. Such models could also provide information on the temporal profile for tissue exposure *in vivo*, which can then be used to design the most appropriate *in vitro* experimental protocol (Blaauboer et al., 1999).

BG2 suggested two main testing strategies appropriate for research and development activities. One strategy was a simple method of using chemical-specific partitioning information and the other was a one-compartment model to estimate the oral dose equivalent to the *in vitro* cytotoxicity value. Research and development activities would involve collecting partitioning information for a number of chemicals, making such oral dose estimations, and then comparing the estimations to empirical values to develop a prediction model.

The other testing strategy BG2 recommended for research and development was a tiered approach for using *in vitro* cytotoxicity assays to evaluate the role of metabolism in the production of acute toxicity due to chemical exposure. The first step would be to estimate hepatocyte metabolism at a relatively low concentration (e.g., 10 µM).

If the rate of metabolism (Vmax/Km) is low, then basal cytotoxicity information could be relied upon to predict *in vivo* toxicity. If the metabolism rate is high, then the responsible enzyme system could be identified with *in vitro* studies. If the primary enzyme system is oxidative or reductive, then metabolic activation may be producing toxicity and a hepatocyte cytotoxicity assay should be performed.

If the IC50 value for hepatocytes is much lower than that for basal cytotoxicity, then the concentration-response for metabolism should be characterized to predict the *in vivo* doses that might be associated with toxicity. If the primary metabolism is detoxification (conjugation, sulfation, etc.), then the basal cytotoxicity results could be used with some confidence to predict the LD50 value.

BG2 also recommended identifying the compounds that represent the outliers in the MEIC correlations of *in vitro* basal cytotoxicity assays with LD50 values. By determining the physico-chemical properties of these compounds and their target tissues, it may be possible to identify factors that could improve the correlation between predicted oral LD50 values in rodents and empirical values. Such an exercise would help define a "predictive range" for various chemical properties over which *in vitro* basal cytotoxicity assays might be expected to provide reasonable LD50 estimates, as well as exclusion rules for

identifying compounds for which *in vitro* assays are not reliable.

Other research recommendations made by BG2 include developing validated, stable human hepatocyte systems and *in vitro* systems for key transporters (renal, biliary, etc.). Such data would provide a mechanistic description of barrier functions that could be incorporated into template physiologically-based biokinetic (PBBK) models for various classes of chemicals. Specific QSPR applications need to be developed to provide other information such as metabolic constants, binding, etc., required by PBBK models.

The interaction between kinetics and dynamics also needs to be explored. For example, the effect of toxicity on the metabolism and excretion of a chemical or, conversely, the effect of metabolism or reabsorption on the toxicity of a chemical must be taken into account. The time dimension in the conduct of these assays should be analyzed rigorously to account for duration and frequency of exposure. Other recommendations for research include:

- Understand the relationship between molecular structure, physical-chemical properties, and kinetic behavior of chemicals in biological systems;
- Develop algorithms to determine the optimum kinetic model for a particular chemical;
- Conduct research on modeling of fundamental kinetic mechanisms;
- Develop mathematical modeling techniques to describe complex kinetic systems;
- Develop mathematical modeling techniques for tissue modeling (anatomically correct models);
- Develop an optimal battery of *in vitro* assays to evaluate chemical-specific kinetic parameters;
- Establish a database of chemical-independent parameters (mouse, rat, human);
- Develop a library of generic models that are acceptable for regulatory risk assessments;
- Understand and model the mechanisms regulating the expression of proteins involved in kinetic processes (metabolizing enzymes, transport enzymes, metallothionein, membrane channels, etc.);
- Understand and model effects of changes in physiological processes on kinetics of chemicals;
- Develop mathematical modeling techniques to describe complex dynamic systems and genetic networks at the cellular and at the systemic level;
- Develop mathematical modeling techniques to describe individual variability (genetic background);
- Develop *in vitro* biological models that are equivalent to *in vivo* tissues (i.e., models that maintain specified differentiated functions that are important for the toxicological phenomena under study);
- Establish lines of differentiated human cells (e.g., derived from stem cells);
- Understand and model mechanisms of multi-cellular interactions in development of toxic responses (co-cultures);
- Understand and model relationships between cellular responses and biomarkers of systemic responses;
- Compare genomic differences or species-specific expression differences between species and within species (e.g., polymorphisms in biotransformation enzymes);
- Perform high dose to low dose extrapolation.

***In Vitro* Methods for Organ-Specific Toxicity**

Breakout Group 3 reviewed *in vitro* methods that can be used to predict specific organ toxicity or toxicity associated with alteration of specific cellular or organ functions and developed recommendations for priority research efforts necessary to support the development of methods that can accurately assess target organ toxicity.

In identifying needs, reviewing current status, and suggesting future directions, BG3 focused on the major organ systems most likely to be affected by acute systemic toxicity: liver, central nervous system, kidney, heart, hematopoietic system, and lung.

- Currently it is possible to assess the potential for hepatic metabolism in high throughput screening assay systems when identification of the specific metabolites is not needed. Future work should include development of a system that will be able to recognize the effect of products of hepatic metabolism on other organ systems in a dose responsive manner. A worldwide database is needed to compare human *in vitro* and *in vivo* data for hepatic toxicity.
- Some endpoints, assays, and cell models for the more general endpoints for *in vitro* neurotoxicity have been studied and used extensively and are ready for formal validation. However, most assays and cell models determining effects on special functions still need significant basic research before they can be used as screening systems.
- Several *in vitro* models to assess BBB function are currently being evaluated in a prevalidation study sponsored by the European Centre for the Validation of Alternative Methods (ECVAM). Models being studied include immortalized endothelial cell lines of both human and animal origin, primary bovine endothelial cells co-cultured with glial cells, and barrier-forming continuous cell lines of non-endothelial origin. Preliminary results from the prevalidation study show that the rate of penetration of compounds that pass the BBB by simple diffusion can be estimated by the determination of log P, or by the use of any cell system that forms a barrier. To assess the impairment of the transporter functions of the BBB, an *in vitro* system with a high degree of differentiation is required, including the significant expression of all transporter proteins representing species-specific properties. At present, this can only be achieved in primary cultures of brain endothelial cells co-cultured with brain glial cells.
- To assess kidney function, *in vitro* systems will need to utilize metabolically competent kidney tubular cells and be able to evaluate the barrier function of the kidney. A system to assess this parameter is currently being studied in Europe with support from ECVAM. In addition, *in vitro* systems will need to assess specific transport functions. More research is needed in this area to develop mechanistically based test systems.
- The Group's review of *in vitro* models for cardiovascular toxicity concluded that none have been validated. The likely candidate *in vitro* systems for an acute cardiotoxicity testing scheme could include: (a) short term single-cell suspensions of adult rat myocytes to measure products of oxidation; (b) primary cultures of neonatal myocytes to measure changes in beating rates and plasma membrane potentials; (c) co-culture of smooth muscle cells or endothelial cells with macrophages to examine rate of wound healing (DNA synthesis); and (d) an immortalized cell line (e.g., the human fetal cardiac myocyte line) to measure classical cytotoxic endpoints. It also may be important to include the perfused heart preparation for a comparison with other *in vitro* models since this system is more representative of the *in vivo* situation than cell culture systems.
- Regarding the status of *in vitro* methods for assessing toxicity on the hematopoietic system, ECVAM is supporting a validation study of the use of colony-forming assays to test for the development of neutropenia. Methods to assess effects on thombocytopoiesis and erythropoiesis are also available and can be considered for validation. ECVAM is also supporting a new project to develop and prevalidate *in vitro* assays for the prediction of thrombocytopenia. A preliminary study by ECVAM's laboratories confirmed the usefulness of

the *in vitro* test for screening drug toxicity to megakaryocyte progenitors. The study also showed that cord blood cells (CBC) can be used as a human source, are more suitable for this purpose, and provide a means of avoiding ethical problems connected with the collection of human bone marrow cells (BMC).

- *In vitro* evaluation of acute respiratory toxicity should consider several cell types since the tracheal-bronchial epithelial lining consists of stratified epithelium and diverse populations of other cell types, including ciliated, secretory (e.g., mucous, Clara, serous), and non-secretory cells. BG3 reviewed a number of models that could be used to indicate chemical-induced cell damage or death. The cells of the airways are relatively accessible to brushing, biopsy, and lavage, and therefore lend themselves for harvesting and use as primary cells (Larivee et al., 1990; Werle et al., 1994). The most useful markers are those that relate to the basic mechanisms by which airway epithelia respond to toxic exposure. However, most assays and cell models for determining effects on special functions still need significant basic research before they can be used as screening systems.

BG3 indicated that specific organ toxicity data would not be needed routinely to assess acute systemic toxicity and recommended a tiered approach to assess the acute systemic toxicity potential of xenobiotics. The first step involves physico-chemical characterization and initial biokinetic modeling for the chemical of interest. Such information should be used to compare the test material with chemicals that have a similar structure or properties and for which toxicity data exist that may be useful for predicting organ distribution. The second step is to conduct a basal cytotoxicity assay. The third step is to determine the potential for metabolism-mediated toxicity. The next two steps can be done in either order. Step 4 involves assessing the effect of the test substance on energy metabolism by using a neuronal cell line that expresses good aerobic energy metabolism. Results from this system will help determine if the nervous or cardiovascular systems are likely targets. If there is evidence of metabolism (from Step 3), Step 4 must be done with both the parent compound and the metabolite(s). The fifth step is to assess the ability of the compound to disrupt epithelial cell barrier function using a transepithelial resistance assay across a membrane. The results from such a system will help determine if organs (e.g., brain, and kidney) that depend on barriers for defense against toxic insult are likely to be targets. If the compound causes disruption of barrier function at a concentration lower than the basal cytotoxicity, the endpoint used in determining the effect on the organism might need to be lowered to take this into consideration. If there is evidence of metabolism in Step 3, Step 5 must be done with both the parent compound and the metabolite(s).

Chemical Data Sets for Validation of *In Vitro* Toxicity Tests

Breakout Group 4 defined the chemical data sets required for validation studies, identified existing resources, and recommended approaches for using existing data sets and/or compiling or developing new data sets.

Rather than develop specific lists of chemicals, BG4 developed criteria for establishing a database of chemicals to use to validate individual tests or prediction models. In identifying needs, BG4 noted that chemicals chosen for use in a validation study should be distributed uniformly across a broad range of toxicity. Two sets of chemicals are needed: 1) training sets that can be used for method development and 2) validation sets that can be used to confirm the predictive capacity of the tests. In selecting chemicals for use in validation studies, needs of the user communities must be met. The performance parameters of the *in vivo* tests must be clearly defined prior to chemical selection if the results of these tests are to serve as a baseline for judging success.

To evaluate the current status of chemical data sets for prevalidation and validation activities, a number of databases were discussed. The NTP database would be a useful component of any primary database of chemicals for validation. The

high production volume (HPV) database, containing predominantly industrial chemicals, might not meet the needs of all user communities. The U.S. Environmental Protection Agency pesticides database and the U.S. Food and Drug Administration drugs and food additive databases contain associated LD50 data of good quality, but accessibility of the data may be impeded by confidentiality claims by the sponsors.

For future activities, BG4 recommended convening an expert committee to assemble a reference set of test chemicals from existing databases according to the following criteria:

- Chemicals selected must be consistent with the test protocol and its prediction model, be physically and chemically compatible with the test system, and include the relevant chemical classes.
  - — The definition of chemical class is context-specific.
  - — The developers of the test must specify the parameters that define the class.
  - — The chemicals must be chosen independently.
- The toxicity must cover the range of response with uniform distribution.
- The number of chemicals used in the subset will depend on the nature of the test and the questions being asked, and should be determined with statistical advice.

BG4 also recommended undertaking a study of existing databases to determine the variation in rodent LD50 results introduced by different laboratories and by different protocols used by various regulatory agencies.

To build upon the MEIC foundation, BG4 recommended that an expert panel review the MEIC approach for measuring acute toxicity parameters in humans. The Group agreed that a standard approach for measuring acute toxicity parameters is necessary and that existing sources of information should be searched carefully to ensure that all human data are obtained.

1.0 INTRODUCTION

This report summarizes the proceedings and outcome of the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, October 17-20, 2000, in Arlington, VA, U.S. This Workshop, the first convened by ICCVAM and NICEATM, evaluated the status of available *in vitro* methods for assessing acute toxicity. These included screening methods such as those that may be used to predict the starting dose for *in vivo* animal studies, and *in vitro* methods for generating information on toxicokinetics, target organ toxicity, and mechanisms of toxicity. The Workshop also developed recommendations for validation efforts necessary to further characterize the usefulness and limitations of these methods and for research and development efforts that might further improve *in vitro* assessments of acute systemic toxicity. Notice of the Workshop and requests for nomination of scientific experts and submission of information on relevant past, current, or future studies were announced in two Federal Register notices (See **Appendix H**).

This introduction briefly summarizes the purpose and history of acute toxicity testing and the purpose and conduct of the Workshop. The final reports from the Breakout Groups are presented in **Sections 2** through **5**. **Section 6** provides a glossary, while **Section 7** contains the Registry of Cytotoxicity (RC) Data, a database of LD50 values and *in vitro* cytotoxicity IC50 values, and a regression analysis between the two values. **Section 8** contains all references cited in the Breakout Group reports and appendices. The Appendices provide supplementary materials, including the Workshop agenda, a summary of the plenary sessions, guidance for the Breakout Groups, the background document provided to Workshop participants, the NICEATM summary of the Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC), regulatory requirements for acute toxicity information, a bibliography, the list of Workshop participants, Federal Register notices regarding the Workshop, and ICCVAM test method recommendations forwarded to Federal agencies.

1.1 History and Purpose of Acute Toxicity Testing

Acute oral systemic toxicity testing is conducted to determine the hazard potential of a single oral exposure to various chemicals and products. Four regulatory agencies in the United States, the Department of Transportation (DOT), the Consumer Product Safety Commission (CPSC), the Occupational Safety and Health Administration (OSHA), and the U.S. Environmental Protection Agency (EPA) require industry to label chemicals and products with hazard information based on LD50 estimates. DOT requires oral lethality data to determine the transportation requirements for hazardous substances (49 CFR 173). CPSC requires such information for labeling hazardous substances so as to protect consumers when such products are used in the home, the school, and recreational facilities (16 CFR 1500). OSHA requires the use of acute lethality data to implement labeling requirements for the hazard communication program to protect employees (29 CFR 1910). Certain EPA regulatory programs also require the submission or generation of acute toxicity data for hazard classification purposes (40 CFR 156). During acute toxicity testing, non-lethal endpoints may also be evaluated to identify potential target organ toxicity, toxicokinetic parameters, and/or dose-response relationships.

As shown in Table 1, the international community also uses acute oral toxicity data as the basis for hazard classification and the labeling of chemicals for their manufacture, transport, and use (OECD, 1998a). Other potential uses for acute toxicity testing data include:

- Establishing dosing levels for repeated-dose toxicity studies;
- Generating information on the specific organs affected;
- Providing information related to the mode of toxic action;
- Aiding in the diagnosis and treatment of toxic reactions;
- Providing information for comparison of toxicity and dose response among

- substances in a specific chemical or product class;
- Aiding in the standardization of biological products;
- Aiding in judging the consequences of single, high accidental exposures in the workplace, home, or from accidental release;
- Serving as a standard for evaluating alternatives to animal tests.

Table 1.1 OECD Harmonized Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances—Oral Toxicity (OECD, 1998a)

Acute Toxicity Route	Toxicity Class 1	Toxicity Class 2	Toxicity Class 3	Toxicity Class 4	Toxicity Class 5
Oral LD50 Values (mg/kg) [approximate]	5	50	300	2000	5000

Historically, lethality has been the primary toxicological endpoint in acute toxicity tests. Trevan (1927) was the first to attempt to standardize a method for assessing the toxicity of potent biological toxicants, the progenitor of the "lethal dose, 50% (LD50) test". The classical LD50 test procedure that evolved from this innovation in the 1970s and early 1980s used from 100 to 200 animals per test substance (Galson, 2000). Although other information, such as the slope of the dose-response curve, confidence interval for the LD50, and toxic signs, could also be obtained from this test, the procedure was severely criticized for both scientific and animal welfare reasons (Zbinden and Flury-Roversi, 1981). These criticisms eventually resulted in the proposal and adoption of a new guideline (OECD TG 401; OECD, 1987) that reduced the required number of animals to 20. This has become the most widely used method for defining the acute toxicity of a chemical and a mandatory-testing requirement for new chemicals. More recently, the acute toxicity test procedure has been modified in various ways to refine and further reduce the number of animals used to a maximum of 16 (OECD, 1992; 1996; 1998b). The Globally Harmonized Scheme for Hazard Classification prompted a re-assessment of all of the OECD *in vivo* test guidelines for acute toxicity (i.e., fixed dose, up and down procedure, acute toxic class method) to ensure that regulatory needs are met while minimizing animal usage and maximizing data quality.

Recent studies suggest that *in vitro* methods may be helpful in predicting acute toxicity and reducing the number of animals necessary to assess acute toxicity. 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* basal 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 in validation studies to determine their reliability and relevance for generating information to meet regulatory requirements for acute toxicity testing. Development and

validation of *in vitro* methods that can establish accurate dose-response relationships will be necessary before such methods can be considered for the reduction or replacement of animal use for acute toxicity determinations.

1.2 Purpose and Objectives of the Workshop

The International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity examined the status of available *in vitro* methods for predicting acute toxicity, including screening methods for acute toxicity, and other methods that might be suitable to predict the starting dose for *in vivo* animal studies, and methods for generating information on toxicokinetics, target metabolism organ toxicity, and mechanisms of toxicity. The Workshop developed recommendations for validation efforts necessary to further characterize the usefulness and limitations of these methods. Recommendations were also developed for future mechanism-based research and development efforts that might further improve *in vitro* assessments of acute systemic lethal and non-lethal toxicity.

Specific objectives of the Workshop were to:

- Review the status of *in vitro* methods for predicting acute systemic toxicity:
  - — Review the validation status of available *in vitro* screening methods for their usefulness in estimating *in vivo* acute systemic toxicity;
  - — Review *in vitro* methods for predicting toxicokinetic parameters relevant to acute toxicity (i.e., absorption, distribution, metabolism, elimination);
  - — 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 to adequately characterize the usefulness and limitations of proposed *in vitro* methods;
- Identify reference chemicals for development and validation of *in vitro* methods for assessing *in vivo* acute toxicity;
- Identify priority research efforts necessary to support the development of *in vitro* methods to assess acute systemic toxicity adequately. Such efforts might include incorporation and evaluation of new technologies such as gene microarrays, and development of methods necessary to generate dose response information.

1.3 Conduct of the Workshop

The International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, which was open to the public, was conducted over three and a half days. The final agenda for the meeting is provided in **Appendix A**. As the agenda shows, the Workshop began with a plenary session to frame the purpose and objectives of the Workshop and formulate the problem of using *in vitro* tests to predict *in vivo* acute toxicity. A summary of the opening plenary session is provided in **Appendix B.** The opening plenary session was followed by Breakout Group discussions for two and a half days. Each of the four Breakout Groups was comprised of 12 to 18 individuals who were invited scientific experts or ICCVAM agency participants. Breakout Groups addressed their assigned objectives for the Workshop by developing responses to questions provided in the background materials for the Workshop (See **Appendix C**). Breakout Groups reported on their progress each morning of the second and third days, and gave a final report on the last day of the meeting. Written reports of each Breakout Group's findings, conclusions and recommendations are provided in **Sections 2** through **5**. Public observers were invited to provide comments in both plenary and breakout sessions of the Workshop. A summary of public comments during plenary sessions is provided in **Appendix B.** After the Workshop, ICCVAM reviewed the Breakout Group reports and developed test method recommendations for Federal agencies (see **Appendix I**).

2.0 *IN VITRO* SCREENING METHODS FOR ASSESSING ACUTE TOXICITY

2.1 Introduction

Since the early work of Pomerat and Leake (1954), Eagle and Foley (1956), and Smith and colleagues (1963), research over the last 50 years has been conducted to evaluate the potential use of *in vitro* cell systems for predicting acute toxic effects *in vivo*. Significant correlations between cytotoxicity *in vitro* and animal lethality have been demonstrated on numerous occasions (for reviews see Phillips et al., 1990; Garle et al., 1994), as have correlations between cytotoxicity *in vitro* and systemic and topical effects from acute exposures to chemicals. Several newer initiatives directed toward reducing and replacing the use of laboratory animals for acute toxicity testing have emerged (Curren et al., 1998; Ohno et al., 1998; Spielmann et al., 1999; Ekwall et al., 2000); these initiatives were reviewed as part of the charge given to Breakout Group 1 (*In Vitro* Screening Methods) at this Workshop.

2.1.1 *Charge to the Breakout Group*

Breakout Group 1 (BG1) was asked to evaluate the validation status of available *in vitro* methods for estimating *in vivo* acute toxicity and was requested to identify methods and appropriate validation studies that might be completed within the next one to two years. It was also envisaged that the Breakout Group would evaluate potential uses of QSAR as part of an *in vitro* strategy.

2.1.2 *Objectives*

The specific objectives of the Workshop pertinent to the charge given to BG1 were given as follows:

(1) Review the validation status of available *in vitro* screening methods for their usefulness in estimating *in vivo* acute toxicity.
(2) Recommend candidate methods for future evaluation in prevalidation and validation studies.
(3) Recommend validation study designs that can be used to adequately characterize the usefulness and limitations of proposed *in vitro* methods.
(4) Identify priority research efforts necessary to support the development of mechanism-based *in vitro* methods to assess acute systemic toxicity.

In its opening deliberation on these objectives, BG1 members decided to limit the review to methods for reducing or replacing animal use for determining acute lethality with the understanding that Breakout Group 3 would focus on methods for assessing acute systemic toxicity.

2.2 Background

Cytotoxicity has been defined as the adverse effects resulting from interference with structures and/or processes essential for cell survival, proliferation, and/or function (Ekwall, 1983). 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. Ekwall (1983) described the concept of "basal cell functions" that virtually all cells possess (mitochondria, plasma membrane integrity, etc.) and suggested that, for most chemicals, toxicity is a consequence of non-specific alterations in those cellular functions which may then lead to effects on organ-specific functions and/or death of the organism.

Ekwall drew two important inferences from his early studies: that (a) cell cultures (notably cell lines) can be used to detect basal cytotoxicity; and (b) many chemicals exert cytotoxic effects on these cultures at concentrations which would be lethal in humans. Ekwall recognized that there will be exceptions and ultimately refinements needed in the development of a test battery for predicting human lethality, as, for example, incorporating test strategies for identifying chemicals that produce cell selective (organ specific) toxicity at lower concentrations than “basal” (or general) cytotoxicity.

Others likewise concluded that, since the actions of chemicals that produce injury and death are ultimately exerted at the cellular level, cytotoxicity assays may be useful for the prediction of acute lethal potency (Grisham and Smith, 1984). Based on that premise, a considerable amount of research has been undertaken into the development and evaluation of *in vitro* tests for use as screens and as potential replacements for *in vivo* LD50 tests. Good agreement between cytotoxicity *in vitro* and animal lethality have been reported by numerous groups (see reviews by Phillips et al., 1990; Garle et al., 1994; Guzzie, 1994). However, none of the proposed *in vitro* models have been evaluated in any formal studies for reliability and relevance, and their usefulness and limitations for generating information to meet regulatory requirements for acute toxicity testing have not been assessed.

More recently, Spielmann and colleagues have conducted studies to indicate that, as a first step toward replacement of LD50 tests, *in vitro* cytotoxicity data could be used now to identify the appropriate starting dose for *in vivo* studies, thereby reducing the number of animals necessary for such determinations (Spielmann et al., 1999). Other studies have indicated an association between chemical concentrations inducing cytotoxic effects *in vitro* and human lethal blood concentrations (Ekwall et al., 2000). Several groups have proposed the use of *in vitro* cytotoxicity tests in tiered testing schemes. These tests include proposed strategies for using *in vitro* test data as a basis for classifying and labeling new chemicals, thereby reducing (and possibly replacing) the need for acute toxicity tests in animals (Seibert et al., 1996) and for *in vitro* cytotoxicity data and other information in a tiered approach to replace oral LD50 tests (Curren et al., 1998). Curren and colleagues recognized that the application of their proposal was limited because of insufficient information on the many cellular mechanisms involved in chemical-induced lethality and because the most reliable *in vitro* models for gastrointestinal uptake, blood-brain barrier (BBB) passage, and biotransformation for more precise quantitative *in vivo* toxic dose/exposures were not yet identified.

To summarize, many investigations of the relationship between *in vitro* cytotoxicity and acute toxicity *in vivo* have been reported. Since it was not possible to critically review and discuss all of the published literature in the course of the Workshop, a selection of recent key activities and reports that included the most advanced and extensive efforts to develop alternative methods for lethality was made for consideration by Breakout Group 1 (Appendix D). The most intensive discussions focused on the ZEBET and MEIC approaches, which are outlined below in detail for the reader's reference (Sections 2.2.1-2.2.6 and 2.2.7, respectively).

2.2.1 *Prediction of In Vivo Starting Doses (ZEBET Approach)*

Investigators (Halle et al., 1997; Halle 1998; Spielmann et al., 1999) have proposed a strategy to reduce the number of animals required for acute oral toxicity testing. The strategy is referred to in this document as the ZEBET approach where ZEBET is the acronym for Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergaenzungsmethoden zum Tierversuch (the National Center for Documentation and Evaluation of Alternative Methods to Animal Experiments). The strategy involves using *in vitro* cytotoxicity data to determine the starting dose for *in vivo* testing. They report the findings of an initial study conducted to assess the feasibility of applying the standard regression between mean IC50 values (i.e., IC50*x*, the mean concentration estimated to affect the endpoint in question by 50%) and acute oral LD50 data included in the Register of Cytotoxicity (RC) to estimate the LD50 value which can then be used to determine the *in vivo* starting dose.

The RC is a database of acute oral LD50 data from rats and mice (taken from the NIOSH Registry of Toxic Effects of Chemical Substances [RTECS]) and IC50*x* values of chemicals and drugs from *in vitro* cytotoxicity assays (Halle and Goeres, 1988; Halle and Spielmann, 1992). It

currently contains data on 347 chemicals (Halle, 1998; Spielmann et al., 1999). The main purpose of establishing the RC was to evaluate, with a large amount of non-selected data from various chemicals with different systemic oral toxicities, whether basal cytotoxicity (averaged over various cells, cell lines, and/or toxicity endpoints) is a sufficient predictor for acute systemic toxicity.

Apart from the fact that basal cytotoxicity was an acceptable predictor (i.e., LD50 values localized in the dose range around the regression line by the empirical factor $F_G \leq \log 5$) of the LD50 for 74% of the RC chemicals (Halle and Spielmann, 1992), the predicted LD50 value can be used as a starting dose in acute oral toxicity testing to reduce the number of animals. This concept was first discussed at an ECVAM workshop (Seibert et al., 1996) as it related to refinements of *in vivo* acute toxicity tests by the use of new sequential dosing methods such as the Acute Toxic Class method ([ATC; OECD TG 423] OECD, 1996) and the Up-and-Down Procedure ([UDP; OECD TG 425] OECD, 1998b). In these tests, the number of animals needed depends upon the correct choice of the starting dose, since the number of consecutive dosing steps would be reduced as the starting dose more closely approximates the true toxicity class (ATC), or the true LD50 (UDP) (i.e., the more precisely the starting dose is predicted, the fewer animals that need to be used).

2.2.2 *Characterization of the RC*

The first registry, RC-I (Halle and Göeres, 1988), contained 117 chemicals and served as a training data set to establish a linear regression model for predicting oral LD50 values. A second data set of 230 chemicals, RC-II, verified the regression obtained with RC-I (Halle, 1998). Currently, a third RC of 150 chemicals that will increase the number of chemicals to almost 500 is in preparation. It is important to note that, in order to keep the registry unbiased, published data that were complete and met the acceptance criteria described below were included in the RC without further restriction. Thus, the RC contains data of nonselected chemicals. However, it has to be noted that selecting only published data may be a slight bias in itself because it identifies chemicals of scientific interest, public concern, etc., so that pharmaceuticals, pesticides, consumer products (e.g., cosmetics, food additives, etc.), and biocides are over-represented compared to industrial chemicals; the majority of the latter are of low toxicity (I. Gerner, BgVV, personal communication, as cited in Spielmann et al., [1999]).

The acceptance criteria for the *in vitro* cytotoxicity data were defined as follows:

- At least two different IC50 values were available, either from different cell types, or from different cell lines, or from different cytotoxicity endpoints.
- Only cytotoxicity data obtained with mammalian cells were accepted.
- Cytotoxicity data obtained with hepatocytes were not acceptable.
- The chemical exposure time in the cytotoxicity tests was at least 16-hr.

Only the following cytotoxicity endpoints were accepted:

- Cell proliferation: cell number, cell protein, DNA content, DNA synthesis, colony formation;
- Cell viability, metabolic indicators: MIT-24, MTT, MTS, XTTC;
- Cell viability, membrane indicators: Neutral Red Uptake (NRU), Trypan blue exclusion, cell attachment, cell detachment;
- Differentiation indicators.

The acceptance criteria for the *in vivo* data were defined as follows:

- Only LD50 values published in RTECS were used.
- If different issues of RTECS reported different LD50 values, then the first LD50 value was used for the RC. This value is also the highest value reported, since NIOSH replaces an LD50 value whenever a smaller value is available in the literature. A continuous change of *in*

vivo data in the RC would not have been acceptable because the RC database had to be 'closed' to form a training data set (RC-I) and later a verification data set (RC-II). Therefore, since the beginning of data collection for RC-II, all LD50 values were only taken from the 1983 RTECS issue, and later issues were not used.

The IC50 values from RC-I and RC-II, for a total of 347 chemicals, were obtained from 157 original publications in the literature. In the regression analysis for 347 chemicals, 1,912 single IC50 values were averaged (geometric means) per chemical to one IC50*x* value and then paired with 347 *in vivo* acute oral LD50 values. Whenever obtainable from RTECS, oral *in vivo* LD50 data from the rat were used (282 values). As a second priority, LD50 data from the mouse were used (65 values). Before data of rats and mice were merged in the RC, regression analyses performed separately with rat and mouse data justified this procedure (Halle, 1998). Although, by pairing 347 *in vitro* IC50*x* data with 347 *in vivo* LD50 data, an equal weight is given to each chemical, it has been criticized by reviewers that the IC50*x* is the geometric mean of a few up to many single data [minimum: n = 2, maximum: n = 32] per chemical. However, if the RC regression is recalculated with the means of only the smallest and the largest IC50 values per chemical, there are no differences in the regression function (Halle, personal communication).

To obtain a prediction model, a linear regression was derived from pairs of the log-transformed IC50*x* values and oral LD50 values (in mmol/kg), where 'a' is the intercept and 'b' is the regression coefficient, to produce the regression model [log (LD50) = b x log (IC50*x*) + a] shown graphically in Figure 2.1:

log (LD50) = 0.435 x log (IC50*x*) + 0.625

To allow comparison of the predictive value of the RC (or parts of the RC) with other similar approaches (prediction of the LD50 from basal cytotoxicity), an empirical linear-shaped prediction interval of a factor (F_G) of ± log 5 was defined (Figure 2.1). The linear-shaped boundaries should not be confused with the curved boundaries of a probability-based confidence interval. Halle defined this interval empirically as an acceptability measure based on information of the required and expected precision of rodent oral LD50 data (Halle and Spielmann,1992).

To evaluate the validity of the regression model, the key parameters of the regression for RC-I, RC-II, and RC-I+II (Table 2.1) were compared with the regression parameters obtained with single mammalian cell lines. Table 2.1 shows that all regression lines have essentially identical intercepts and regression coefficients (slopes) regardless of whether single parts of the RC or the whole RC were analyzed, or whether data from single studies with only one cell line were used. In addition, the percentage of data within the defined prediction interval (± log 5) is almost constant (73%-77%). In summary, the regression function derived from the RC, and from the RC subsets, seems to be a reliable description of the general relationship between basal cytotoxicity and rodent oral systemic LD50 values. This relationship can consequently be used as a mathematical model for prediction of rodent oral LD50 values from basal cytotoxicity.

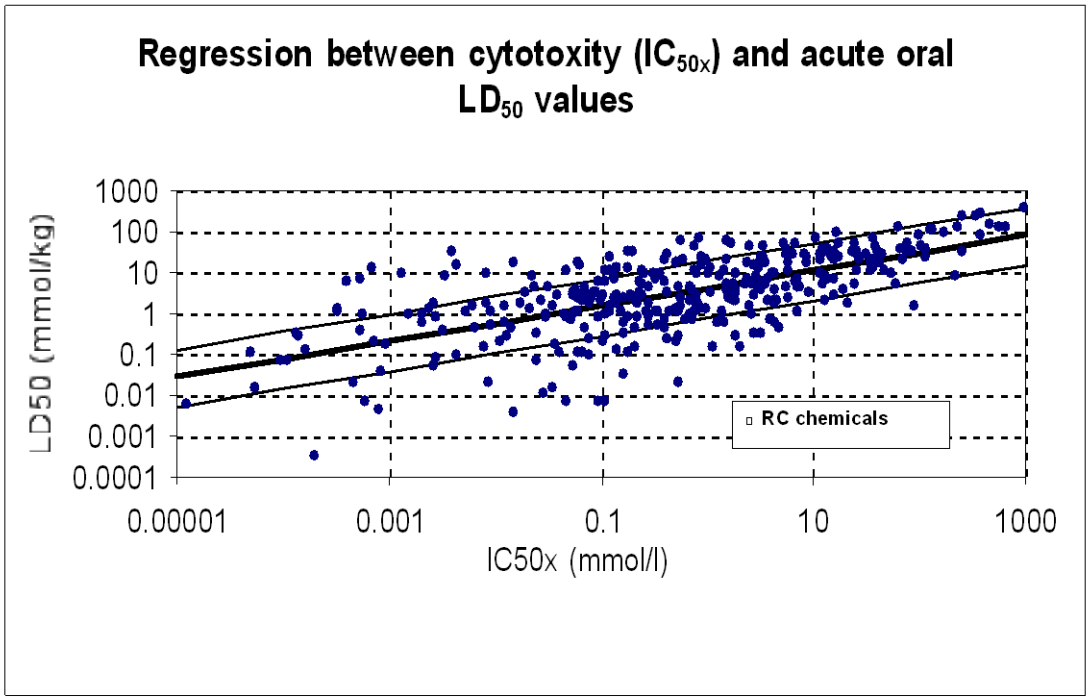


Figure 2.1. Registry of Cytotoxicity regression between cytotoxicity (IC50*x*) and rodent acute oral LD50 values of 347 chemicals

The heavy line represents the fit of the data to a linear regression model (r=0.67); the two additional lines represent the boundaries of ± log 5, an acceptance interval for this prediction model (Halle and Spielmann, 1992). This factor, $F_G = \pm \log 5$, was established based on information of the required and expected precision of LD50 values from rodent studies. The equation of the regression line (prediction model) reads: log (LD50) = 0.435 x log (IC50*x*) + 0.625.

Table 2.1. Linear regression parameters of two RC issues and two single studies using one cell line and one cytotoxicity endpoint

RC or Cell line**	Number of Chemicals (n)	Correlation Coefficient (r)	Intercept (a)	Regression Coefficient (b)	% Chemicals in Prediction Interval[a]	Reference[b]
RC-I *	117	0.667	0.637	0.477	74	1
RC-II *	230	0.666	0.634	0.414	73	2
RC-I+II *	347	0.672	0.625	0.435	73	2, 3, 4
BCL-D1**	22	0.720	0.536	0.633	77	5
3T3-L1 **	91	0.720	0.631	0.427	74	6

[a]Prediction interval for regression line is $\pm F_G \leq \log 5$.

[b]References: 1 = Halle and Göeres, 1988; 2 = Halle, 1998; 3 = Halle et al., 1997; 4 = Spielmann et al., 1999; 5 = Knox et al., 1986; 6 = Clothier et al., 1988.

2.2.3 *Influence of the Starting Dose in the Acute Toxic Class (ATC) Method.*

Introductory note: The current accepted version of the ATC is the version adopted by the OECD in 1996 (OECD TG 423; OECD, 1996). Several updated drafts have been created since the OECD endorsed a new Globally Harmonized System (GHS) for the classification of chemicals in November 1998 (OECD, 1998a). The most recent draft of TG 423 was issued after the ICCVAM Workshop was held (OECD, October, 2000; http://www.oecd.org/ehs/test/health.htm). Consequently, the following analysis focuses on the 1996 OECD version of TG 423, but also attempts to address recent developments.

Following a national and an international experimental validation study of the ATC Method (Schlede et al., 1992, 1994; Diener et al., 1995), the ATC was accepted by the OECD (OECD TG 423; OECD, 1996) as an alternative to the classical LD50 test for acute oral toxicity. In the TG 423 procedure, a substance is tested in a stepwise dosing procedure with each step using three animals of a single sex at the same time. The proportion of survivors dosed at one step determines the next step, which is: (a) no further testing, or (b) dose three additional animals with the same dose, or (c) dose three additional animals at the next higher or the next lower dose. Originally, the method was developed and experimentally validated with two sexes and three different fixed starting doses (25, 200, and 2000 mg/kg body weight [b.w.]) reflecting the European Union (EU) hazard classification system. A thorough biometrical analysis (Diener et al., 1995) showed that the ATC is applicable to all hazard classifications currently in use.

Figure 2.2 shows, for example, that to classify a chemical as "toxic" or "very toxic", 1-2 consecutive steps could be saved if 25 mg/kg b.w. was used as the starting dose instead of the medium dose. With increasing distance between the true toxicity class and the starting dose, the number of dosing steps increases. This effect is shown in more detail in Table 2.2, which shows the expected number of animals used and the number that died in relation to starting dose and true LD50 for a dose-mortality slope of $\beta = 2$. Biometrical calculations with other slopes (from $\beta = 1$ to $\beta = 6$) revealed the dependency in Table 2.2 is only slightly affected by the dose-mortality slope (for details see Diener et al., 1995).

In summary, one to three dosing steps can be avoided if the optimum starting dose can be predicted from a preceding cytotoxicity test. Taking into account that approximately 75% of the LD50 values predicted from basal cytotoxicity tests are expected to fall within the prediction interval of $\pm \log 5$ (see Table 2.1), and, moreover, that the space between the three starting doses (25, 200, 2000 mg/kg b.w.) is a factor of about 10, it was anticipated that, for most chemicals, the starting dose predicted from cytotoxicity would have been the dose requiring the fewest consecutive steps to reach a classification.

In November 1998, the GHS for the classification of chemicals, which uses four toxicity classes instead of the three used by the current EU system, was endorsed by the OECD (OECD, 1998a). A fifth toxicity class (>2000–5000 mg/kg b.w.) was additionally introduced for special regulatory purposes. As a consequence, the current updated Draft OECD TG 423 (OECD, October, 2000; http://www.oecd.org/ehs/test/health.htm) now uses four different starting doses (5, 50, 300, and 2000 mg/kg b.w.), but the upper boundary of the fifth class of 5000 mg/kg b.w. is not used as a starting dose. Figure 2.3 shows the proposed revision of the ATC.

For the version of the revised ATC to be consistent with the OECD GHS classification system, biometrical calculations of the expected number of animals used and dead in relation to starting dose, true LD50, and dose-mortality slope, have been published (Diener and Schlede, 1999). While any increase in the number of possible starting doses theoretically increases the potential to save dosing steps when using the optimal starting dose, only a small decrease in

animal numbers is expected compared to the current ATC method because (a) the number of starting doses has been increased at the toxic end of the scale, where the prediction of the LD50 by IC50 is less accurate than at the non-toxic end of the scale, and (b) the entire scale is still about the same length.

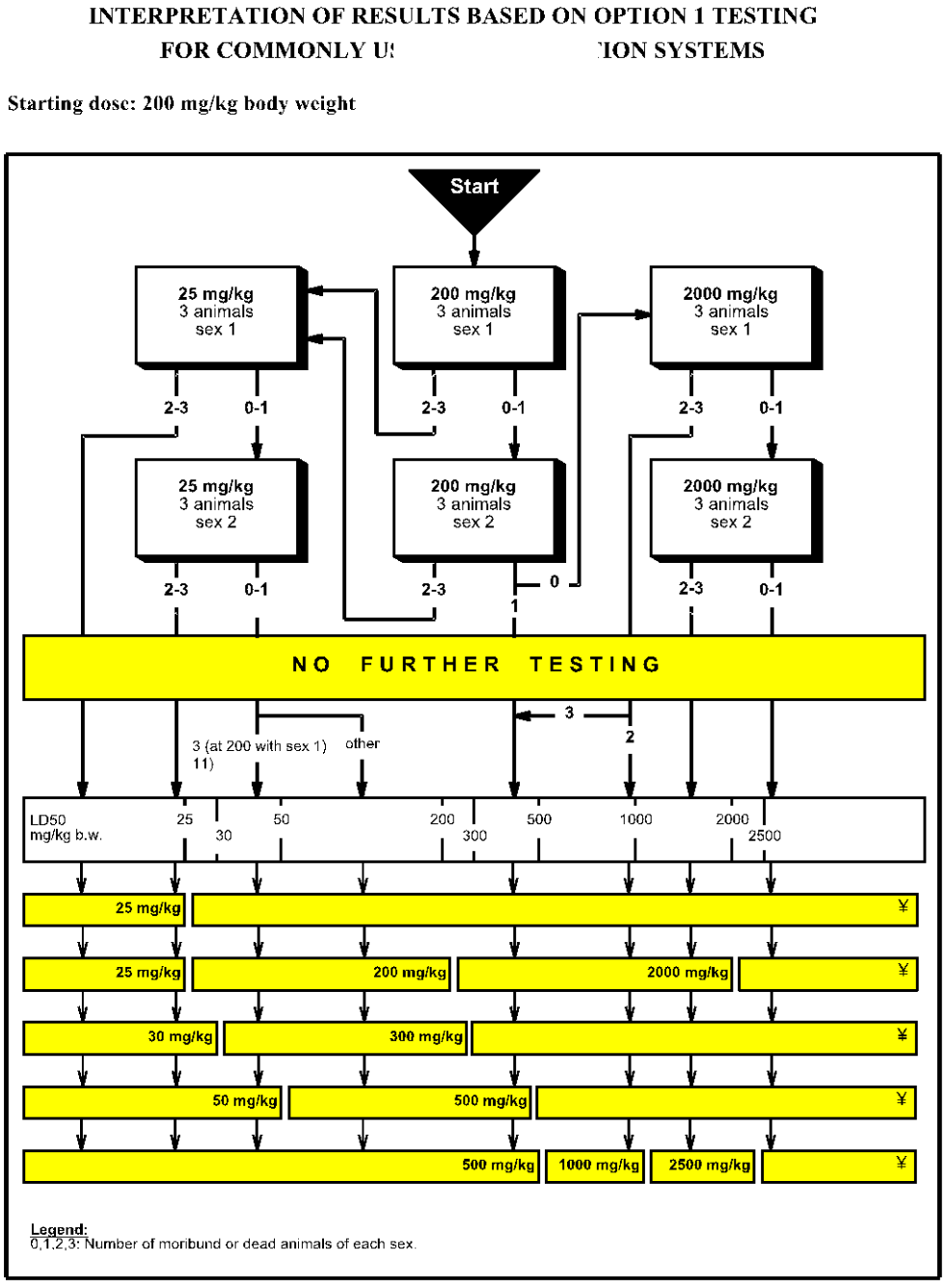


Figure 2.2 Principle of the Acute Toxic Class (ATC) method: medium starting dose
Source: OECD TG 423, Annex 3b (OECD, 1996). Example shows the possible dosing steps when 200 mg/kg b.w. is used as the starting dose. Depending on the toxicity of the test substance, 2 to 4 steps may be necessary to reach a classification according to hazard classification systems currently in use.

Table 2.2. Influence of the ATC starting dose on total number of animals (used and dead) in relation to the true LD50 for slope = 2[a]

	Starting dose in mg/kg body weight					
	25		200		2000	
True LD50	Used	Dead	Used	Dead	Used	Dead
1	3.0	3.0	6.0	6.0	9.0	9.0
2	3.0	3.0	6.0	6.0	9.0	9.0
5	3.1	2.8	6.1	5.8	9.1	8.8
10	3.4	2.7	6.4	5.6	9.4	8.6
20	4.6	2.8	7.2	5.3	10.2	8.3
50	7.5	3.3	8.6	4.2	11.6	7.2
100	9.3	3.2	9.3	3.3	12.2	6.2
200	11.2	3.2	9.7	3.1	12.0	5.3
500	14.0	3.3	9.3	3.3	10.0	3.9
1000	14.9	2.6	9.1	2.6	9.2	2,7
2000	15.4	1.8	9.4	1.8	9.3	1.8
5000	16.5	1.0	10.5	1.0	9.0	1.0
10000	17.3	0.4	11.3	0.4	7.7	0.4
20000	17.8	0.1	11.8	0.1	6.6	0.1
50000	18.0	0.0	12.0	0.0	6.1	0.0
100000	18.0	0.0	12.0	0.0	6.0	0.0

[a]Presented by W. Diener at the OECD ad hoc expert meeting on evaluation of the ATC in Berlin, Germany, 1994.

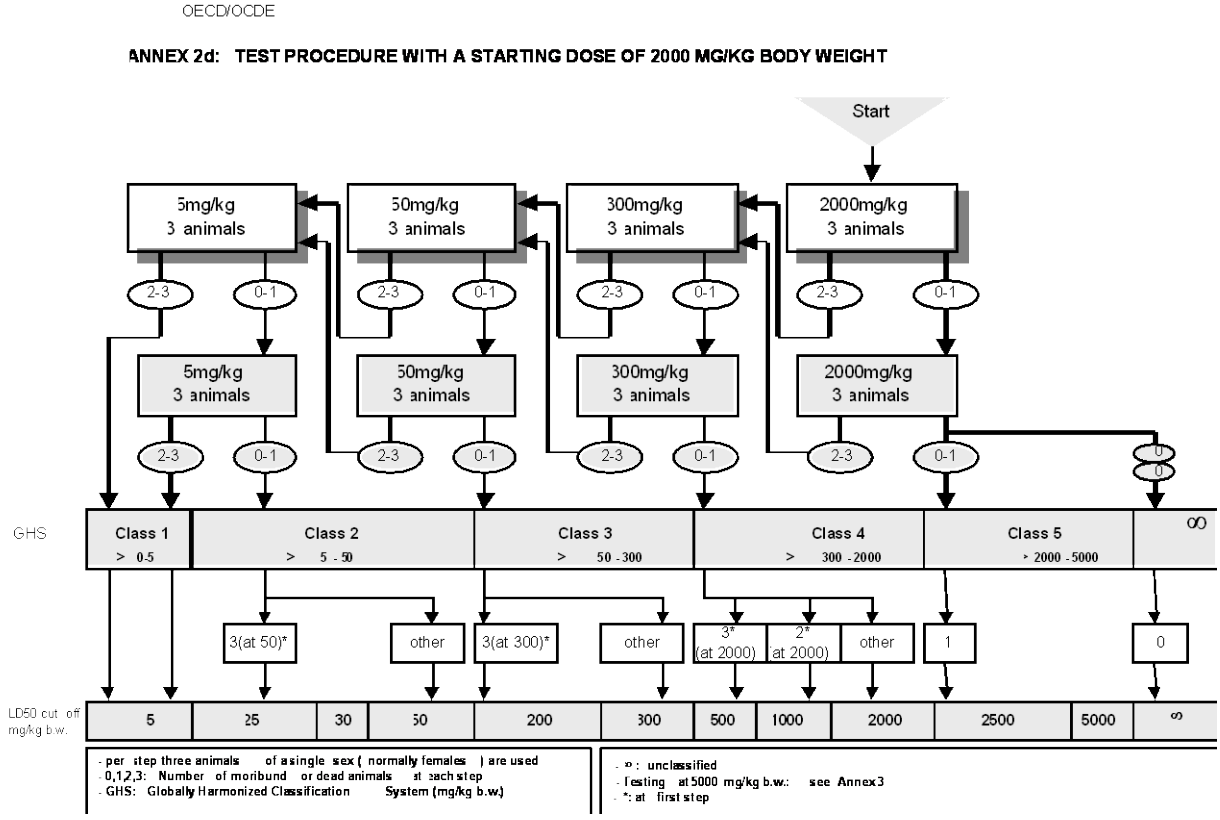


Figure 2.3. Proposed revision of the ATC to meet requirements of the OECD GHS
Source: OECD, Draft TG 423 (OECD, 2000). The number of new starting doses and spaces between have been changed so that the results from this test will allow a substance to be ranked and classified according to the GHS for the classification of chemicals which cause acute toxicity (OECD, 1998a).

2.2.4 *Influence of the Starting Dose in the Up-and-Down-Procedure (UDP)*

Introductory note: The current accepted version of the UDP is the version adopted by the OECD in 1998 (OECD TG 425; OECD, 1998b). Updated drafts of TG 425 have been created to allow for assessment of the confidence interval for the LD50 point estimate, and to include the application of new stopping rules and a larger dose progression factor, both of which tailor the UDP to the most efficient use of animals and improve the point estimate obtained. The most recent draft of TG 425 was issued after the ICCVAM Workshop was held (OECD, October 2000; http://www.oecd.org/ehs/test/health.htm). The analysis of the possible number of animals saved in a tiered approach is therefore based on the currently adopted 1998 OECD version of TG 425, but the significance for both versions can be assumed.

The concept of the up-and-down testing approach was first described by Dixon and Mood (Dixon and Mood, 1948; Dixon, 1965; 1991a, 1991b) and was later proposed to be used for the determination of acute toxicity of chemicals (Bruce, 1985). Apart from many biometrical publications refining the method (not cited here), a key review paper (Lipnick et al., 1995a) compared the results obtained with the UDP, the conventional LD50 test ([TG 401] OECD, 1981) and the Fixed Dose Procedure ([FDP; TG 420] OECD, 1992).

In principle, all versions of the UDP are stepwise procedures that use (as opposed to the ATC) single animals with the first animal receiving a dose at the best estimate of the LD50 (adopted TG 425, OECD 1998b), or one dosing step below the best estimate of the LD50 (most recent draft TG 425). Depending on the outcome for the first animal, the dose for the next is increased or decreased, either by a factor of 1.3 (adopted TG 425), or by a factor of 3.2 (recent draft TG 425). This sequence continues until there is a reversal of the initial outcome (i.e., the point where an increasing dose results in death rather than survival, or decreasing dose results in survival rather than death). After reaching the first reversal of the initial outcome, four additional animals are dosed following the up-down principle according to the adopted TG 425 (OECD, 1998b). In the most recent draft, however, a combination of stopping criteria is used to keep the number of animals to a minimum, while adjusting the dosing pattern to reduce the effect of a poor starting value or low slope. When one of the following criteria is satisfied, dosing is stopped and estimates of the LD50 and confidence interval are calculated according to the maximum likelihood method.

Three stopping criteria are defined in the draft UDP test guideline as follows:

(1) Three consecutive animals survive at the upper bound;
(2) Five reversals occur in any six consecutive animals tested (not just the first six);
(3) At least four animals have followed the first reversal and the specified likelihood-ratios exceed the critical value. (Calculations are made at each dose following the fourth animal after the first reversal.)

Under certain circumstances, which are defined in the draft Guideline, statistical computation will not be possible or will likely give erroneous results. For most applications, testing will be completed with only four to six animals after an or the initial reversal in animal outcome [stopping rule (c)]

Since the UDP test guideline ([TG 425] OECD, 1998b) clearly states that the test performance of the method is optimal if the investigator's best estimate is used as a starting dose, Spielmann et al. (1999) have investigated the quality of LD50 estimates derived from the RC (Halle, 1998) for several chemicals used to validate the UDP (Lipnick et al., 1995a). Of the 35 chemicals used in the UDP validation study (Lipnick et al., 1995a), nine chemicals were also part of the RC (acetonitrile, p-aminophenol, caffeine, coumarin, dimethyl-formamide, mercury (II) chloride, nicotine, phenylthiourea and resorcinol). For four chemicals, the LD50 values predicted by the RC

were almost exactly the same as those determined with the UDP *in vivo*, (i.e., the LD50 values determined in the UDP were on the regression line of the RC) (see Figure 1 in Spielmann et al., 1999). For three chemicals, the predicted LD50 values were within the prediction interval of ± log 5, and for two chemicals (p-aminophenol and caffeine), the predicted LD50 values differed from the *in vivo* LD50 values by one order of magnitude (Spielmann et al., 1999). Thus, even in this small set of data, the 'basic rule' derived from the RC that about 75% of the LD50 values predicted from cytotoxicity (see Section 2.2.2, Table 2.1) are acceptable, was confirmed. This indicates that cytotoxicity assays could be successfully used to determine starting doses, and can reduce the number of animals for *in vivo* studies, particularly the UDP.

To date, no computer simulations have been performed to estimate the possible reduction in animal numbers if the combined *in vitro*/*in vivo* approach is applied to the UDP. Thus, the Workshop discussions were based on computations taken from the ICCVAM background document for the peer review of a recent revision of the UDP (ICCVAM, 2000) which are shown in a slightly improved way in Figure 2.4a and Figure 2.4b. Figure 2.4a applies to the stopping rule defined in the adopted TG 425 (OECD, 1998b), and Figure 2.4b shows the effect when the likelihood-ratio (LR) stopping-rule (current draft OECD TG 425) applies.

Since the LR rule is only one out of three stopping rules that should be applied in an adaptive way, additional computation will be needed to assess the influence of the starting dose on animal usage. The upper curves of both figures depict the numbers of animals used if the starting dose is two logs from the true LD50 (1/100 LD50) while the lower curves show the number of animals used if the true LD50 is used as a starting dose. The percentage of animals saved when the starting dose equals the true LD50 value is about 30% in Figure 2.4a, and independent of the dose mortality slope; whereas in the case of the LR stopping rule (Figure 2.4b), 25 to 40% fewer animals may be used, depending on the slope.

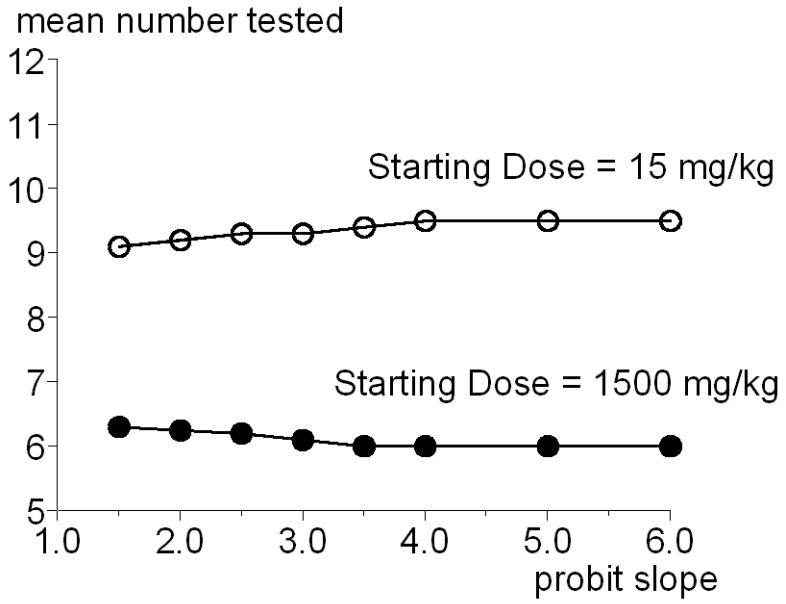


Figure 2.4a. Number of animals needed in relation to the starting dose for UDP adopted TG 425 (OECD 1998b) for LD50 = 1,500 mg/kg b.w.

The figure shows the number of animals needed if the LD50 is used as starting dose (lower curve), or if 1/100 of the LD50 is used as starting dose (upper curve). For details on the stopping rule applied see text.

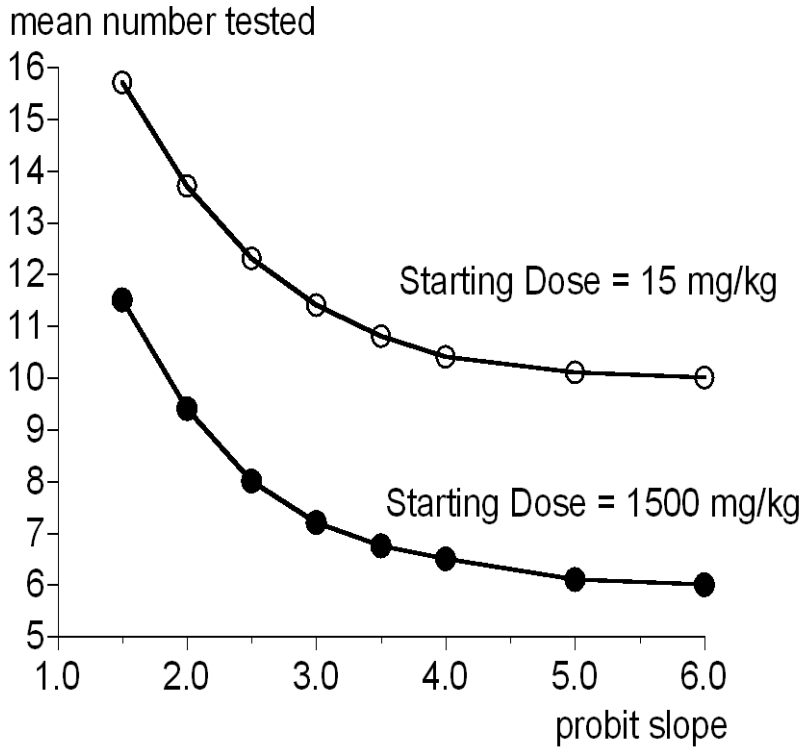


Figure 2.4b. Number of animals needed in relation to the starting dose for UDP draft TG 425 (OECD, 2000) for LD50 = 1,500 mg/kg b.w.

Figure shows the number of animals needed if the LD50 is used as starting dose (lower curve), or, if 1/100 of the LD50 is used as starting dose (upper curve) if the LR stopping rule singularly applies. For details see text.

2.2.5 *Prediction of a Limit Test Value from Basal Cytotoxicity Data*

According to a personal communication (Ingrid Gerner, BgVV) published by Spielmann et al. (1999), the notification process of new chemicals in the EU since 1982 revealed an unbalanced frequency distribution of the toxicity of industrial chemicals. No chemicals were classified "very toxic" (LD50 $\leq$ 25 mg/kg). Only 3% of the chemicals were classified "toxic" (LD50 > 25-200 mg/kg), while 21% were classified "harmful" (LD50 >200-2000 mg/kg), and the vast majority (76%) remained unclassified (LD50 > 2000 mg/kg). In other words, in the world of new industrial chemicals a clear majority are candidates for performing a 'limit test' where only the defined highest dose (2000 mg/kg most often, and occasionally 5000 mg/kg) is applied and no or marginal mortality occurs. Limit tests are defined in all OECD guidelines for acute oral toxicity testing (TG 401, TG 420, TG 423, and TG 425).

It must be emphasized that, if the limit dose defined in these guidelines is applied to all chemicals without knowledge of their toxicity, it would be correct for 76% of the chemicals, while 24% of the chemicals would cause avoidable deaths. It is therefore recommended to perform a limit test only if the prediction from a preceding basal cytotoxicity test suggests an LD50 value larger than the defined limit test dose. Special notice should be given to the fact that the precision of the prediction of low systemic toxicity from cytotoxicity data is much better than

the precision of high systemic toxicity. This is empirically supported by data from the RC (Halle, 1998) shown in Figure 2.1. The main factors affecting a strict log-linear relationship between basal cytotoxicity and systemic toxicity, bioavailability, and in some cases, biotransformation, play a minor role if a chemical is of low basal cytotoxicity.

2.2.6 *Evaluation of a Cytotoxicity Test Intended to be Used for Prediction of a Starting Dose*

This section describes how basal cytotoxicity data can be used to predict a starting dose for an *in vivo* lethality assay. Theoretically, any *in vitro* test that is capable of determining basal cytotoxicity could be used for determining the best estimate of a starting dose for acute testing in the UDP and ATC method. In addition, if the LD50 value predicted from cytotoxicity is high (≥ 2000 mg/kg b.w.), any of the currently used *in vivo* test protocols, including the FDP (OECD, 1992), would allow for performing an *in vivo* limit test without a proceeding sighting study.

In order to apply predictions of LD50 values obtained with experimental cytotoxicity data in the proposed tiered testing strategy as starting doses for the ATC or UDP methods, Spielmann et al. (1999) suggested a procedure shown in Figure 2.5. The authors suggested selecting 10-20 reference chemicals from the RC (Halle, 1998) and testing them in a standardized cytotoxicity test (Figure 2.5, Step 1). A promising candidate would be the BALB/c 3T3 NRU test that has proved robust in several validation studies. To allow comparison of the regression obtained with the in-house test (Figure 2.5, Step 2), reference chemicals should be selected to cover the entire range of cytotoxicity and to be as close as possible to the RC regression line.

Next, the in-house regression equation should be calculated by linear regression (least square method) using the new in-house IC50 values for the reference chemicals and the corresponding LD50 values from the RC. The resulting regression is then compared with the RC regression (Figure 2.5, Step 3). If the regression function obtained with the in-house cytotoxicity test is parallel to the RC regression and within the defined prediction interval, then the test is regarded suitable to be used without modification in applying the RC regression for future predictions of starting doses (Figure 2.5, Step 4). If the in-house regression shows a significantly higher or lower slope, then it may be possible to adjust the in-house test to a higher or lower sensitivity. However, it is likely that a more efficient approach would be to use a cell line and protocol, which have produced results that closely reproduce the RC data (recommended in the Guidance Document, ICCVAM, 2001).

The procedure of evaluating the usability of an in-house cytotoxicity test is explained in full detail in a special Guidance Document from this Workshop (ICCVAM, 2001), in which a set of 11 well-selected reference chemicals from the RC is recommended, and new experimental data obtained by testing the chemicals are presented. The data confirm that an in-house NRU cytotoxicity test, performed either with normal human keratinocytes (NHK) or with BALB/c 3T3 mouse cells, produces a regression line which matched the RC regression line (R^2> 0.9).

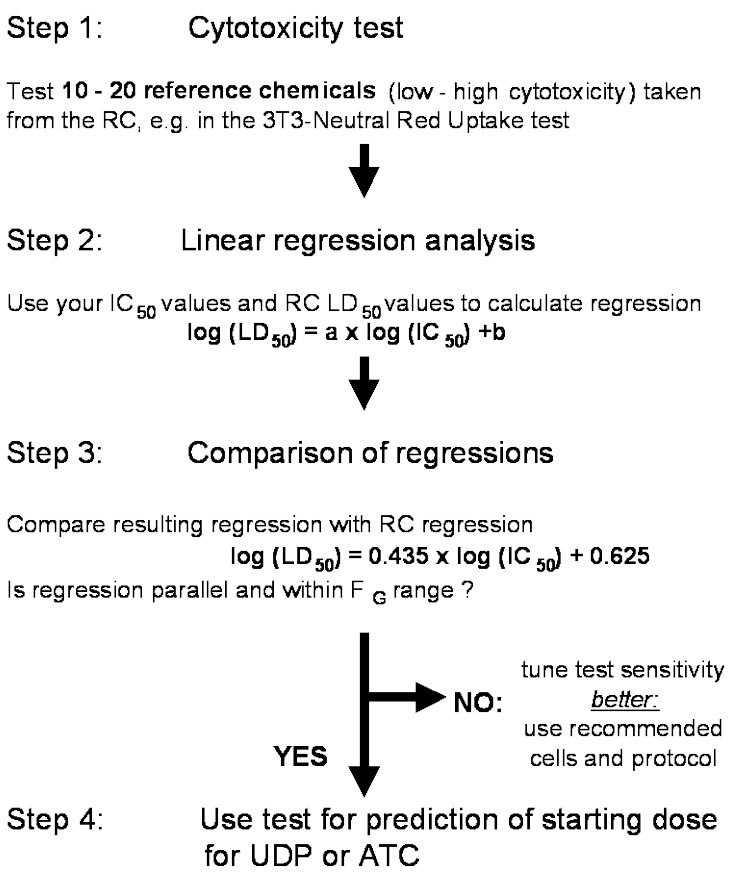


Figure 2.5. Procedure for evaluating a cytotoxicity test for tiered *in vitro*/*in vivo* testing for acute oral toxicity testing (slightly modified version of the scheme presented by Spielmann and colleagues). Note: based on the expectation that many valid cytotoxicity tests would match with the RC regression, Spielmann et al. (1999) defined only the "yes" option between steps 3 and 4. A "no" option has been added here for clarity.

2.2.7 *Multicenter Evaluation of In Vitro Cytotoxicity (MEIC Approach)*

The MEIC program was established by the Scandinavian Society for Cell Toxicology in 1989. The intention of the program was to investigate the relevance of *in vitro* test results for predicting the acute toxic action of chemicals in humans directly rather than in rodents. Batteries of existing *in vitro* tests that have the potential to serve as replacements for acute toxicity tests were identified. The program was designed as an open study with all interested laboratories worldwide invited to participate and test 50 preselected reference chemicals in their particular *in vitro* toxicity assays (Bondesson et al., 1989). Minimal methodological directives were provided in order to maximize protocol diversity among the laboratories. Eventually, some 96 laboratories participated in this voluntary undertaking.

The 50 reference chemicals were selected to represent different classes of chemicals, with the availability of good data on acute toxicity (lethal blood [or serum] concentrations [LC] in humans; oral LD50 values in rats and mice) being a key determinant. Since the LC data available from clinical toxicology handbooks are average values with a wide variation, they were found to be sub-optimal for comparative purposes. Therefore, during 1995-97, the MEIC management team collected case reports from human poisonings

with the reference chemicals to provide LC data with known times between ingestion and sampling/death. The aim was to compile enough case reports to be able to construct time-related LC curves for comparison with the IC50 values for different incubation times *in vitro.* The results were presented and analyzed in a series of 50 MEIC Monographs (referred to as the MEMO subproject by the organizers).

When the MEIC project finished in 1996, all 50 reference chemicals had been tested in 61 different *in vitro* assays. Twenty of these assays used human-derived cells, 18 of which were cell lines and two were primary cell cultures. In 21 of the assays, the cells were of animal origin (12 cell lines and 9 primary cell cultures). Eighteen of the assays were ecotoxicological tests, and two were cell-free test systems. The majority of the assays were based on measurement of effects on cell viability or cell growth (or a combination of the two).

The test results submitted to MEIC were analyzed statistically using analysis of variance (ANOVA), principal component analysis (PCA), and partial least square analysis (PLS) techniques. The analyses conducted were based on *in vitro* cytotoxicity data presented as IC50 values. The predictability of *in vivo* acute toxicity from the *in vitro* IC50 data was assessed against human lethal blood concentrations compiled from three different data sets: clinically measured acute lethal serum concentrations, acute lethal blood concentrations measured post-mortem, and peak lethal concentrations derived from approximate LC50 curves over time after exposure (Ekwall et al., 1998a).

Statistical analysis of results from the 61 assays using the PLS model predicted the three sets of lethal blood concentrations well (R^2 = 0.77, 0.76 and 0.83, Q^2 = 0.74, 0.72, and 0.81, respectively, where R^2 is the determination coefficient and Q^2 is the predicted variance according to cross-validation in the PLS model used) (Ekwall et al., 2000). A two-component PLS model of the prediction of lethal doses in humans from published oral rodent LD50 values for the 50 MEIC compounds was less effective (R^2 = 0.65, Q^2 = 0.64) (Ekwall et al., 1998a; Ekwall et al., 2000).

The analysis showed that *in vitro* assays that were among the most predictive generally used human cell lines (6 of the 18 assays using them gave the highest determination coefficients, vs. 1 of 12 rat cell line assays that performed comparably). Two of 9 non-human primary cell assays analyzed also performed well. Assays that did not perform well were primarily ecotoxicological assays using bacteria or plant cells and, in general, assays with very short exposure times (up to a few hours). Two human primary cell assays, both of which utilized PMN leukocytes and involved 3-hour exposure times, also performed relatively poorly. These results led the authors to note that human-derived cells appeared to be the most predictive for human acute toxicity.

The exposure time for the *in vitro* assays was most often 24 hours, but ranged from 5 minutes to 6 weeks. For 22 of the 50 reference chemicals, the toxicity *in vitro* increased with increasing exposure time. However, high predictivity was generally observed in vertebrate cell assays with 24 to 168 hours exposure. The actual endpoint measurements (cell viability assays) used with the *in vitro* tests were not crucial. Typically, different endpoint measurements gave approximately the same result, suggesting that basal (general) cytotoxicity can be assessed using many mammalian cell lines and almost any growth/viability endpoint.

To select an optimal battery for predicting acute toxicity in humans, the MEIC management team further evaluated various combinations of assays using PLS models and 38 chemicals deemed to have the most reliable and relevant lethal peak concentration data (see Ekwall et al., 2000, for the detailed procedure). From their analysis, the most predictive and cost-effective test battery consisted 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. The test battery (designated 1,5,9/16) was found to be highly predictive of the peak human lethal blood concentrations of all 50 chemicals (R^2 = 0.79, Q^2

= 0.76) when incorporated into an algorithm developed by the team. The R^2 value was further improved to 0.83 when information on BBB penetration was added to the battery results.

It was noted that passage across the BBB can be predicted from the chemical formula and/or physico-chemical properties, or from *in vitro* tests in appropriate model systems; however those methods were not used in the MEIC analysis. The MEIC team proposed that the cell battery they identified could be used immediately for many non-regulatory purposes in a multistep testing strategy and urged its formal validation (and/or that of other promising cell assays also identified in the MEIC program) as soon as possible (Ekwall et al., 2000). Test protocols for evaluating the proposed assays in a validation exercise remain to be developed and optimized.

In summarizing, the MEIC team concluded that their study yielded a limited battery of *in vitro* assays using human cell lines that showed very good performance and were cost effective for predicting acute lethality in humans (Ekwall et al., 2000). However, to further improve the predictive capability of this proposed battery, and to take into account non-basal cytotoxicity factors as a full replacement for acute animal tests, further, targeted development of *in vitro* methods for other particular endpoints is needed. An evaluation-guided development of new *in vitro* tests (EDIT) has been proposed to address these requirements (Ekwall et al., 1999), which includes, as most urgently needed, *in vitro* assays for:

- Assessing passage through the BBB;
- Predicting gut absorption;
- Distribution volume;
- Biotransformation.

The results of the MEIC program have appeared in a series of publications in the open literature (Clemedson et al., 1996a; Clemedson et al., 1996b; Clemedson et al., 1998a; Clemedson et al., 1998b; Ekwall et al., 1998a; Ekwall et al., 1998b; Ekwall et al., 1999; Clemedson et al., 2000; Ekwall et al., 2000). Additional information about MEIC, MEMO and EDIT, as well as the MEMO database, can be found at the following Internet address:
http://www.cctoxconsulting.a.se/nica.htm

2.3 Identifying Needs

In the area of human health effects, the overall aim is to reliably and accurately predict the potential for human acute toxicity. The Breakout Group noted that there is extensive documentation showing that human outcomes from chemical exposure are not predicted well by studies in rodent species (see, e.g., Ekwall et al. [2000] and the recent survey by Olson et al. [2000] on target organ toxicity). Consequently, it was agreed that the long-term goal (the ideal approach) should be the use a battery of *in vitro* tests employing human (rather than rodent or other animal) cells and tissues to provide data which when combined with information derived from other sources (e.g., on key physico-chemical parameters, kinetics, and dynamics) could more accurately predict human acute toxic effects including lethality. However, in the near term, the Breakout Group considered it appropriate and more pragmatic to concentrate on ways to reduce and replace animal use in acute oral toxicity tests as detailed in OECD TG401, TG420, TG423, and TG425.

The Breakout Group was fully aware that rather more information than just an (approximate) LD50 value can be obtained and used from a properly conducted rodent acute toxicity test (such as clinical signs, dose-response relationships, possible target organs, etc.); however, it received reassurance from the U.S. regulatory agencies represented at the Workshop that if there was a validated *in vitro* cytotoxicity test which could accurately predict the approximate rodent LD50 value *in vivo*, then its implementation would result in a significant reduction in animal use. Thus, the primary focus of Breakout Group 1 was to identify and evaluate candidate *in vitro* cytotoxicity tests that could possibly serve as reduction and replacement alternatives for current rodent acute oral toxicity tests for determining LD50 values.

2.3.1 *Near-term (< 2 years) Goals and Potentially Attainable Objectives*

The Breakout Group participants started from the premise that it is biologically plausible that cell death (cytotoxicity) *in vitro* could be used to predict acute lethality. The many studies that show relatively good correlations between *in vitro* IC50 values and *in vivo* LD50 data support this view (e.g., Phillips et al., 1990; Garle et al., 1994). Thus, the near-term focus should be on conducting studies aimed at reducing and replacing animal use for determining LD50 values of chemical substances.

The Breakout Group agreed that standardized *in vitro* test protocols were available but probably not optimized, and that prediction models were needed for predicting acute oral LD50 values. Consequently, a prevalidation study, which would include several promising candidate *in vitro* cytotoxicity tests, would have to be undertaken in order to determine which tests should go forward to the validation stage. Partly because of this, the development of a practical replacement test will take time. As a parallel activity, the ZEBET method for generating cytotoxicity data to help establish the starting dose for *in vivo* testing of new chemical substances (Spielmann et al., 1999) should be seriously considered as an interim measure to potentially reduce the numbers of animals used in the *in vivo* tests.

2.3.2 *In Vitro Endpoints for Assessing In Vivo Acute Toxicity*

There is considerable literature covering a large variety of endpoints and endpoint measurements that have been evaluated for *in vitro* cytotoxicity testing (e.g., Phillips et al., 1990; Balls and Fentem, 1992; Garle et al., 1994; Itagaki et al., 1998a; 1998b; Ohno et al., 1998a; 1998b; 1998c; Tanaka et al., 1998; Clemedson and Ekwall, 1999; Ekwall, 1999). Some of these citations were provided to the Breakout Group members for reference, but time did not allow a systematic assessment of the literature on this topic. It was noted nevertheless that, in practice, basal function endpoints (such as NRU or MTT reduction and/or inhibition of cell proliferation), even though they may measure different cellular functions, have been commonly used with a reasonable degree of success; where cell lines are concerned, the endpoints typically assess a combination of both cell death and cell growth/proliferation. Since the events are based on cellular events that have circumstantial if not direct relevance to cellular responses to chemicals *in vivo*, model cell systems incorporating these "nonspecific" endpoints may satisfy requirements for fidelity and discrimination for alternative methods that have been set forth earlier (Blaauboer et al., 1998). The need for cell-specific or functional endpoints in acute toxicity assays was considered to be on a case-by-case basis and more relevant to studying target organ-specific toxicities (Breakout Group 3's charge).

2.3.3 *Other Issues for Selecting Protocols*

The key components of the protocols for *in vitro* cytotoxicity tests were considered to be the appropriate choice of: (a) cell type (human or animal, cell line or primary cultures) and its characteristics (stability, origin, characterization, availability); (b) exposure period(s) – (i.e., duration cells are exposed to the test chemical); and (c) endpoint measurement(s) – (i.e., cell viability assays such as NRU, lactate dehydrogenase [LDH] leakage, ATP content) (Borenfreund and Puerner, 1986; Riddell et al., 1986; Phillips et al., 1990; Balls and Fentem, 1992; Garle et al., 1994; Ekwall, 1999; Ohno et al., 1998a; Ekwall, 1999; Ekwall et al., 2000). In addition, the inclusion of a prediction model, evidence of repeatability, and facility of transfer between laboratories are important considerations (Balls et al., 1995; Bruner et al., 1996; Archer et al., 1997; ICCVAM, 1997). Ease of automation/high throughput where applicable should offer attractive additional cost benefits but is not a requirement for validation purposes.

2.3.4 *QSAR Models for Predicting Acute Toxicity*

The Breakout Group was requested to assess the role of QSAR, or related models such as structure-activity relationships (SAR) in predicting acute toxicity. While SAR methods involve qualitative assessment of chemical features that confer biological properties, QSAR approaches develop a quantitative relationship between physico-chemical or structural properties

and biological activity (Albert, 1985; Barratt et al., 1995). 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 fit the correlation poorly, and thus their effects would not be predicted accurately. Although defining chemical classes or commonality of mechanisms of action are not trivial due to the multidimensional nature of both characteristics, a review of QSAR studies for predicting LD50 values concluded that QSAR methods have shown some success in relating LD50 values to certain physico-chemical properties of a compound, especially lipophilicity (Phillips et al., 1990).

In contrast, QSAR approaches appear to be less successful in correlating electronic properties of molecules (related to reactivity), or structural variables, with LD50 values, and their use with certain important chemical classes, (e.g., pesticides), is problematic. However, the Breakout Group felt that it lacked sufficient expertise in the field to evaluate the potential of QSAR as a replacement test for lethality and suggested that the topic be reviewed more thoroughly by a more appropriate scientific body. The review should include coverage of commercially available models (e.g., TOPKAT, CASE).

The Breakout Group did recognize that these methods might play key roles as adjuncts to improve LD50 predictions and to reduce animal usage. As noted by others (e.g., Barratt et al., 1998; Lipnick et al., 1995b), QSAR can aid in a number of areas, including the selection of test chemicals for validation studies, the interpretation of outliers, and the grouping of chemicals by structure and biological mechanisms. In addition, looking to future requirements to improve the predictive capability of *in vitro* cytotoxicity data for *in vivo* LD50 values, the Breakout Group agrees with Breakout Group 2 in recommending a more thorough evaluation of QSARs for predicting gut absorption and passage across the BBB. These applications were discussed at length by Breakout Group 2.

The Breakout Group noted that, in principle, expert systems, neural networks, and classical structure-activity approaches might be developed and validated for predicting specific systemic effects (Barratt, 2000; Dearden et al., 1997; Phillips et al., 1990). Requirements for the successful development and use of QSAR methods have been identified and include the following:

- A well-defined mechanism of action for the compound(s) used to derive the QSAR model;
- Use of congeneric, pure compounds and not mixtures;
- A common site of action for the biological effect;
- For comparative purposes, expressing concentrations or doses in molar (not weight) units;
- Validation of each model by investigating its predictive capability using a different set of compounds from its learning (i.e., training) set;
- Use of the same ranges of parameter space as the original test chemicals; and
- The QSAR should not be applied outside of its domain of validity (Phillips et al., 1990; Barratt et al., 1995; Worth et al., 1998).

The limitations or general applicability of each model for different chemical classes will need to be established. The application of QSAR procedures for identifying potential systemic effects was considered by Breakout Group 2.

2.4 Current Status

Many investigations of the relationship between *in vitro* cytotoxicity and acute toxicity *in vivo* have been reported. It was not possible to critically review and discuss all of the literature during the course of the Workshop, so the Workshop organizers made a selection of recent key activities and reports for consideration by Breakout Group 1. The Breakout Group made note of the fact that many of these recent initiatives build upon the conclusions of studies conducted, in particular, during the 1980s (e.g.,

Balls et al., 1992; Balls and Clothier, 1992; Balls and Fentem, 1992; Borenfreund and Puerner, 1986; Clothier et al., 1987; Dierickx, 1989; Ekwall, 1983; Ekwall et al., 2000; Fentem et al., 1993; Fry et al., 1988; Fry et al., 1990; Garle et al., 1987; Garle et al., 1994; Gülden et al., 1994; Guzzie, 1994; Halle and Spielmann, 1992; Hopkinson et al., 1993; Hulme et al., 1987; Ohno et al., 1998a; Phillips et al., 1990; Riddell et al., 1986; Seibert et al., 1996; Spielmann et al., 1999; Wakuri et al., 1993; Zanetti et al., 1992).

The studies and approaches considered were:

- Studies conducted by FRAME and partners (e.g., Balls et al., 1992; Fry et al., 1990; Hulme et al., 1987; Riddell et al., 1986);
- The MEIC scheme (e.g., Clemedson and Ekwall, 1999; Ekwall et al., 2000);
- Japanese Society of Alternatives to Animal Experiments (JSAAE) activities (e.g., Ohno et al., 1998a);
- The ZEBET approach for predicting *in vivo* starting doses (Halle et al., 2000; Halle and Goeres, 1988; Spielmann et al., 1999);
- Testing strategy outlined in ECVAM Workshop Report 16 (Seibert et al., 1996);
- Testing framework proposed under the auspices of SGOMSEC (Curren et al., 1998);
- TestSmart acute systemic toxicity initiative to determine whether cellular changes can predict acute system failure *in vivo* (A. Goldberg, personal communication).

The MEIC and ZEBET approaches were presented to the Breakout Group as specific proposals for adoption as alternative methodologies by regulatory authorities, and therefore received the most attention.

2.4.1 *In Vitro Methods for Estimating Acute In Vivo Toxicity*

There are more than 80 variations of *in vitro* basal cytotoxicity tests, employing a variety of cell lines (e.g., HeLa, HL-60, BALB/c 3T3, Chang cells) and endpoint measurements (e.g., MTT reduction, NRU, ATP content, LDH leakage). From the results of the MEIC and ZEBET programs it appears that basal cytotoxicity can be determined using almost any cell line and almost any toxicity endpoint measurement that correlates well with cell death and/or growth inhibition. Standard protocols are available for some of these methods (e.g., via the *INVITTOX* database run by ECVAM, from the JSAAE validation study, and by slight modification of test protocols used for other purposes such as phototoxicity or eye irritation testing), but these have not necessarily been optimized for predicting rodent oral LD50 values.

Typically, prediction models have not been explicitly defined, although they are usually based on the IC50 value derived in the *in vitro* cytotoxicity assay. Some of these initiatives made note of that and tried to define useful testing strategies that incorporated *in vitro* assays. An example was the ECVAM Workshop report, which to some extent was based on work from the University of Kiel, recognizing the importance of including biokinetic parameters alongside *in vitro* cytotoxicity data to improve the predictions (Seibert et al., 1996).

2.4.2 *Strengths and Limitations of Available In Vitro Cytotoxicity Assays*

Sufficient information was presented to the Breakout Group for evaluating the merits of the MEIC and ZEBET proposals and the JSAAE study in that the information could be adapted and utilized for evaluating assays designed to predict acute lethality.

The MEIC proposal was that a battery of three human cell-based tests (HepG2, protein content, 24 hr exposure; HL-60, ATP content, 24-hr exposure; Chang liver cell morphology, 24 and 168-hr exposure) could be used to predict human lethal blood concentrations and be a surrogate for the LD50 test (Ekwall et al., 2000). Although the MEIC program was not set up as a validation study and assessing reproducibility was not an objective, the Breakout Group agreed with the following MEIC conclusions:

(1) There is a strong correlation between concentrations of chemicals causing cytotoxicity *in vitro* and human lethal serum concentrations.
(2) Metabolism may not play a role *in vivo* as frequently as thought.
(3) Specificity of action requiring many types of differentiated cells is not as significant a problem as may initially have been envisaged.
(4) Some simple corrections of the data, such as for BBB passage, improve the correlations observed.

The key strengths of the MEIC approach are the comparison of acute cytotoxicity data with human exposure data and the database on human lethal concentrations, kinetic profiles, etc., which has been generated and is available as MEMO monographs for others to evaluate and use. The Breakout Group agreed that attempts be made to extend this human database, and that it should be subjected to independent peer review. The outcome of the MEIC program in general was considered to provide strong support for the concept of basal cytotoxicity first proposed by Ekwall in 1983.

Several issues were raised concerning the MEIC proposal and the use of such an approach as an alternative to animal tests. Various limitations of the approach were cited, including the following:

(1) Because the program was not intended to be a validation study, it was not conducted under controlled conditions.
(2) Replicate assays were generally not performed, hence there is limited information on intra-laboratory assay repeatability and inter-laboratory reproducibility. Nevertheless, there is a large body of evidence from other validation studies that *in vitro* cytotoxicity assays are highly reproducible and relatively easy to transfer between laboratories.
(3) The chemicals tested in the different laboratories were probably from different batches and sources (allowed by MEIC for practical purposes, and because the human case exposures likely involved different materials and sources also).
(4) Statistical analyses were often performed on groups of tests rather than on individual assays.
(5) In many of the assays, not all 50 chemicals were tested. This impacts on the conclusions being made on the basis of correlation coefficients;
(6) There is a tendency for the data to be over-interpreted and some of the conclusions have been over-stated in the publications.
(7) Prediction models were not defined for any of the *in vitro* assays. This would be a pre-requisite for a validation study.

There were also specific confounding factors in relation to the 1, 9, 5/16 battery proposed by Ekwall and colleagues (Ekwall et al., 2000). The assay battery was selected using data from 38 of the 50 MEIC chemicals, and the predictivity for all 50 chemicals reassessed by PLS analysis. The values obtained were: R^2=0.84, 38 chemicals; R^2=0.77, 50 chemicals; R^2=0.88, 38 chemicals + BBB correction; R^2=0.83, 50 chemicals + BBB correction. However, it was noted that: (a) results for test 1 were reported for only 45 chemicals, and 3 of the missing 5 results were for chemicals included in the first set of 38, thus n=35 and n=45; in addition, three other *in vitro* tests employing HepG2 cells and a 24-hr exposure time were evaluated in the MEIC program, and the data vary considerably, particularly for some of the reference chemicals; (b) results for test 9 were reported for only 46 chemicals, and all 4 of the missing results are for chemicals included in the first set of 38, thus n=34 and n=46; and (c) tests 5/16 used Chang liver cells, which are known to possess several HeLa markers. In addition, only single data points for each combination of *in vitro* test and chemical have been reported, meaning that there is no way to evaluate the variability in the assay results which would necessarily impact upon the robustness of the conclusions drawn by the MEIC management team.

A major strength of the ZEBET RC approach is the extensive database underpinning the strategy proposed (Spielmann et al., 1999). The database

includes IC50 values derived from numerous *in vitro* cytotoxicity tests on more than 300 chemicals. The actual data are used in a very defined way in trying to predict starting doses for *in vivo* testing, and the simplicity of the concept, flexibility in choice of potentially useful cell systems, and ease of validating and applying the cell systems in practice are attractive features of the approach.

One disadvantage of the ZEBET approach at the present time is the lack of information on the variability in both the *in vitro* and *in vivo* data. In addition, the use of LD50 values from RTECS is perhaps a problem because of this. The Breakout Group suggested that several follow-up actions be undertaken immediately after the Workshop to update and improve the understanding of the applicability of this approach: (a) the examples shown for using *in vitro* cytotoxicity data to identify the starting dose for the ATC or UDP *in vivo* study should be updated to bring them in line with the new draft guidelines, which have now been modified to incorporate the OECD harmonized hazard classification system (OECD, 1998a); and (b) additional simulation modeling should be undertaken to demonstrate the actual reduction in animal use which is expected to be achieved by implementing the approach, and real-life worked examples should be provided to serve as guidance for those adopting and evaluating the approach in the future (See Section 2.6).

2.4.3 *Validation Status of Available In Vitro Screening Methods*

The Breakout Group considered the validation status of the *in vitro* cytotoxicity assays evaluated in the MEIC program, and those used to generate the data included in the RC, relative to the ICCVAM Validation Criteria (ICCVAM, 1997) and the ICCVAM Evaluation Guidelines (ICCVAM, 1999; Section 11, Appendix E). It was concluded that no single *in vitro* cytotoxicity test, or test battery, has yet been formally validated for the specific purpose of replacing the rodent LD50 test. Upon completion of the MEIC study, Ekwall suggested that the battery of three tests proposed should now undergo formal validation (Ekwall et al., 2000). Typically, data on the intra- and inter-laboratory reproducibility of the *in vitro* assays, generated in a structured manner, are lacking, and further work is still needed to fully evaluate the predictive ability of *in vitro* cytotoxicity tests for acute toxicity *in vivo*.

Since several *in vitro* cytotoxicity assays have been included in formal validation studies on eye irritation and phototoxicity (e.g., various test protocols using BALB/c 3T3 mouse fibroblasts or keratinocytes and NRU as the endpoint measurement [Balls et al., 1995b; Brantom et al., 1997; Spielmann et al., 1996; Spielmann et al., 1998]), objective data on the intra-laboratory and inter-laboratory reproducibility of these tests are available for test materials which were coded and tested in at least three laboratories. The Breakout Group proposed that a Working Group be established to evaluate this information and to undertake a paper exercise to determine the capability of these particular *in vitro* cytotoxicity tests for predicting rodent LD50 values rather than Draize rabbit eye irritation scores. It was envisaged that LD50 data would be available for most of the chemicals tested in the EC/HO and BgVV eye irritation validation studies.

A validation study on five *in vitro* cytotoxicity tests (endpoint measurements: colony formation, crystal violet staining, LDH release, MTT, and NRU) has been conducted under the auspices of the JSAAE (Ohno et al., 1998a). Six chemicals (Tween 20, Tween 80, sucrose fatty acid ester, propylene glycol, cetylpyridinium chloride, and sodium lauryl sulfate) were tested. The LDH release endpoint measurement was not reproducible, and the crystal violet staining assay was deemed to be the most reliable of the *in vitro* cytotoxicity tests evaluated (Ohno et al., 1998a). The colony formation assay in HeLa S3 (SC) and BALB/c 3T3 A31-1-1 cell lines was reported to be the most sensitive, but also showed the largest variation (Tanaka et al., 1998).

Disadvantages of the colony formation assay are that it is time-consuming (7 to 13 days culture time, depending on the cell line) and cannot be conducted in 96-well plates and, hence, cannot be readily automated. Although the focus of the

study was on comparisons with Draize eye irritation scores and not acute lethality *in vivo*, the study does provide another source of objective information on the general reproducibility and transferability of *in vitro* cytotoxicity tests (Ohno et al., 1998a). In that sense, the Working Group should also examine the data from this study for how well they predict rodent LD50 values for the test chemicals.

Based on consideration of the studies referred to in previous sections, it was concluded that none of the available *in vitro* methods or proposed testing strategies had been adequately evaluated for implementation to reduce and/or replace animal use for acute systemic toxicity testing. However, it was suggested that the ZEBET approach, using *in vitro* cytotoxicity data to predict *in vivo* starting doses, should be implemented relatively quickly once a guidance document had been prepared (see Section 2.6). The rapid adoption of the ZEBET approach into general practice would enable data to be generated in a relatively short time to fully establish its usefulness and accuracy with a large number of test chemicals.

2.4.4 *Selection of the Most Appropriate Cell Type*

The selection of the most appropriate cell type depends on the objective. Thus, for the prediction of rodent LD50 values in a replacement test, one would conceptually favor a rodent cell line; for the human situation, human cell lines would be more appropriate. Although the MEIC results tend to support this view, the Breakout Group did not feel the data were strong enough (for the reasons given above) to come to a definitive conclusion on this point. Further evidence of this was provided by an analysis of the ZEBET RC data relative to IC50 data generated using a human cell line evaluated in the MEIC program (Clemedson et al., 1998a; Clemedson et al., 1998b). The correlation between the IC50*x* (RC) and IC50*m* (MEIC human cell line) values for the 50 MEIC chemicals was extremely high (R^2=0.90; see Addendum to this report). Consequently, where the objective is to reduce animal numbers required for lethality tests, the apparent difference is too small to rule out the use of a human cell line if that cell line offers other particular advantages or performs acceptably for that purpose.

The current *in vitro* basal cytotoxicity tests do not take into account metabolism-mediated toxicity. It is widely accepted that simple predictive systems (*in vitro* or *in silico*) will need to be developed for early identification of those substances likely to be metabolized to more toxic or less toxic species than the parent chemical (e.g., Fentem et al., 1993; Seibert et al., 1996; Curren et al., 1998; Ekwall et al., 1999). It should be noted that in Ekwall's early studies, approximately 20% of the chemicals assayed in HeLa cell cultures did not fit the basal cytotoxicity concept (Ekwall, 1983). It is expected from the existing literature that "biotransformation screens" will provide valuable data to supplement *in vitro* cytotoxicity results for improving predictions of LD50 values for a significant fraction of those chemicals.

2.5 Future Directions

The Breakout Group concentrated its efforts mainly on short-term approaches to reduce and replace animal use in acute oral toxicity tests, leaving the discussion of longer-term research needs and priorities to Breakout Groups 2 (biokinetics) and 3 (specific organ toxicity and mechanisms). However, it was agreed that the long-term goal (i.e., the ideal approach) should be to develop and use a battery of *in vitro* tests employing human cells and tissues, and integrate this information with that derived from other sources (e.g., on key physico-chemical parameters, kinetics, and dynamics) to predict human acute toxicity, including systemic target organ effects.

2.5.1 *Most Promising In Vitro Methods for Further Evaluation to Reduce and/or Refine Animal Use for Acute Toxicity*

The Breakout Group considered that, in the absence of other information which enables the dose to be set with confidence (e.g., acute toxicity data on structurally related chemicals, physico-chemical or other information), *in vitro*

cytotoxicity data generated using the proposed ZEBET approach should be useful for predicting starting doses for *in vivo* studies. The proponents presented supporting data indicating that this approach would result in a further reduction and refinement in animal use for acute toxicity testing. By judicious use of time and resources, initial cytotoxicity assays need not slow the overall developmental or evaluation processes and in fact may actually expedite it where several chemicals can be tested *in vitro* at the same time.

To use the approach, test laboratories should evaluate and compare the performance of several *in vitro* cytotoxicity tests with the existing RC data (Figure 2.1). For example, a protocol employing the BALB/c 3T3 mouse fibroblast cell line, a 24-hour exposure time, and NRU as the endpoint measurement is appropriate, but other cell lines and cell viability assays could serve the same purpose equally well. The main considerations are:

- The selection of cell type for assessing general cytotoxicity (e.g., rodent fibroblast cell line, human epithelial cell line; monolayer or suspension [e.g., HL60 human acute leukemia cell line] cultures);
- Exposure period (a minimum of 24 hours, but consideration of longer exposures [e.g., 72 hours] as well, if appropriate);
- Endpoint (cell viability/growth);
- Endpoint measurement (e.g., NRU, MTT, ATP, protein).

Since the choice of endpoint measurement does not appear to be critical to the correlative power of the tests (Garle et al., 1994; Ohno et al., 1998a; Spielmann et al., 1999; Ekwall et al., 2000), the simplest, cheapest, most reproducible, with least interference by test chemicals, and, especially where large numbers of chemicals or materials are to be tested, most easily automated endpoint measurements would be the most practical option.

An *in vitro* cytotoxicity test could be implemented in a tiered testing strategy (in the context of predicting starting doses for a subsequent *in vivo* test) in the short-term, without needing to await the outcome of formal validation activities (Section 2.5.2; see below). The main prerequisite would be the production of a guidance document, including details of test protocols considered to be appropriate, and worked examples illustrating the practical application of the strategy.

2.5.2 *Most Promising In Vitro Methods for Further Evaluation to Replace In Vivo Acute Toxicity Test Methods*

The Breakout Group did not evaluate individual test protocols or proposals as candidates for replacement of *in vivo* acute toxicity tests and therefore could not address this question directly. As noted earlier, *in vitro* tests do not currently provide all the information that can be obtained from an *in vivo* study. However, the accumulated results of many cytotoxicity studies and the ZEBET/MEIC initiatives do suggest that, in general, we may be able to obtain reasonable estimates of LD50 values if this parameter is the primary one required for regulatory decisions. Certainly by applying one or more reasonably predictive assays of the LD50 to test the considerable number of chemicals on which such risk assessment data are needed, (e.g., high production volume [HPV] chemicals), it should be possible to make a truly significant reduction in animal usage.

The Breakout Group agreed that a prevalidation study should be initiated at the earliest possible date to identify the most promising *in vitro* cytotoxicity tests for further validation. The study should include a comparison of different cell types (as a minimum, one rodent and one human cell line), exposure periods, and endpoint measurements. Regarding exposure times to evaluate, it was evident from the data available that a minimum exposure of 24 hours should be recommended (Garle et al., 1994; Hopkinson et al., 1993; Riddell et al., 1986), plus an additional "expression" period during which the previously treated cells are cultured in the absence of test material. There may be a need to evaluate several exposure times, as the most appropriate will depend on the cell type chosen, the kinetics of the test chemical, and the sensitivity of the endpoint measured (e.g., Ohno et al., 1998a).

The Breakout Group urged that a Working Group be established to follow up on its conclusions and recommendations at this Workshop (Section 2.6), and specifically, to define the details of the test protocols to be included in any prevalidation study. The selection of basal cytotoxicity tests to be included should be justified with reference to the scientific literature. It was also suggested that the statistical analyses of the MEIC program results be reviewed, so that the basis for the selection of the test battery is fully transparent.

The Breakout Group anticipates that the general performance of the assay or combination/battery of cytotoxicity assays determined from the validation study to be the best predictor of *in vivo* lethality can be enhanced further by supplementation with other information or data. In this respect, immediate research and development needs of particular importance relate to identifying, standardizing, and validating simple predictive systems for gut absorption, BBB passage, kinetics, and metabolism. These are all important parameters which have been identified as improving the predictive ability of *in vitro* cytotoxicity data for *in vivo* LD50 values (Curren et al., 1998; Seibert et al., 1996; Ekwall et al., 1999). A new initiative on acute systemic toxicity, being undertaken as part of the TestSmart activities, has been established to address the question "can one measure cellular changes that will predict acute system failure?" The successful development of this system would complement basal cytotoxicity assays for predicting acute toxicity *in vivo* (Goldberg, personal communication).

In the longer-term, preferably undertaken as a parallel activity, the focus should be on the development and validation of human test systems for predicting human acute toxicity, integrating the approaches suggested by Breakout Groups 2 and 3. In this respect, there are numerous mechanism-based endpoints that need to be identified and evaluated in future studies.

The Breakout Group recognizes the potential impact genomics and proteomics technologies may have in many areas of toxicology, but feels these technologies could only lead to the identification of new endpoints and screening methods in the long-term, and that acute toxicity testing is not currently an area of high priority for the application of these new technologies. Investigations of changes in gene expression (e.g., using microarrays) are better targeted to more specific toxicological effects rather than general responses such as acute lethality.

2.5.3 *Ways to Evaluate the Usefulness of In Vitro Assays in an Overall Acute Toxicity Testing Strategy*

The evaluation of the usefulness of *in vitro* cytotoxicity assays in the overall testing strategy can be achieved in two ways, as indicated above. Firstly, a prospective evaluation "in practice" (in this case by implementing the use of an *in vitro* cytotoxicity test in the strategy proposed by ZEBET [Spielmann et al., 1999]) can be made once the necessary guidance document, including worked examples, has been produced. Once a sufficient body of data has been collected, the *in vitro* cytotoxicity tests can be evaluated retrospectively to determine the validity and practical usefulness of the strategy and to assess whether the predicted starting dose for an *in vivo* study is accurate for a sufficiently large enough percentage of test chemicals to continue its use.

Secondly, a formal validation activity (of which prevalidation would be an initial step; Curren et al., 1995; ICCVAM, 1997) could be conducted in which the test protocols and prediction models are evaluated independently in a multi-laboratory study involving testing of coded chemicals for the reproducibility of their responses, within and among laboratories, and the ability to predict rodent LD50 values (Balls et al., 1995a; ICCVAM, 1997).

2.6 Summary

2.6.1 *Conclusions*

The Breakout Group agreed that its primary objective was to identify and evaluate candidate *in vitro* cytotoxicity tests that could possibly serve as reduction and replacement alternatives for rodent acute oral toxicity tests for determining LD50 values. Despite the considerable research

efforts by a large number of laboratories from different sectors, no standardized *in vitro* cytotoxicity assays, with optimized protocols and prediction models for the determination of LD50 values, have yet been validated. It appears from the number of studies showing positive correlations between cytotoxicity results *in vitro* and acute toxic effects *in vivo* that the application of such *in vitro* methods does have the potential to reduce and refine, and, if properly developed, ultimately replace the use of laboratory animals in acute lethality tests.

A strategy was devised by the Breakout Group that was considered to offer realistic short-term and long-term solutions to address the need for prevalidation and validation of *in vitro* cytotoxicity tests (Figure 2.6). In the short-term, the Breakout Group concluded that the ZEBET approach (Section 2.2.1) had the potential to produce modest reductions in animal use in the ATC and UDP (OECD TG 423 and TG 425) *in vivo* tests (and in the FDP [OECD TG 420] to obviate the need for any initial sighting study). Thus, it is suggested that an *in vitro* cytotoxicity test be used in a tiered testing scheme as proposed by Spielmann et al, (1999).

The Breakout Group concluded that a guidance document with test protocol details, supporting information, and worked examples should be produced and disseminated as quickly as possible. The testing strategy should be implemented as soon as this guidance was available, without the need for a validation study. This conclusion is based on the Breakout Group's awareness of the large database on *in vitro* cytotoxicity and its demonstrated correlative power with rat acute oral LD50 values, particularly the MEIC and RC approaches. The validity of the *in vitro* cytotoxicity data in establishing appropriate starting doses for *in vivo* studies (and hence its direct predictive capability for the LD50) should be assessed retrospectively by evaluating the data generated on a sufficiently large number of substances according to pre-defined criteria for judging the acceptability of the approach. The implementation of such a testing strategy was considered to be relatively inexpensive and simple, and would not compromise the actual outcome of the *in vivo* test.

In vitro assays to replace animal tests for acute lethality will require more time to implement. The information and time available to the Breakout Group was inadequate to recommend specific cytotoxicity assays for prevalidation and validation, although the major considerations and suggestions for possible assays (e.g., a BALB/c 3T3 mouse fibroblast NRU assay) have been documented (Section 2.5.1). An additional Working Group will need to be convened for this purpose at the earliest possible date to maintain momentum and to make progress in the near term.

The scheme conceptualizing the Breakout Group's conclusions as to how cytotoxicity tests can reduce/refine and ultimately replace animal use for acute toxicity (LD50) testing (Figure 2.6) indicates what needs to be done and the projected timings for reaching that point. Each pathway involves a stepwise approach to addressing the issue. Step 1 in any testing scheme would be the collection and integration of information on the physical/chemical properties of a compound, including literature reviews and analysis of structure-activity relationships whenever possible. Most companies currently do this as a preliminary step in their evaluation of new candidate compounds for commercial development. In addition, the likelihood that acute toxicity could be metabolism-mediated needs to be considered at this early stage, and here it would be useful to integrate data derived from simple *in vitro* or *in silico* screens for biotransformation (bioactivation or detoxification). Step 2 would involve conducting an *in vitro* basal cytotoxicity test to provide data, either for correct selection of the *in vivo* starting dose (enabling an immediate reduction and refinement of animal use in the interim) or in lieu of animal testing for estimating rodent LD50 values (once the battery of *in vitro* tests required to do this had been validated for this purpose).

In the left-hand pathway in Figure 2.6, *in vivo* studies are still performed and provide supplementary information on dose response, clinical signs, and target organ effects from acute

exposure for those agencies or organizations that need this additional information. However, it is anticipated that conducting a preliminary cytotoxicity test for starting dose selection would result in a modest, but cumulatively appreciable, reduction in animal numbers at minimal cost and with negligible impact on chemical or product development time. It is further projected that the ZEBET approach can be proved effective in a straightforward exercise, and Guidance for applying the approach prepared within a short period of time (i.e., 2 to 3 months).

In the right-hand pathway of Figure 2.6, the steps required for validating one or more *in vitro* cytotoxicity assays to replace animal testing for acute lethality are shown (Balls et al., 1995; ICCVAM, 1997). This goal will take longer to achieve in light of the current state of the art. It will first be necessary to design and conduct a prevalidation study on those *in vitro* assays that are considered promising (Curren et al., 1995). Then the *in vitro* test protocol(s) and prediction models would be subjected to full validation studies to provide the necessary supporting data for assay evaluation, and eventual regulatory acceptance.

It was considered that, if the commitment to conducting a formal validation study was strong enough, the scientific resources could be harnessed for this effort with facility and the *in vitro* tests studied proved good enough, a replacement test battery might be achieved in as short a time as 2-3 years. However, past experience indicates that the formal acceptance of this battery might require substantial additional time. All prevalidation and validation studies should be conducted in compliance with the ICCVAM and ECVAM guidelines (Balls et al., 1995; ICCVAM, 1997), following the designs of similar validation studies conducted on *in vitro* tests for eye irritation (e.g., Brantom et al., 1997), skin corrosion (Fentem et al., 1998) phototoxicity (Spielmann et al., 1998), and a prevalidation study for skin irritation (Fentem et al., 2001).

In summary, it was concluded that initially a prevalidation study should be undertaken for several promising candidate *in vitro* cytotoxicity tests. Meanwhile, as a parallel activity, the generation of *in vitro* cytotoxicity data to help establish the starting dose for *in vivo* testing of new chemical substances (Spielmann et al., 1999) should be strongly encouraged as a means to potentially reduce the numbers of animals used in LD50 tests (Figure 2.6).

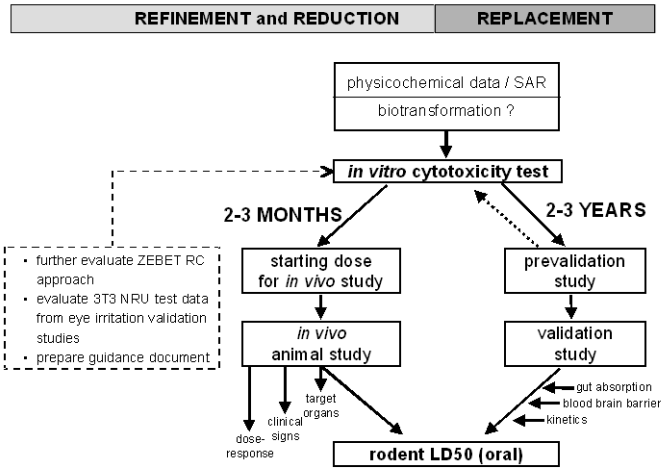


Figure 2.6. Strategy for the reduction, refinement and replacement of animals in acute LD50 testing

2.7 Recommendations

Breakout Group 1 made the following recommendations for the prevalidation, validation, and future development of *in vitro* assays for acute lethal toxicity.

2.7.1 *Short-term Activities*

- A guidance document on the application of *in vitro* cytotoxicity data for predicting *in vivo* starting doses, to include details of current test protocols considered appropriate and their application, and worked examples, should be prepared.
- A Working Group of scientific experts should be established to identify and/or define specific test protocols for inclusion in a prevalidation study. The Working Group should design and plan the study in detail. This Group should take into account the suggestions on cell type, exposure period, and endpoint measurement made by BG1 in this report.

2.7.2 *Intermediate-term Activities*

- It is anticipated that simple systems that predict gut absorption, BBB passage, key kinetic parameters, and metabolism will be needed to improve the capability of *in vitro* cytotoxicity assays to predict rodent LD50 values, or any *in vivo* toxic effects. Continued development and optimization of such systems for this application is encouraged and should receive regulatory support.
- QSAR approaches, including expert systems and neural networks, could be developed and validated as adjunct systems for predicting acute systemic toxicity. The development of commercial QSAR packages should be encouraged. As an initial step in the development of these approaches, an up-to-date review of current QSAR systems for predicting rodent oral LD50 values should be undertaken. In addition, QSARs for predicting gut absorption, metabolism, and BBB passage should be developed and evaluated.

2.7.3 *Longer-term Activities*

- The ultimate objective is the prediction of acute toxicity in humans. For this purpose, the development of simple predictive models for human acute toxicity should be a major focus.
- The evaluation and ultimate acceptance of *in vitro* assays for human acute toxicity will need a larger reference database than is presently available for validation purposes. The MEIC human database should be peer-reviewed, modified if needed, and expanded as soon as possible in order to have the data available for future validation studies.
- Other mechanism-based *in vitro* methods or endpoints, in particular resulting from the application of genomics/proteomics, may provide data that enhances the information that can be derived from cytotoxicity tests. Such research efforts should continue to be encouraged and financially supported.

2.8 References

Albert, A. 1985. Selective Toxicity, 7th edition. Chapman and Hall, London.

Archer, G., M. Balls, L.H. Bruner, R.D. Curren, J.H. Fentem, H-G. Holzhütter, M. Liebsch, D.P Lovell, and J.A. Southee. 1997. The Validation of Toxicological Prediction Models. ATLA 25: 505-516.

Balls, M., and R.H. Clothier. 1992. Cytotoxicity Assays for Intrinsic Toxicity and Irritancy. In: *In Vitro* Methods of Toxicology. (R.R. Watson, ed). CRC Press, Boca Raton, FL. pp. 37-52.

Balls, M., and J.H. Fentem. 1992. The Use of Basal Cytotoxicity and Target Organ Toxicity Tests in Hazard Identification and Risk Assessment. ATLA 20: 368-388.

Balls, M., C. Atterwill, J.Fentem, M. Garle, and F. Wiebel. 1992. Evaluation of Alternatives to Animal Tests for Assessing the Acute Lethal Potency and Neurotoxic Potential of Chemicals. A report prepared for DGXI, CEC; Contract Number B4-3081/91/8678. FRAME, Nottingham.

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. 1995a. Practical Aspects of the Validation of Toxicity Test Procedures. The report and recommendations of ECVAM Workshop 5. ATLA 23: 129-147.

Balls, M., P.A.Botham, L.H. Bruner, and H. Spielmann. 1995b. The EC/HO International Validation Study on Alternatives to the Draize Eye Irritation Test. Toxicol. *In Vitro* 9: 871-929.

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.

Barratt, M.D., P.G. Brantom, J.H. Fentem, I. Gerner, A.P. Walker, and A.P. Worth. 1998. The ECVAM International Validation Study on *In Vitro* Tests for Skin Corrosivity. 1. Selection and distribution of the test chemicals. Toxicol. *In Vitro* 12: 471-482.

Barratt, M. 2000. Prediction of toxicity from chemical structure. Cell Biol. Toxicol. 16: 1-13.

Blaauboer, B.J., Balls, M., Barratt, M., Casati, S., Coecke, S, Mohamed, M.K., Moore, J., Rall, D., Smith, K.R., Tennant, R., Schwetz, B.A., Stokes, W.S., Younes, M. 1998. 13th meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC): Alternative testing methodologies and conceptual issues. Environ. Health Persp. 106 (Suppl. 2): 413-418.

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.

Borenfreund, E., and J.A. Puerner. 1986. Cytotoxicity of Metals, Metal-Metal and Metal-Chelator Combinations Assayed *In Vitro.* Toxicology 39: 121-124.

Brantom, P.G., L.H. Bruner, M. Chamberlain, O. DeSilva, J. Dupuis, L.K. Earl, D.P. Lovell, W.J.W. Pape, M. Uttley, D.M. Bagley, F.W. Baker, M. Bracher, P.Courtellemont, I. Declercq, S. Freeman, W. Steiling, A.P. Walker, G.J. Carr, N. Dami, G. Thomas, J. Harbell, P.A. Jones, U. Pfannenbecker, J.A. Southee, M. Tcheng, H. Argembeaux, D. Castelli, R. Clothier, D.J. Esdaile, H. Itigaki, K. Jung, Y. Kasai, H.Kojima, U. Kristen, M. Larnicol, R.W. Lewis, K. Marenus, O. Moreno, A. Peterson, E.S. Rasmussen, C. Robles, and M. Stern. 1997. A Summary Report of the COLIPA International Validation Study on Alternatives to the Draize Rabbit Eye Irritation Test. Toxicol. *In Vitro* 11: 141-179.

Bruce R.D. 1985. An Up-and-Down Procedure for Acute Toxicity Testing. Fundam. Appl. Tox. 5: 151-157.

Bruner, L.H., G.J. Carr, M. Chamberlain, and R.D. Curren. 1996. Validation of Alternative Methods for Toxicity Testing. Toxicol. *In Vitro* 10: 479-501.

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. 1996a. 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. 1996b. 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. 1998a. 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. 1998b. 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.

Clothier, R.H., L.M.Hulme, M. Smith, and M. Balls. 1987. Comparison of the *In Vitro* Cytotoxicities and Acute *In Vivo* Toxicities of 59 Chemicals. Molecular Toxicol. 1: 571-577.

Clothier, R.H., L.M.Hulme, A.B. Ahmed, H.L. Reeves, M. Smith, and M. Balls. 1988. *In Vitro* Cytotoxicity of 150 Chemicals to 3T3-L1 Cells Assessed by the FRAME Kenacid Blue Method. ATLA 16: 84-95.

Curren, R.D., J.A. Southee, H. Spielmann, M. Liebsch, J.H. Fentem, and M. Balls. 1995. The Role of Prevalidation in the Development, Validation and Acceptance of Alternative Methods. ATLA 23: 211-217.

Curren, R., L. Bruner, A. Goldberg, and E. Walum. 1998. 13th meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC): Validation and acute toxicity testing. Environ. Hlth Perspect. 106 (Suppl. 2): 419-425.

Dearden, J.C., M.D. Barratt, R. Benigni, D.W. Bristol, R.D. Combes, M.T.D.Cronin, P.N. Judson, M.P. Payne, A.M. Richard, M. Tichy, A.P. Worth, and J.J. Yourick. 1997. The Development and Validation of Expert Systems for Predicting Toxicity. The report and recommendations of an ECVAM/ECB workshop (ECVAM workshop 24). ATLA 25: 223-252.

Diener, W., U. Mischke, D. Kayser, and E. Schlede . 1995. The Biometric Evaluation of the OECD Modified Version of the Acute-Toxic-Class Method (Oral). Arch. Toxicol. 69: 729-734.

Diener W. and E. Schlede. 1999. Acute toxic class methods: alternatives to LD/LC_{50} tests. ALTEX 16: 129-134.

Diener, W., and E. Schlede. 2000. Acute Toxic Class Methods: Biometric Evaluations and Test Procedures for the New International Classification Systems. Manuscript in preparation.

Dierickx, P.J. 1989. Cytotoxicity Testing of 114 Compounds by the Determination of Protein Content in HepG2 Cell Cultures. Toxicol. *In Vitro* 3: 189-193.

Dixon, W.J., and A.M. Mood. 1948. A Method for Obtaining and Analyzing Sensitivity Data. J. Amer. Statist. Assoc. 43: 109-126.

Dixon, W.J. 1965. The Up-and-Down Method for Small Samples. J. Amer. Statist. Assoc. 60: 967-978.

Dixon, W.J. 1991. Staircase Bioassay: The Up-and-Down Method. Neurosci. Biobehav. Rev. 15: 47-50.

Dixon, W.J. 1991. Design and Analysis of Quantal Dose-Response Experiments (with Emphasis on Staircase Designs). Dixon Statistical Associates, Los Angeles CA, USA.

Eagle, H., and G.E. Foley. 1956. The Cytotoxic Action of Carcinolytic Agents in Tissue Culture. Amer. Jour. of Med. 21: 739-745.

Ekwall, B. 1983. Screening of Toxic Compounds in Mammalian Cell Cultures. Ann. New York Acad. Sci. 407: 64-77.

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: 665-673.

Ekwall, B., C. Clemedson, B. Crafoord, Ba. Ekwall, S. Hallander, E. Walum, and I. Bondesson. 1998a. 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. 1998b. 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.

Ekwall, B., C. Clemedson, B. Ekwall, P. Ring, and L. Romert. 1999. EDIT: A new international multicentre programme to develop and evaluate batteries on *in vitro* tests for acute and chronic systemic toxicity. ATLA 27: 339-349.

Ekwall, B., B. Ekwall, and M. Sjostrom. 2000. MEIC Evaluation of Acute Systemic Toxicity. Part VIII. Multivariate partial least squares evaluation, including the selection of a battery 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.

Fentem, J.H., G.E.B. Archer, M. Balls, P.A. Botham, R.D. Curren, L.K.Earl, D.J.Esdaile, H-G. Holzhütter, and M. Liebsch. 1998. The ECVAM International Validation Study on *In Vitro* Tests for Skin Corrosivity. 2. Results and evaluation by the Management Team. Toxicology *In Vitro* 12: 483-524.

Fentem, J.H., D. Briggs, C.Chesné, G.R. Elliott, J.W. Harbell, J.R. Heylings, P. Portes, R. Roguet, J.J.M. van de Sandt, and P.A.Botham. 2001. A Prevalidation Study on *In Vitro* Tests for Acute Skin Irritation: results and evaluation by the Management Team. Toxicology *In vitro* 15:57-93.

Fry, J.R., M.J. Garle, and A.H. Hammond. 1988. Choice of Acute Toxicity Measures for Comparison of *In Vivo-In Vitro* Toxicity. ATLA 16: 175-179.

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: 175-178.

Galson, S. 2000. Historical and current regulatory perspectives. Opening Plenary Session, ICCVAM International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, October 17-20, 2000.

Garle, M.J., A.H. Hammond, and J. R. Fry. 1987. The Cytotoxicity of 27 Chemicals to V79 Chinese Hamster Cells. ATLA 15: 30-32.

Garle, M., 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.
Grisham, J.W., and G.J. Smith. 1984. Predictive and Mechanistic Evaluation of Toxic Responses in Mammalian Cell Culture Systems. Pharmacolog. Rev. 36 (Suppl.): 151S-171S.

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.

Guzzie, P.J. 1994. Lethality Testing. In: *In Vitro Toxicology*. (S.C. Gad, ed). Raven Press, New York. pp. 57-86.

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.

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

Halle, W., M. Liebsch, D. Traue, and H. Spielmann. 1997. Reduktion der Tierzahlen bei der Einstufung von Stoffen in die EU-Toxizitätsklassen für akute orale Toxizität mit Hilfe von Daten aus dem Register der Zytotoxizität (RC). ALTEX 14: 8-15.

Halle, W. 1998. Toxizitätsprüfungen in Zellkulturen für eine Vorhersage der akuten Toxizität (LD50) zur Einsparung von Tierversuchen. Life Sciences/ Lebens-wissenschaften, Volume 1, 94 pp. Jülich: Forschungszentrum Jülich.

Halle, W., H. Spielmann, and M. Liebsch. 2000. Prediction of Human Lethal Concentrations by Cytotoxicity Data from 50 MEIC Chemicals. ALTEX 17: 75-79.

Hopkinson, D., R. Bourne, and F.A. Barile. 1993. *In vitro* Cytotoxicity Testing: 24-hour and 72-hour studies with cultured lung cells. ATLA 21: 167-172.

Hulme, L.M., H.L. Reeves, R.H. Clothier, M. Smith, and M. Balls. 1987. Assessment of Two Alternative Methods for Predicting the *In Vivo* Toxicities of Metallic Compounds. Molecular Toxicol. 1: 589-596.

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). 1997. Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods. NIH Publication 97-

3981. National Institute of Environmental Health Sciences, Research Triangle Park, NC. Available on the Internet at http://iccvam.niehs.nih.gov/docs/guidelines/validate.pdf.

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). 1999. Evaluation of the validation status of toxicological methods: General Guidelines for Submissions to ICCVAM Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods. NIH Publication 99-4496. National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina. Available on the Internet at http://iccvam.niehs.nih.gov/docs/guidelines/subguide.htm.

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). 2000. The revised Up-and-Down Procedure: a test for determining the acute oral toxicity of chemicals and products. Proposed test method and background review document, April 14, 2000. ICCVAM / NIEHS, Research Triangle Park, NC 27709. Available on the Internet at http://iccvam.niehs.nih.gov/methods/udpdocs/AllBRDlk.pdf.

Itagaki, H., T.Ohno, M. Hatao et al. 1998a. Validation Study on Five Cytotoxicity Assays by JSAAE. V. Details of the crystal violet staining assay. AATEX 5: 87-98.

Itagaki, H., T.Ohno, M. Hatao et al. 1998b. Validation Study on Five Cytotoxicity Assays by JSAAE. VII. Details of the MTT assay. AATEX 5: 119-130.

Klaassen, C.D., and D.L. Eaton. 1991a. Principles of Toxicology. In: Caserett and Doull's Toxicology: The Science of Poisons. 4th Ed (Amdur, M.O., J. Doull, and C.D. Klaassen, eds). Pergamon Press Inc., New York, pp. 16-17.

Klaassen, C.D., and D.L. Eaton. 1991b. Principles of Toxicology. In: Caserett and Doull's Toxicology: The Science of Poisons. 4th Ed (Amdur, M.O., J. Doull, and C.D. Klaassen, eds). Pergamon Press Inc., New York, pp. 22.

Knox, P., P.F. Uphill, J.R. Fry, J. Benford, and M. Balls. 1986. The FRAME Multicentre Project on *In Vitro* Cytotoxicity. Food Chem. Toxicol. 24: 457-463.

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.

Lipnick, R.L., M. Zeeman, and J.A. Cotruvo. 1995b. Structure-activity relationships in the validation of in vitro toxicology tests. In: *Animal Test Alternatives: Refinement Reduction Replacement* . H. Salem, ed., pp 47-55. Marcel Dekker, New York.

MEIC, MEMO, and EDIT information: http://www.cctoxconsulting.a.se/nica.htm

NIEHS (National Institute of Environmental Health Sciences), 2001. Guidance document on using in vitro data to estimate in vivo starting doses for acute toxicity. NIH Publication 01-4500. NIEHS, Research Triangle Park, North Carolina.

OECD. October, 2000; Draft OECD Guideline for Testing of Chemicals, 423: Acute Oral toxicity - Acute Toxic Class Method; http://www.oecd.org/ehs/test/health.htm.

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. OECD, Paris. http://www.oecd.org/ehs/class/HCL6htm

OECD. 1998b. OECD Guideline for Testing of Chemicals. 425. Acute Oral Toxicity - Up-and-Down Procedure. OECD. Paris.

OECD. 1981. OECD Guideline for Testing of Chemicals. 401. Acute Oral Toxicity. OECD, Paris. [updated in 1987]

OECD. 1987. OECD Guideline for Testing of Chemicals. 401. Acute Oral Toxicity. OECD, Paris.

OECD. 1992. OECD Guideline for Testing of Chemicals. 420. Acute Oral Toxicity - Fixed Dose Method. OECD, Paris.

OECD. 1996. OECD Guideline for Testing of Chemicals. 423. Acute Oral Toxicity - Acute Toxic Class Method. OECD, Paris.

Ohno, T., M. Asakura, T. Awogi et al. 1998a. Validation Study on Five Cytotoxicity Assays by JSAAE. I. Overview of the study and analyses of variations of ED50 values. AATEX 5: 1-38.

Ohno, T., Y. Futamura, A. Harihara et al. 1998b. Validation Study on Five Cytotoxicity Assays by JSAAE. VI. Details of the LDH release assay. AATEX 5: 99-118.

Ohno, T., Y. Futamura, A. Harihara et al. 1998c. Validation Study on Five Cytotoxicity Assays by JSAAE. VIII. Details of the Neutral Red Uptake assay. AATEX 5: 131-145.

Olson, H., G. Betton, D. Robinson, K. Thomas, A. Monro, G. Kolaja, P. Lilly, J. Sanders, G. Sipes. 2000. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul. Toxicol. Pharm. 32: 56-67.

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: 375-394.

Pomerat, C., and C.D. Lake. 1954. Short Term Cultures for Drug Assays: general considerations. Ann. New York Acad. Sci. 58: 1110-1128.

Riddell, R.J., D.S.Panacer, S.M. Wilde, R.H. Clothier, and M. Balls. 1986. The Importance of Exposure Period and Cell Type in *In Vitro* Cytotoxicity Tests. ATLA 14: 86-92.

Schlede, E., U. Mischke, R. Roll, and D. Kayser. 1992. A National Validation Study of the Acute-Toxic Class Method - An Alternative to the LD50 Test. Arch. Toxicol. 66: 455-470.
Schlede, E., U. Mischke, W. Diener, and D. Kayser. 1994. The International Validation Study of the Acute-Toxic Class Method (Oral). Arch. Toxicol. 69: 659-670.

Seibert, H., M. Balls, J.H. Fentem, V. Bianchi, R.H. Clothier, P.J. Dierickx, B. Ekwall, M.J. Garle, M.J. Gómez-Lechón, L. Gribaldo, M. Gülden, M. Liebsch, E. Rasmussen, R. Roguet, R. Shrivastava, and E.

Walum. 1996. Acute Toxicity Testing *In Vitro* and the Classification and Labeling of Chemicals: The report and recommendations of ECVAM Workshop 16. ATLA 24: 499-510.

Smith, C.G., J.E. Grady, and J.I. Northam. 1963. Relationship between Cytotoxicity *In Vitro* and Whole Animal Toxicity. Cancer Chemother. Rep. 30: 9-12.

Spielmann, H., M. Liebsch, and S. Kalweits. 1996. Results of a Validation Study in Germany on Two *In Vitro* Alternatives to the Draize Eye Irritation Test, the HET-CAM Test and the 3T3-NRU Cytotoxicity Test. ATLA 24: 741-858.

Spielmann, H., M. Balls, J. Dupuis, W.J.W. Pape, G. Pechovitch, O. de Silva, H-G. Holzhütter, R. Clothier, P. Desolle, F. Gerberick, M. Liebsch, W.W. Lovell, T. Maurer, U. Pfannenbecker, J.M. Potthast, M. Csato, D. Sladowski, W. Steiling, and P. Brantom. 1998. EU/COLIPA "*In Vitro* Phototoxicity" Validation Study, Results of Phase II (blind trial), Part 1: the 3T3 NRU phototoxicity test. Toxicol. *In Vitro* 12: 305-327.

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: 957-966.

Tanaka, N. M. Asakura, C. Hattori et al. 1998. Validation Study on Five Cytotoxicity Assays by JSAAE. IV. Details of the colony formation assay. AATEX 5: 74-86.

Trevan, J.W. 1927. The Error of Determination of Toxicity. Proceedings of the Royal Society (London). Series B 101: 483-514.

Wakuri, S., J. Izumi, K. Sasaki, N. Tanaka, and H. Ono. 1993. Cytotoxicity Study of 32 MEIC Chemicals by Colony Formation and ATP Assays. Toxicol. *In Vitro* 7: 517-521.

Worth, A.P., M.D. Barratt, and J.B. Houston. 1998. The Validation of Computational Prediction Techniques. ATLA 26: 241-247.

Zanetti, C., I. De Angelis, A-L. Stammati, and F. Zucco. 1992. Evaluation of Toxicity Testing of 20 MEIC Chemicals on Hep-2 Cells Using Two Viability Endpoints. ATLA 20: 120-125.

Zbinden, G., and M. Flury-Roversi. 1981. Significance of the LD50 Ttest for the Toxicological Evaluation of Chemical Substances. Arch. of Toxicol. 47: 77-99.

ADDENDUM

Combined analyses of the ZEBET Register of Cytotoxicity (RC) and MEIC data

The predictions of acute lethality *in vivo* from the RC and MEIC cytotoxicity data have been analyzed. The correlation for the 50 MEIC chemicals (IC50 *in vitro* vs rodent oral LD50 *in vivo*), including the RC cytotoxicity data for various mammalian cell lines (dark triangles, dark linear regression line) and the MEIC program cytotoxicity data for various human cell lines (circles, gray linear regression line; taken from Clemedson et al., 1998a; Clemedson et al., 1998b), are shown in Figure A.1. Similar standard regression lines, with comparable data fits, were obtained for the RC values (mean IC50*x* data) and the MEIC values (IC50m) for the 50 chemicals (Table A.1).

A similar comparison of the correlations for the 50 MEIC chemicals (RC mammalian *in vitro* values and MEIC human *in vitro* values from Clemedson et al. [1998a; 1998b]) was also undertaken for *in vitro* IC50 vs human peak lethal blood concentrations *in vivo* (Ekwall et al., 1998a). Again, similar standard regression lines, with comparable fits, were obtained (Table A.1):

RC: log (peak concentration) =
0.822 x log (IC50*x*) - 0.437; r=0.81; R^2=0.66

MEIC: log (peak concentration) =
0.913 x log (IC50*m*) - 0.702; r=0.86; R^2=0.74

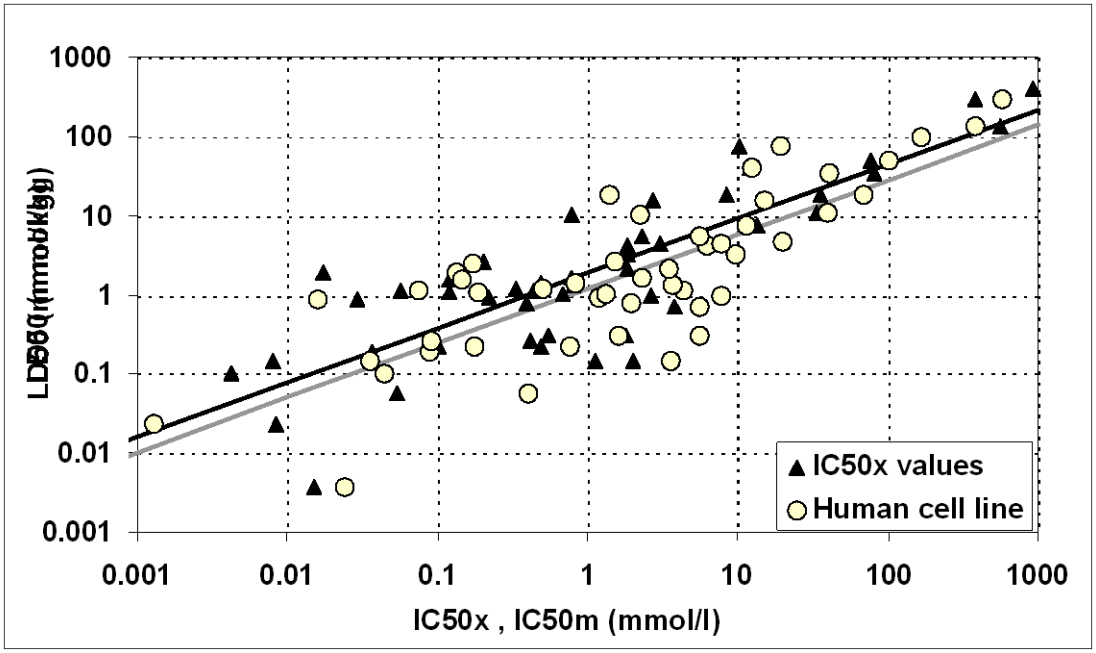


Figure A.1. Regression between Cytotoxicity (IC50) and rodent acute oral LD50 for the 50 MEIC chemicals
RC: log (LD50) = 0.689 x log (IC50*x*) + 0.276; r=0.84; R^2=0.71
MEIC: log (LD50) = 0.690 x log (IC50*m*) + 0.080; r=0.81; R^2=0.66

Table A.1. Summary of linear regression analyses (RC vs MEIC)

Chemicals	x	y	slope	constant	r	R^2
347 non-selected (RC)	IC50*x*	LD50	0.435	0.625	0.67	0.45
50 MEIC (RC)	IC50*x*	LD50	0.689	0.276	0.84	0.71
50 MEIC (human cell lines)	IC50*m*	LD50	0.690	0.080	0.81	0.66
50 MEIC (RC)	IC50*x*	human lethal	0.822	- 0.437	0.81	0.66
50 MEIC (human cell lines)	IC50*m*	human lethal	0.913	- 0.702	0.86	0.74
50 MEIC	LD50	human lethal	0.879	- 0.669	0.71	0.50

To set these results in context, the predictivity of the rat LD50 for human peak lethal concentration was assessed for the MEIC chemicals (Figure A.2; Table A.1). The correlation was not as good as that found with the IC50 values.

The 50 MEIC chemicals are a subset of the RC; the overall predictivity of the entire RC (347 chemicals) for rodent LD50 values is lower than that of the 50 MEIC chemicals (Figure A.3; Table A.1). The relationship between *in vitro* IC50 values and *in vivo* LD50 values should be investigated further by employing multiple regression techniques rather than simple linear regression. In addition, cluster analysis could also be undertaken.

To investigate how basal cytotoxicity data obtained from various human cell lines (IC50m) in the MEIC program (part III and IV) compares with basal cytotoxicity data from various mammalian cell lines (IC50*x*), the correlation between IC50*x* and IC50m is shown in Figure A.4. The correlation is judged very high by R^2 = 0.90, and suggests that basal cytotoxicity data obtained with either human cells or other mammalian cells may be similar and equivalent for the prediction of *in vivo* lethality measures.

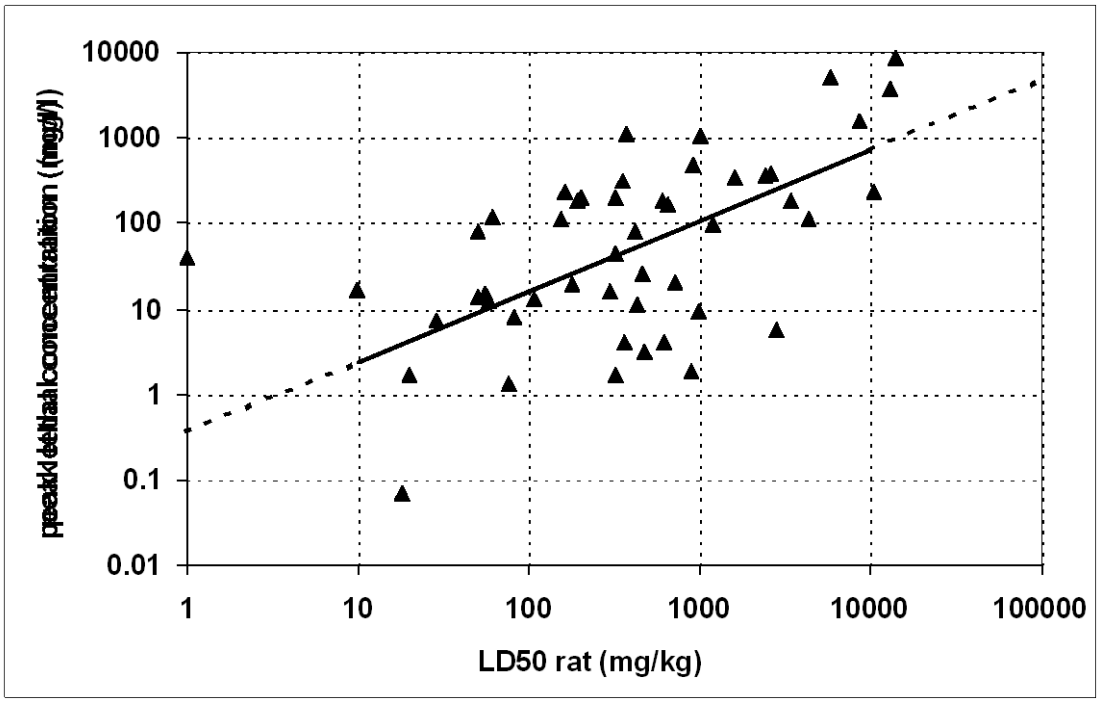


Figure A.2. Regression between rodent acute oral LD50 values and human peak lethal concentrations for the 50 MEIC chemicals.
Regression equation: log (peak conc.) = 0.879 x log (LD50) – 0.669; r=0.71; R^2=0.50.

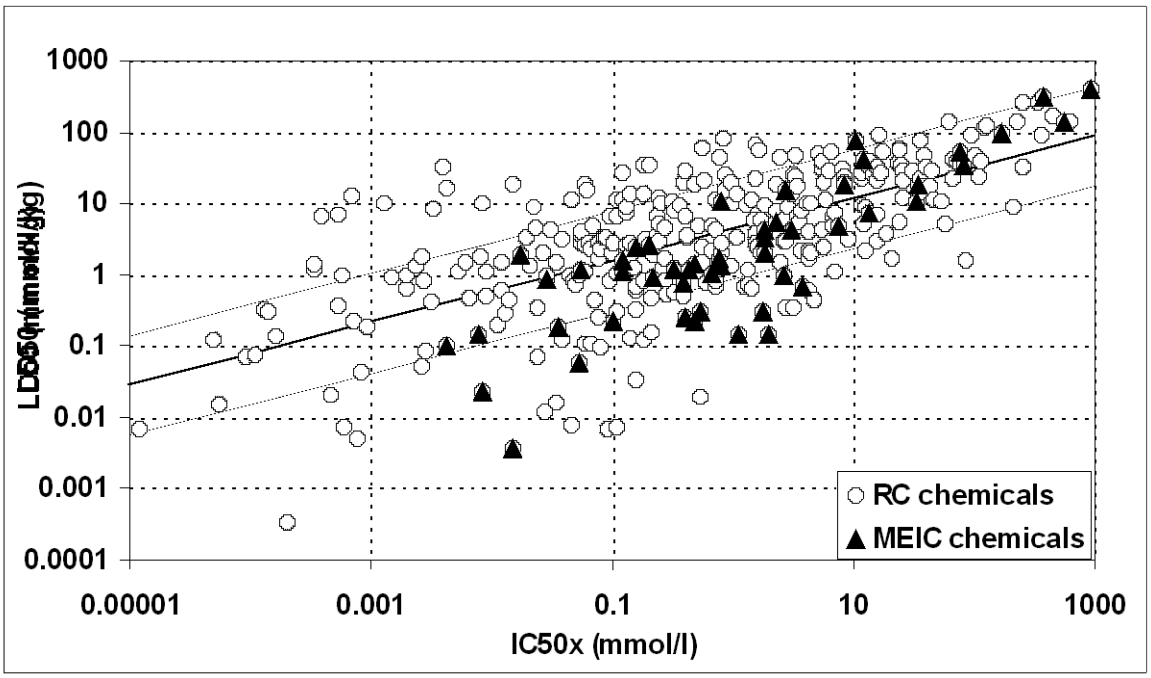


Figure A.3. Regression between Cytotoxicity (IC50) and rodent acute oral LD50 values for the RC database showing the 50 MEIC chemicals as a subset of the 347 chemicals in the RC

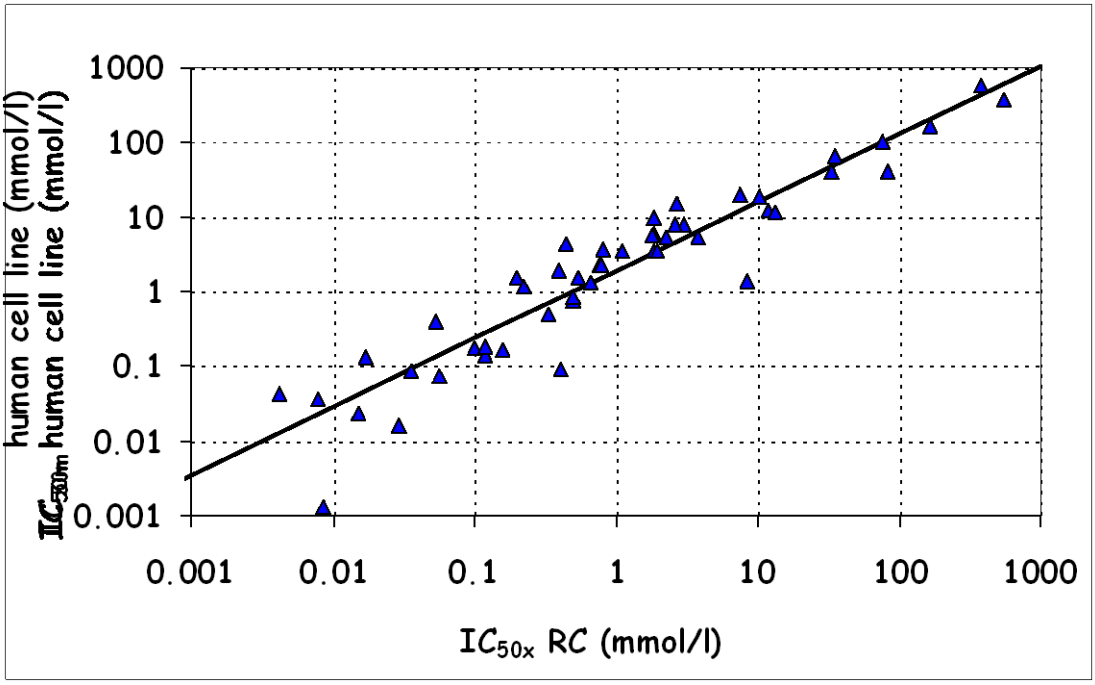


Figure A.4. Correlation between IC50*x* (averaged from various mammalian cell lines) of the RC and IC50m (from various human cell lines) is shown for the 50 MEIC chemicals
The linear correlation coefficient is high (r = 0.95) and judged by an $R^2 = 0.90$.

3.0 *IN VITRO* METHODS FOR ASSESSING ACUTE TOXICITY: BIOKINETIC DETERMINATIONS

3.1 Introduction

The biokinetics determinations Breakout Group (Breakout Group 2) was given the task of discussing and evaluating the capabilities of *in vitro* methods for providing biokinetic information (i.e., on absorption, distribution, metabolism, and excretion) that can be used to estimate target-organ dosimetry for acute toxicity testing. The Breakout Group was asked to identify future research needs in the area of biokinetics that will enable *in vitro* methods to more accurately predict acute toxicity *in vivo*. The role of quantitative structure-activity relationships (QSAR) and quantitative structure-property relationships (QSPR) in biokinetic determinations was also to be considered.

The Breakout Group was asked to answer a number of questions in three areas:

(1) The identification of the need for specific knowledge in the field of biokinetics;
(2) The current status of knowledge and technology in the field;
(3) Future directions for research.

The group discussions followed general lectures given in the Workshop's opening plenary session. A presentation to the Breakout Group entitled "An integrated approach for predicting systemic toxicity" was particularly relevant to the Breakout Group's responsibilities, demonstrating the central role of biokinetic modeling in the prediction of systemic toxicity using *in vitro* data (Blaauboer et al., 2000).

3.1.1 *General Discussion*

The goals for the Workshop were presented and the following specific questions were posed:

(1) What *in vitro* systems are available and how can these systems be applied and/or improved?
(2) What research requirements can be formulated?
(3) Which priorities can be set for research?

The discussions of the Breakout Group centered on the role of the kinetics of a chemical *in vivo* in its acute systemic toxicity. The following summary was developed as a point of departure for the Breakout Group's deliberations:

> Results obtained from *in vitro* studies in general are often not directly applicable to the *in vivo* situation. One of the most obvious differences between the situation *in vitro* and *in vivo* is the absence of processes regarding absorption, distribution, metabolism and excretion (i.e., biokinetics) that govern the exposure of the target tissue in the intact organism. The concentrations to which *in vitro* systems are exposed may not correspond to the actual situation at the target tissue after *in vivo* exposure. In addition, the occurrence of metabolic activation and/or saturation of specific metabolic pathways or absorption and elimination mechanisms may also become relevant for the toxicity of a compound *in vivo*. This may lead to misinterpretation of *in vitro* data if such information is not taken into account. Therefore, predictive studies on biological activity of compounds require the integration of data on the mechanisms of action with data on biokinetic behavior. Over the last decade, the feasibility of using mathematical models for interpretation of *in vivo* biokinetics has grown substantially. This development has been facilitated by the increasing availability of computer-based techniques for numerical solution of differential equation sets that characterize biokinetic processes (Blaauboer et al., 2000).

The Breakout Group also reached consensus on some terminology: the word "toxicokinetics" should be replaced by "kinetics" or "biokinetics". Problem areas in predicting kinetics of chemicals were noted in: (a) biotransformation (value of *in vitro* systems for determining biotransformation,

interpretation of *in vitro* data, scaling up to the *in vivo* situation); and (b) the passage across special barrier systems (e.g., in the gastrointestinal [GI] tract, the blood-brain barrier [BBB], and the kidney).

Short presentations on the following were provided as a focal point for Breakout Group discussions:

- Biokinetic modeling of acute exposure;
- QSAR/QSPR;
- BBB;
- Kidney barrier systems;
- Intestinal barrier;
- Metabolic activation, including different systems available for the liver (and extrahepatic tissue);
- Skin as a barrier;
- Microarray alternatives;
- Information from NIEHS Microarray Center;
- Expert systems for making predictions of a compound's partitioning and toxicity.

After the presentation on the use of Physiologically-Based Biokinetic (PBBK) models, the Breakout Group concluded that kinetics play a crucial role in estimating a compound's acute systemic toxicity. The use of these physiologically determined models has proven to be very useful in many aspects. Over the last ten years, the feasibility of this modeling approach has been greatly enhanced due to the availability of computer techniques that allow for the simultaneous numerical solution of differential equations. While species-specific anatomical and physiological data are generally available from the literature (e.g., Arms and Travis, 1988; Brown et al., 1997), compound-specific parameters for PBBK models (e.g., tissue-blood partition coefficients and the Michaelis-Menten constants Vmax and Km) are often still obtained by fitting these parameters to experimental data obtained *in vivo*. Proper use of PBBK models in itself can contribute to reduction and refinement of animal studies by optimization of study design through identification of critical parameters and time frames in kinetic behavior. In addition, incorporation of *in vitro*-derived parameters will lead to a further reduction of large-scale animal studies for quantitative assessment of the biological activity of xenobiotics.

The Breakout Group concluded that a distinction can be made between the goals to be achieved:

- Short-term: improvement of the interpretation of *in vitro* toxicity data for estimating rodent LD50 values;
- Long-term: using *in vitro* data for estimating/predicting sublethal acute toxic effects caused by chemicals in humans (e.g., represented by a TD10 value, i.e., the dose at which mild toxicity could be expected in no more than 10% of the exposed humans).

It will be obvious that the latter goal is of greater interest for the risk evaluation of chemicals, where the protection of humans with regard to toxic effects is the highest priority.

These different goals need different scientific activities; different groups of chemicals will need different approaches for modeling the kinetics. In some cases, a great deal of information is available (e.g., on low molecular weight; volatile lipophilic compounds). For these compounds, reasonable estimates can be obtained for their partitioning in the organism based on their physico-chemical properties. Many kinetic parameters (e.g., Vd and k_e) are also determined by the size of the dose (i.e., the amount of compound available for systemic circulation) because of capacity-limited processes in metabolism and transport.

3.1.2 *Subjects of Discussion*

The intestinal barriers, the role of the gut flora, first pass metabolism, and (counter) transport systems were discussed. A number of cell lines are available to estimate absorption through the gut barrier. BBB and skin absorption models were also addressed. *In vitro* methods for these systems exist, but none reflects the full metabolic and transport capacity seen *in vivo*.

The current status of systems to estimate the kidney epithelia as a barrier was discussed. These

systems include the use of renal cell lines, such as LLC-PK_1 cells and MDCK cells. The former cells form low resistance epithelial monolayers when grown on permeable supports; the latter form extremely high resistance. However, these cell lines do not express all the relevant transporters found *in vivo*. The lack of the organic anion transporter is particularly problematic and cell lines transfected with these transporters may be more appropriate. Currently, an ECVAM prevalidation study is under way of trans-epithelial resistance and inulin permeability as endpoints in *in vitro* nephrotoxicity testing.

The ability to estimate biotransformation reactions of chemicals is of particular interest since acute toxicity may be mediated through the bioactivation or deactivation of chemicals. *In vitro* systems designed to address this possibility include:

- Liver homogenates;
- Microsomal preparations;
- Isolated cells;
- Primary monolayer cultures;
- More complicated cell cultures (co-cultures, 3D cultures);
- Transgenic cell lines.

QSAR systems have also been proposed for modeling the metabolic biotransformation of chemicals. The use of QSAR/QSPR and the development of software systems to predict "chemical functionalities" of compounds which may be used to estimate kinetic behavior (including protein binding) and the toxicodynamics were also discussed.

3.2 Identifying Needs

3.2.1 *In Vitro Methods for Evaluating Chemical Kinetics*

As mentioned above, the Breakout Group recognized a short-term and a longer-term goal for using *in vitro* or other non-animal techniques for predicting acute systemic toxicity. First, one focuses on the longer-term goal: how to use these techniques for the evaluation of a chemical's kinetics and the ultimate prediction of sublethal acute toxic effects in humans. Section 3.4.4 concentrates on the short-term (interim) goal: how to improve the prediction of acute lethal effects in rodents. *In vitro* methods, in combination with knowledge of a chemical's structural properties, can be used to predict/determine the chemical's absorption, distribution, metabolism, and elimination in an intact organism. However, it will be a major challenge for the field of *in vitro* toxicology to identify the particular target tissue(s) or cells and the time course of clinical toxicity in the absence of *in vivo* observations.

In the short-term, physico-chemical properties can be used to predict/determine partition. QSAR (or QPPR) can be helpful for this determination (DeJongh et al., 1997). *In vitro* determinations of rates of metabolism and of passage of a chemical across membrane barriers (e.g., GI $\Rightarrow$ blood; blood $\Rightarrow$ brain) will improve the kinetic modeling. Taken together, these may be able to be used to calculate an LD50 value (as administered to an intact organism) from the LC50 value in a basal cytotoxicity test. Presentation of any such predicted LD50 value also requires concurrent presentation of the quantitative uncertainties attendant to that value. In the long-term, knowledge of a chemical's kinetics will need to include a comparison of the kinetic and the toxicodynamic time-profiles. Moreover, knowledge of kinetics assists in determining the mode of toxic action and vice versa (Ekwall et al., 2000; Liebsch et al., 2000). [see MEIC evaluation of acute systemic toxicity, Appendix E].

3.2.2 *Biokinetics in the Overall Toxicological Evaluation*

Biokinetics is essential for relating administered dose of toxicant to concentration at the target tissue(s). Tissue-specific concentration of the toxicant is one of the mechanisms that can result in organ-selective toxicity. In addition, biokinetics can establish whether metabolism plays a role in modulating the toxicity. Such modulation can either attenuate or enhance the toxicity.

3.2.3 *Biokinetic Techniques as In Vitro Assays*

The following are techniques that need further development:

(1) *In vitro* determination of partition coefficients, metabolism, protein binding, and stability;
(2) Characterization of biotransformation enzymology;
(3) Structural knowledge and its translation into "chemical functionalities"; estimation of partition coefficients, metabolism, etc. ("*in silico*", including QSAR/QSPR);
(4) Biokinetic modeling, including the integration of toxicodynamic and biokinetic modeling in predicting systemic toxicity.

3.3 Current Status

3.3.1 *Prediction of Biotransformation*

Biotransformation can be carried out using human or animal hepatic subcellular fractions, human or animal primary hepatocytes, or human or animal hepatic precision-cut slices. The use of primary human hepatocytes in suspensions or culture requires specific expertise and may not be appropriate for use in all laboratories. Human or animal hepatic subcellular fractions can be cryopreserved and used at a later time to provide qualitative kinetic data, but these fractions may not reflect the integrated routes (activation and detoxification) of metabolism of a compound.

The selective use of cofactors can aid the determination of routes of metabolism. There is a need for standardization of the conditions for the preparation and incubation of rat hepatocytes. Rat hepatocyte incubations may overestimate the metabolic clearance of a compound. It is essential to quantify the rate of disappearance of the parent compound and desirable to quantify the rate of metabolite formation.

3.3.2 *Systems for Estimating Gastrointestinal Absorption*

Apparent membrane permeability and aqueous solubility are reasonably predictive of the fraction of a dose that will be absorbed through the GI tract. Several *in vitro* systems for measuring intestinal absorption include measuring apparent permeability constants in either intestinal tissue segments or cell monolayers that have been grown on a porous support. Cell lines used for this purpose include the human colon carcinoma cell line Caco-2, the canine kidney cell line MDCK, and the porcine kidney cell line LLC-PK_1. All systems are widely used in the pharmaceutical industry in the oral drug discovery process. Each system has advantages and disadvantages which may or may not be relevant depending on the chemical under study.

Cell lines do not require the use of animals. However, they often lack or have non-physiological levels of uptake and efflux transporters that are present *in vivo*. These transporters can dramatically affect the extent of bioavailability at low doses. The nature and extent of species differences in transporter activity/affinity is presently unknown. The Breakout Group consensus was that in the absence of data to the contrary, it would be appropriate to assume that an administered dose would be completely absorbed. This is a public health conservative approach. For those compounds where such an assumption is not appropriate, the above-mentioned *in vitro* systems can be used to provide experimental data on the fraction absorbed.

3.3.3 *Prediction of Renal Clearance/Accumulation*

Glomerular filtration and reabsorption in the proximal tubule determine the renal excretion of most compounds. These parameters can be predicted from the physico-chemical properties of the compound and its plasma protein (albumin) binding. These parameters are less predictable where active secretion or reabsorption and saturation kinetics are involved. Many of the currently available renal cell lines or renal cell primary cultures lack specific transporters (in particular, the organic anion transporter) which are implicated in the accumulation of several nephrotoxic compounds. The substrate specificity of other proximal tubular transporters is poorly defined.

3.4 Future Directions

3.4.1 *Proposed Approach for Consideration of Kinetics in the Estimation of Acute Oral Toxicity*

The diagram presented in Figure 3.1 illustrates a conceptual structure for the use of kinetic information in the estimation of acute oral toxicity. Under this scheme, available *in vitro* data on the absorption, tissue partitioning, metabolism, and excretion of a test material would be used to parameterize a chemical-specific biokinetic model (Clewell, 1993). In many cases, currently available QSPR/QSAR techniques could be used to estimate chemical properties and kinetics when the specific data for that chemical is lacking. For example, simple empirical correlations have been developed for estimating the tissue partitioning of a chemical from its water solubility, vapor pressure, and octanol/water partitioning (Paterson and Mackay, 1989; DeJongh et al., 1997). Emerging QSAR techniques (e.g., knowledge-based systems) may eventually prove useful in predicting potential target tissues for toxicity so that the appropriate assays of *in vitro* dynamics (response) could be selected. These target tissue assays would, in turn, provide information on the nature and location of the toxicity produced by the chemical (DeJongh et al., 1999).

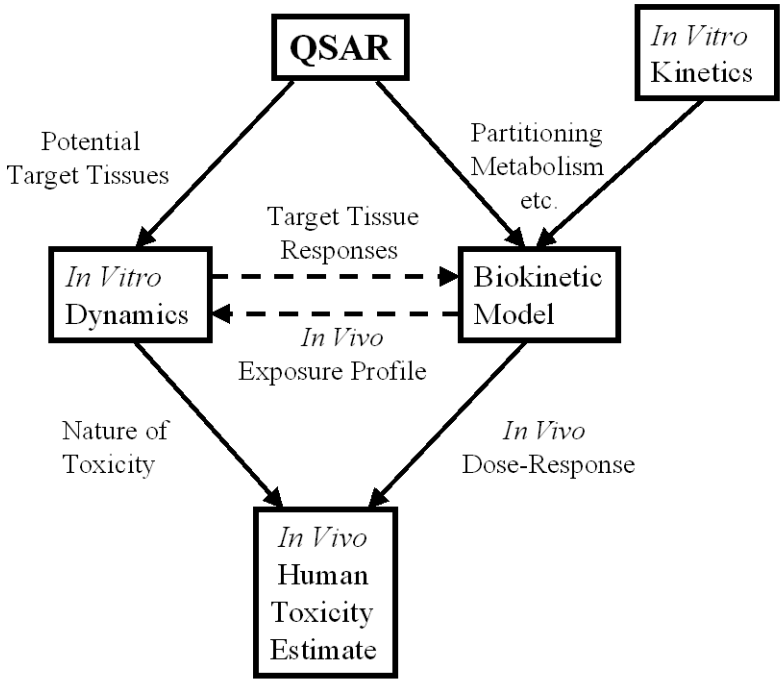


Figure 3.1. A recommended scheme for incorporation of QSAR (QSPR) data, *in vitro* data on kinetics and dynamics, and kinetic modeling in the estimation of human (or animal) toxicity

3.4.2 *Classification of Compounds Based on Their Physico-Chemical Properties*

The complexity of the biokinetic model would depend on the physico-chemical and biochemical characteristics of the chemical. In the specific case of acute toxicity, a simple one-compartment description of the administered chemical may suffice for many chemicals. The volume of distribution for such a model could be estimated

from the volume-weighted average of the estimated partitioning into various tissues, and estimates of fractional absorption and rate of clearance could be based on data for structurally similar compounds.

Each of these assumptions or predictions, however, introduces its own associated uncertainty into the result of the lethality risk estimate. Even with such a simple model, it may be possible to estimate the systemic concentrations that could be expected to result from an *in vivo* exposure to a given dose (DeJongh et al., 1999). Thus, the model could be used to relate the concentrations at which toxicity is observed in an *in vitro* toxicity assay to the equivalent dose that would be expected to be associated with toxicity for *in vivo* exposure. These models can also provide information on the temporal profile for tissue exposure *in vivo*, which can then be used in the design of the most appropriate *in vitro* experimental protocol (Blaauboer et al., 1999).

There are chemical classes for which a one-compartment description would not be expected to be adequate. However, the physiological mammalian structure (tissue volumes, blood flows, ventilation rate, glomerular filtration rate, etc.) is well characterized, and there is no difficulty in describing tissues separately. As mentioned above, techniques exist for estimating tissue-specific partitioning. Other data required would depend on the class of chemical. For volatile chemicals, ventilatory clearance can be estimated from the blood-air partition. For water-soluble chemicals, urinary clearance can be estimated from the glomerular filtration rate or the renal blood flow (for secreted compounds). For some classes of chemicals, it would also be necessary to determine the fractional binding of the chemical to plasma proteins or the partitioning of the chemical into red blood cells.

The greatest challenge in parameterizing the biokinetic model remains the estimation of metabolic clearance. The possibility is increasing to use *in vitro*-determined metabolic parameters (Vmax and Km) in order to accurately predict total body metabolic clearance (Houston and Carlile, 1997). Currently, it would be necessary to perform *in vitro* assays of the dose-response (capacity and affinity) for metabolic clearance (Kedderis, 1997; Kedderis and Held 1996; Kedderis et al., 1993). These assays are generally more expensive than the dynamic (toxicity) assays, since they necessarily involve the development of an analytical method for quantifying the concentration of the parent compound and its metabolite(s) in each tissue of interest over time. Quantification of the concentration of compound in the dynamic assays should also be preferred, but it is not absolutely necessary in that case. Eventually, as data accumulate for a large number of structurally-diverse materials, it might be possible to predict metabolism and disposition using knowledge-based systems.

An important underpinning of this process is that the kind of information necessary for a particular test material depends on its structure and physico-chemical properties. It seems reasonable to expect that chemicals could be categorized into classes based on their properties, and that this categorization would simplify the process of determining the data needed for a particular compound. This concept is illustrated in Figure 3.2. As noted above, the key physico-chemical properties of a test material involves its volatility (reflected in its blood-air partition, Hb/g), its water solubility (Sw), and its lipophilicity (reflected in its octanol-water partition, Ko/w). Compounds with similar properties can be grouped, and data from similar compounds can be used to fill gaps in the knowledge of a particular compound.

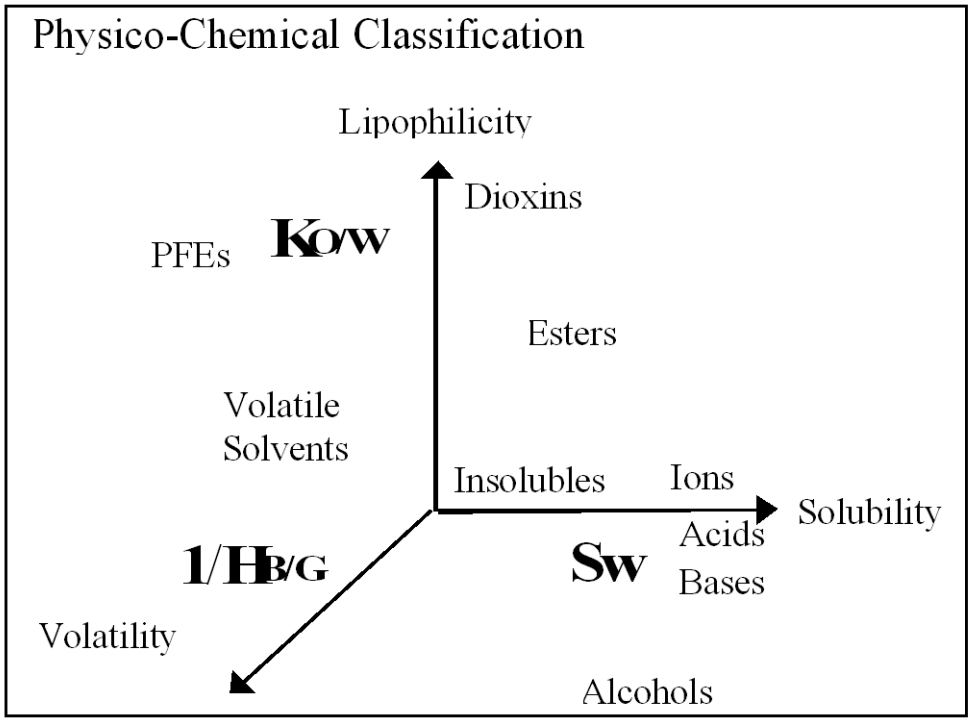


Figure 3.2. Classification of compounds based on their physico-chemical properties

There are two advantages of this *in vitro*/modeling approach over the traditional *in vivo* LD50 test. First, the *in vitro*/modeling approach can provide more extensive information than a traditional oral LD50 value provides. As information accumulates across chemicals, QSAR techniques could play a correspondingly greater role in the prediction of both kinetic and dynamic information. It is likely that QSAR techniques would be more successful for these fundamental processes and simple *in vitro* assays than they have been for the prediction of the *in vivo* assay. Secondly, all of these assays should be performed using human cell systems. The Breakout Group consensus was that *in vitro* testing should, when possible, be performed with human cells rather than rodent cells. This obviates the need, inherent in the rodent LD50 test, to extrapolate from rodents to humans. The uncertainties with the current approach of extrapolating *in vitro* derived data employing human cell cultures to the situation in the intact situation in humans will generally be smaller than those uncertainties for extrapolating data from animal cell experiments to humans.

Classification of chemicals according to their physico-chemical properties has been done extensively in the past. This approach has proven to be useful to predict effects, particularly within closely related classes of chemicals. However, this approach has limitations; it should not be used outside the boundaries of the prediction model used (i.e., the effects that can be predicted should be within the scope of the model assumptions).

If the focus is on the use of *in vitro*-derived data, then the importance of using specific cell systems becomes more important if one is looking at more specific forms of toxicity. Then the biological properties of the cells used become more important. Ultimately, there are two questions that coexist all the time: What does the chemical do to the cell?; and what does the cell do to the chemical? From this conceptual point of departure, the rate-determining step and more

often the rate-limiting steps need to be identified for mathematical modeling.

This problem and part of its solution can be illustrated based on central nervous system (CNS) vs. liver effects of solvents (limit it to small molecular weight chlorinated aliphatics). It is known from the Meyer-Overton rule (Meyer, 1937) that these anesthetic chemicals are very predictive of one another's CNS effects *in vivo*. However, these predictions do not hold for chronic liver effects and vice versa. This is understandable since the two effects have nothing in common, kinetics being the rate-determining step for anesthesia (wake-up driven by elimination of the chemical) vs. dynamics being the rate-determining step for liver cancer (slow reversibility of preneoplastic foci after complete elimination of the solvent). However, an acute endpoint such as reduced flicker fusion reflex is a much more sensitive endpoint of impairment than is chronic liver cancer. Therefore, people will be protected from cancer if regulation is based on the acute effect without the need for elaborate PBBK models based on metabolism in the liver.

The acute toxicity of all these solvents consists of CNS depression leading to respiratory failure without regard to the route of administration. These considerations will become more important when one moves away from the prediction of acute lethal toxicity towards predicting more subtile sublethal (acute) effects. However, these points are essential for modeling (sub)-chronic toxicity.

3.4.3 *Kinetic Support of Interim Rat LD50 Estimate*

In developing the approach just described, the focus of the Breakout Group was on the prediction of human TD10 values (i.e., the dose at which mild toxicity could be expected in no more than 10% of exposed humans). However, the Breakout Group acknowledged that there will be a need in the short-term for the estimation of rodent LD50 values under the HPV chemical program. The following discussion describes the application of the approach described above for this latter need.

3.4.3.1 ***Research and Development Needs***

In the first step, estimates of key kinetic parameters can be obtained either from data available on the chemical or from the use of QSPR techniques (which are based on physico-chemical properties of the compound). QSPR techniques can be used as a first approximation of key kinetic parameters such as absorption, partition, etc. If one can use kinetic data that are actually measured, then these data will prevail.

- Octanol/water partition coefficient;
- Water solubility;
- Saturation vapor pressure or blood-air partition;
- PKa;
- Molecular weight/volume (for estimating gastrointestinal absorption);
- Hydrogen bond donors/acceptors (for estimating gastrointestinal absorption).

This prior knowledge on kinetic parameters or the estimation on the basis of QSPR data can then be used to evaluate the *in vitro* LC50 values for a chemical. The assumption is that this LC50 value is equal to the concentration in the intact organism at which cells die *in vivo*. Depending on the chemical's physico-chemical properties, the kinetic model to be used for this estimation may be simple or more complex. For many (e.g., water-soluble compounds) a simple one-compartment model can be used to estimate the oral dose that would result in an average systemic exposure equivalent to the *in vitro* LC50 value over the time period of interest. The key factors needed for the model would be estimates of the oral bioavailability, tissue partitioning (to obtain the volume of distribution), and total clearance. Depending on the properties of the compound, the clearance could be dominated by metabolism, urinary excretion, or pulmonary ventilation. In most cases, metabolic clearance will have to be determined empirically.

A key problem for this near-term application is that many HPV chemicals may not have adequate analytical methods yet developed. Therefore, metabolism assays may be too expensive and time-consuming for high-throughput LD50

estimation. However, a simple, conservative estimate for the oral dose resulting in systemic exposure equivalent to an *in vitro* LC50 value could be obtained by assuming 100% bioavailability, ignoring metabolic clearance, and simply estimating tissue partitioning to obtain the volume of distribution (Vd). For example, a commonly used default for the volume of distribution for water-soluble chemicals as a function of body weight (b.w.) is:

$$Vd = 0.65 * b.w.$$

In this simple approximation, the relationship between the *in vivo* and *in vitro* assays could be described by the formula:

$$LD50 = LC50 * Vd / b.w..$$

Other adjustments could be made to this approach for chemicals where ventilatory or urinary clearance would be important, as described in the previous section. In addition, if data on bioavailability are available, such information could be factored in to obtain a more accurate LD50 estimate. An additional benefit of this approach is that similar calculations could be used to convert the *in vitro* LC50 value to an *in vivo* LC50 value for acute inhalation. These assumptions, however, introduce inherent uncertainties into the resulting calculation of the oral LD50 value and depending upon the material of concern, may result in substantial inaccuracies.

It is not certain that the approach described here is actually viable; in particular, it needs to be determined whether sufficient information is available on the compounds of interest to support the necessary calculations. A first step would be to characterize the HPV chemicals in terms of their physico-chemical properties and determining the range and most frequent combinations of physico-chemical properties. This would provide a basis for the selection of "proof of concept" chemicals (not necessarily HPV chemicals) that could be used to evaluate the kinetic parameter estimation paradigm described here.

Another useful exercise would be to identify the compounds that represent the outliers in the RC correlations of *in vitro* basal cytotoxicity assays with LD50 values. By determining the physico-chemical properties of these compounds, and knowing their target tissues, it might be possible to identify factors that could improve the correlation (e.g., consideration of BBB penetration) between predicted oral LD50 values in rodents and empirical values. In this way it might be possible to define a "predictive range" for various chemical properties over which the *in vitro* assay might be expected to provide reasonable LD50 estimates. Also, exclusion rules for identifying compounds for which the results of the *in vitro* assay should not be relied upon might be defined.

3.4.3.2 ***Tiered Approach for Evaluating Acute Toxicity***

A particular problem area in terms of the predictive value of the currently available *in vitro* toxicity assays is where toxicity is secondary to metabolic activation. In particular, it is possible that rapid oxidative or reductive metabolism could result in acute liver toxicity from oral exposure. Examples of such toxicity is the production of phosgene by the oxidative metabolism of chloroform and the acute liver necrosis seen after carbon tetrachloride exposure. Such toxicity would not be observed in *in vitro* assays using basal cells with little or no metabolic competence.

One possible approach for dealing with this problem is illustrated in Figure 3.3. The first step would be to estimate hepatocyte metabolism at a relatively low concentration (e.g., 10 micromolar). If the rate of metabolism (Vmax/Km) observed is low, then the basal cell LC50 value could be relied upon. If, however, the rate is high, then it would be necessary to identify the responsible enzyme system. This identification could be performed, for example, by using a microsomal (S9) fraction with selective addition of cofactors or inhibitors. If these studies indicate that the primary enzyme system is oxidative or reductive, then the possibility of toxicity associated with metabolic activation exists. In this case it would be necessary to perform a hepatocyte cytotoxicity assay. If the LC50 value for the hepatocytes was much lower than for the basal cells, it would be necessary to characterize the concentration-response for metabolism in order to predict the *in vivo* doses that might be associated with toxicity. On the other hand, if the primary metabolism represents detoxication (conjugation, sulfation, etc.), then the (acute) toxicity of the metabolites

will generally be much lower and, therefore, the basal cell assay results for the parent compound could be used with some confidence to calculate the LD50 value.

An alternative approach, suggested by Breakout Group 3, would be to begin with a basal cell cytotoxicity assay (to screen out highly toxic compounds) and then perform a toxicity assay with a hepatocyte primary culture. If similar LC50 values were obtained in both assays, the concern for toxicity secondary to metabolic activation could be effectively ruled out. In such cases, a much less extensive characterization of metabolism would be needed to support an estimate of clearance. On the other hand, if the toxicity in the hepatocyte assay was strikingly greater than that for the basal cells, the more complete characterization of metabolism discussed above would be justified.

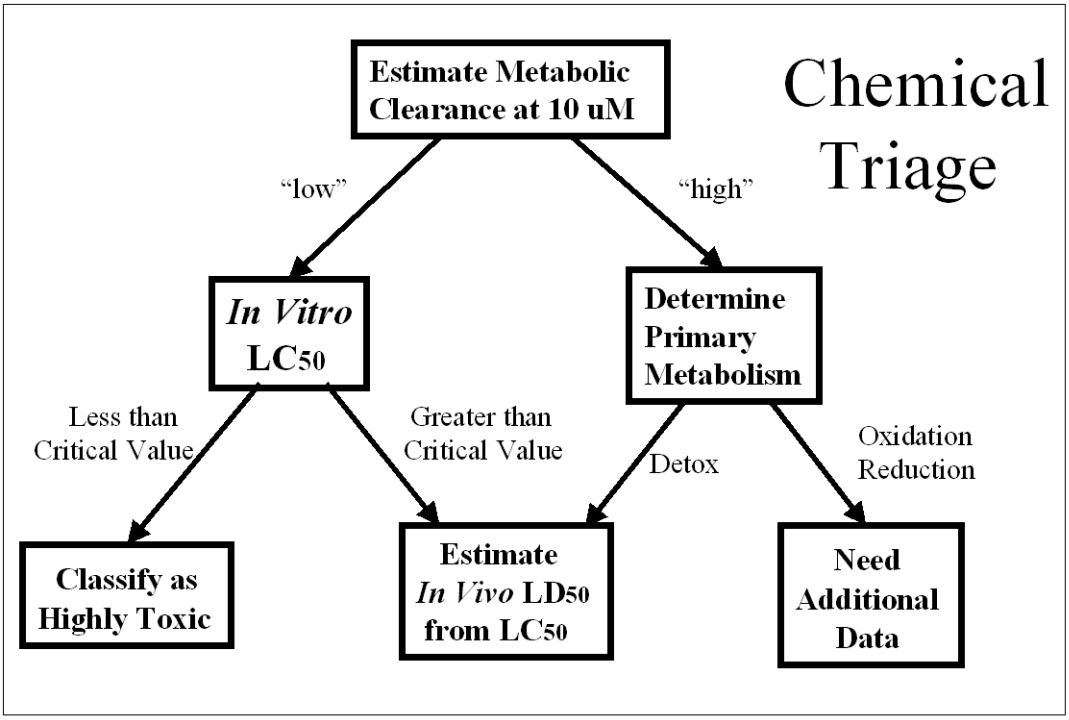


Figure 3.3: Tiered approach for evaluating acute toxicity

3.5 Recommendations

Table 3.1 (Section 3.5.2) lists a number of specific research areas in the area of biokinetics that the Breakout Group felt would improve the ability to use *in vitro* information in the prediction of acute toxicity. The following discussion highlights some of these research areas and illuminates some concerns emphasized by the Breakout Group.

3.5.1 *Long-Term Research Needs*

3.5.1.1 ***Metabolites and Acute Toxicity***

In some cases, a circulating metabolite can be responsible for acute toxicity in a tissue remote from its generation. Kidney toxicity from some chlorinated alkenes has been shown to result from the production of a GST conjugate (in the liver) which is converted to the cysteine conjugate in the

kidney, and then activated to a toxic mercaptan by beta-lyase. Another example: the CNS effects of chloral hydrate result from the metabolite trichlorethanol, which is produced in the liver. In cases such as these, metabolite-specific kinetic data are necessary to estimate target tissue exposure, and *in vitro* toxicity assays would have to be conducted with the metabolite(s) responsible for the observed toxicities. The latter, requires structural identification and synthesis of the metabolite(s) of concern in sufficient quantities to conduct these studies.

Other important research areas include the development of validated, stable human hepatocyte systems, as well as *in vitro* systems for key transporters (renal, biliary, etc.). A long-range goal should be the development of template PBBK models for the various classes of chemicals. Target tissues evaluated by *in vitro* assays would be included explicitly in the physiological structure of these models. The models would provide a mechanistic description of barrier functions (gut, bile, kidney, blood-brain barrier, skin), so that the data obtained from transporter assays could be readily incorporated.

3.5.1.2 ***QSPR Applications***

At the same time, specific QSPR applications need to be developed to provide the kind of information required by PBBK models (metabolism constants, binding, etc.). Unfortunately, the principal limitation in the development of useful QSPR applications appears to be the dearth of suitable data available for training knowledge-based systems.

3.5.1.3 ***Kinetics and Dynamics***

The interaction between kinetics and dynamics needs to be explored. For example, the effect of toxicity on the metabolism and excretion of a chemical or, conversely, the effect of metabolism or reabsorption on the toxicity of a chemical must be taken into account. Rigorous analyses of the time dimension in the conduct of these assays to account for duration and frequency of exposure is also an area that needs to be addressed. Because of cell viability issues, it may not be possible to reproduce the time frame of *in vivo* tissue exposure using *in vitro* systems. Also, the time frame for the appearance of toxicity may be quite different from the time frame for exposure to the chemical (Soni et al., 1999).

It is important to recognize that the proposed schemes (Figures. 3.1 and 3.2), and the discussion above, concern only the approximation and prediction of acute oral toxicity. It was neither the intent nor the purpose of the Breakout Group that these conclusions could be extended in any way to other types of toxicity that are relevant to public health risk assessment (e.g., developmental toxicity, sensitization, carcinogenesis, etc.). In the final analysis, *in vivo* exposure captures the effects of many potentially complex interactions that may be difficult to reproduce with *in vitro* systems.

3.5.2 *Research Needs for the Application of In Vitro Methods to the Prediction of Acute Chemical Toxicity*

Table 3.1 Biokinetic Research Needs

Kinetics	Kinetics-Dynamics Interface (Feedback)	Dynamics	Extrapolation
Understand the relationship between molecular structure, physical-chemical properties, and kinetic behavior of chemicals in biological systems. Develop mathematical modeling techniques to describe complex kinetic systems. Develop mathematical modeling techniques for tissue modeling (anatomically correct models). Develop algorithms to determine the optimum kinetic model for a particular chemical. Conduct research on modeling of fundamental kinetic mechanisms. Develop an optimal battery of *in vitro* assays to evaluate chemical-specific kinetic parameters. Develop QSAR models to predict kinetic parameters. Develop a library of generic models that are acceptable for regulatory risk assessments. Establish a database of chemical-independent parameters (mouse, rat, human).	Understand and model the mechanisms regulating the expression of proteins involved in kinetic processes – (metabolizing enzymes, transport enzymes, metallothionein, membrane channels, etc.). Understand and model effects of changes in physiological processes on kinetics of chemicals.	Develop *in vitro* biological models that are equivalent to *in vivo* tissues (i.e., models that maintain specified differentiated functions that are important for the toxicological phenomena under study). Develop mathematical modeling techniques to describe individual variability (genetic background). Develop mathematical modeling techniques to describe complex dynamic systems and genetic networks at the cellular and at the systemic level. Establish lines of differentiated human cells (e.g., derived from stem cells). Understand and model mechanisms of multi-cellular interactions in development of toxic responses (co-cultures). Understand and model relationships between cellular responses and biomarkers of systemic responses.	Inter- and intra-species extrapolation; comparison of genomic differences, or species-specific expression differences between species and within one species (e.g. polymorphisms in biotransformation enzymes). High dose - low dose extrapolation

3.6 References

Arms, A.D., and C.C. Travis. 1988. Reference physiological parameters in pharmacokinetics modeling. U.S. Environmental Protection Agency (EPA).

Blaauboer, B.J., M.D. Barratt, J.B. Houston. 1999. The Integrated Use of Alternative Methods in Toxicological Risk Evaluation. ECVAM Integrated Testing Strategies Task Force Report 1. ATLA 27: 229-237.

Blaauboer, B.J., A. Forsby, J.B. Houston, M. Beckman, R.D. Combes, and J. DeJongh. 2000. An Integrated Approach to the Prediction of Systemic Toxicity using Biokinetic Models and Biological *In Vitro* Test Methods. In: Progress in the Reduction Refinement and Replacement of Animal Experimentation. (Balls, M., A-M van Zeller, and M.E. Halder, eds). Elsevier, Amsterdam, pp. 525-536.

Brown, R. P., M. D. Delp, S.L. Lindstedt, L.R. Rhomberg, and R.P. Beliles. 1997. Physiological Parameter Values of Physiologically Based Pharmacokinetic Models. Toxicol. Ind. Health 13: 407-484.

Clewell, H.J. 1993. Coupling of computer modeling with *In vitro* Methodologies to Reduce Animal Usage in Toxicity Testing. Toxicol. Lett. 68: 101-117.

DeJongh, J., H.J.M. Verhaar, and J.L.M. Hermens. 1997. A Quantitative Property-Property Relationship (QPPR) Approach to Estimate *In Vitro* Tissue-Blood Partition Coefficients of Organic Chemicals in Rats and Humans. Archives of Toxicol. 72: 17-25.

DeJongh, J., M. Nordin-Andersson, B.A. Ploeger, and A. Forsby. 1999. Estimation of Systemic Toxicity of Acrylamide by Integration of *In Vitro* Toxicity Data with Kinetic Simulations. Toxicol. Appl. Pharmacol. 158: 261-268.

Ekwall, B., B. Ekwall, and M. Sjostrom. 2000. MEIC Evaluation of Acute Systemic Toxicity. Part 8. 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: 201-234.

Houston, J.B., and D.J. Carlile. 1997. Prediction of Hepatic Clearance from Microsomes, Hepatocytes, and Liver Slices. Drug Metabol. Rev. 29: 891-922.

Kedderis, G.L. 1997. Extrapolation of *In Vitro* Enzyme Induction Data to Humans *In Vivo*. Chem.-Biol. Interact. 107: 109-121.

Kedderis, G.L., and S.D. Held. 1996. Prediction of Furan Pharmacokinetics from Hepatocyte Studies: Comparison of bioactivation and hepatic dosimetry in rats, mice, and humans. Toxicol. Appl. Pharmacol. 140: 124-130.

Kedderis, G.L., M.A. Carfagna, S.D. Held, R. Batra, J.E. Murphy, and M.L. Gargas. 1993. Kinetic Analysis of Furan Biotransformation *In Vivo* and *In Vitro*. Toxicol. Appl. Pharmacol. 123: 274-282.

Liebsch, M., D. Traue, C. Barrabas, H. Spielmann, P. Uphill, S. Wilkins, J.P. McPherson, C. Wiemann, T. Kaufmann, M. Remmele, and H.G. Holzhutter. 2000. The ECVAM Prevalidation Study on the Use of EpiDerm for Skin Corrosivity Testing. ATLA 28: 371-402.

Meyer, K.H. 1937. Contributions to the Theory of Narcosis. *Faraday Soc. Trans.* 33: 1062-1064.

Paterson, S., and D. Mackay. 1989. Correlation of Tissue, Blood, and Air Partition Coefficients of Volatile Organic Chemicals. Brit. J. Indust. Med. 46: 321-328.

Soni, M.G., S.K. Ramaiah, M.M. Mumtaz, H.J. Clewell, and H.M. Mehendale. 1999. Toxicant-Inflicted Injury and Stimulated Tissue Repair Are Opposing Toxicodynamic Forces in Predictive Toxicology. Reg. Toxicol. Pharmacol. 29:165-174.

4.0 *IN VITRO* METHODS FOR ORGAN-SPECIFIC TOXICITY

4.1 Introduction

Breakout Group 3 reviewed *in vitro* methods that can be used to predict specific organ toxicity and toxicity associated with alteration of specific cellular or organ functions. The Breakout Group then developed recommendations for priority research efforts necessary to support the development of methods that can accurately assess acute target organ toxicity.

Knowledge of the effects of acute exposure to unknown materials is needed early in the development of new products and chemicals. Researchers who are using new chemicals in the laboratory need to know what types of safety precautions they need to take when handling these materials. Manufacturers must have some idea of the safe levels of exposure before they can develop the processes and build the facilities to safely manufacture the materials. The toxic doses also define precautions that must be taken when shipping materials, and govern the appropriate response of emergency personnel in case of accidental spills. Planned or inadvertent single-dose exposure of specific human or other populations may also occur, such as from accidental ingestion of common household materials, application of single use pesticides, and some pharmaceuticals.

The Breakout Group was asked to review *in vitro* methods for predicting specific target organ toxicity. Specifically the Breakout Group was asked to do the following: (a) identify the most important areas where *in vitro* methods are needed; (b) review and comment on the current status of *in vitro* methods to predict target organ toxicity; and (c) prioritize the need for future research in this area. In addition, the Breakout Group considered where it would be necessary to include prediction of specific target organ toxicity in developing an *in vitro* program to replace the current acute oral toxicity assays used in hazard classification systems.

The scope of the remit was very broad and the Breakout Group proceeded by identifying the organ systems where failure could lead to lethality after acute exposure. The Breakout Group reviewed each system individually, and then proposed a scheme for including the important endpoints identified into a replacement test battery for acute toxicity.

4.1.1 *Regulation of Industrial Chemicals and Pesticides*

A representative (Dr. Karen Hamernik) of the U.S. EPA related the needs of an agency that regulates industrial/commodity chemicals and pesticides. In addition to their use in assigning an international hazard classification, the results of acute toxicity tests are used to set doses for *in vivo* cytogenetics assays, acute neurotoxicity tests, and, occasionally, for other types of rodent tests. Dose setting may utilize LD50 information and dose response data over a range of doses for a given test material. In addition, information on the effect of single exposures is gathered during acute neurotoxicity tests, developmental toxicity tests, and metabolism studies. In these tests, multiple endpoints may be measured and the results can be used for hazard and risk assessments for single-exposure scenarios.

The U.S. EPA is concerned with organ-specific effects -- including their severity, onset, and duration -- that become apparent from various test material exposure scenarios including acute, sub-chronic, or chronic exposure. Some study protocols provide reversibility-of-effects information. Information on organ-specific effects may have an impact, at least in part, on risk assessment methods depending on the effect of concern, whether a mechanism for toxicity can be proposed or identified, and on the available dose-response information. For instance, organ-specific effects may impact decisions on whether to regulate based on cancer or non-cancer endpoints, to use linear or non-linear models, and whether to use dose-response data or benchmark dose approaches.

How organ-specific effects impact risk assessment depends to some extent on where the effects occur on the dose-response curve, what types of effects are seen and their severity, and the nature of the exposure. Examples include the

presence of clear toxic effects such as necrosis and changes in enzyme activities or elevations in hormone levels that may be considered precursors to possible longer-term toxic, or even carcinogenic, effects. The impact of these effects may depend upon whether they are seen only in adult animals, young or adolescent animals, or during *in utero* exposure. Toxicity data are used for human risk assessment and to provide clues for potential concerns for effects in wildlife.

In the United States, organ-specific effects seen in toxicity studies may trigger Food Quality Protection Act-related issues such as the possibility of grouping chemicals with common modes of action or mechanisms for cumulative risk assessment. Certain organ-specific effects may serve as a starting point to look at questions related to human relevance. The presence of such findings may trigger the need for additional studies to support the suspected toxicological mechanism.

4.1.2 *Regulation of Pharmaceuticals*

A representative (Dr. David Lester) of FDA/CEDR related the needs of an agency that regulates pharmaceutical materials. CEDR does not ask for, nor regulate, non-clinical toxicity testing, and does not use estimates of the LD50 value in its assessments. In general, the agency does not find identification of specific organ toxicity after single-dose acute exposure useful since most pharmaceuticals are given as multiple doses.

The results of acute toxicity tests are not useful in establishing dosing regimes because most pharmaceuticals are developed for multiple use. Acute effects are more important for oncologic drugs because the margins of safety may be smaller. Single-dose studies may also be useful for developing imaging agents where it is important to understand tissue distribution after a single exposure.

In vitro studies are often performed in drug development as part of the effort to understand the disease process or to understand the actions of the drugs on specific cells. In drug development, the risk assessments are based on the total dose of the material given and not on the tissue concentration. *In vitro* studies have been used in setting doses for initial human exposure to cancer therapeutics, but otherwise are rarely used for dose setting because current methods cannot extrapolate from the *in vitro* concentration to the dose that must be given to achieve similar effects *in vivo*. Animal studies may be used for initial dose setting for early clinical studies, but these are usually not acute, single-exposure studies.

4.1.3 *U.S. National Toxicology Program (NTP)*

The Breakout Group also heard a presentation (from Dr. Rajendra Chhabra) on the use of acute oral toxicity data by the National Toxicology Program (NTP). The NTP does not find it necessary to use acute studies to set doses for subchronic studies; instead, researchers go directly to 14- or 90-day studies. If there are sufficient data on the chemical of interest, then they are often able to avoid a 14-day study. The results of 90-day studies in rodents are used to set doses for chronic studies and also to determine what specific types of additional studies may be needed (i.e., reproductive, cancer, neurotoxicology, etc.). To facilitate decision making and reduction of animal use, the NTP adds several endpoints to the 90-day study including sperm morphology, immunotoxicology, neurotoxicology, and a micronucleus test.

The NTP is evaluating a battery of *in vitro* tests that might reduce the need for 14-day dermal toxicity studies. The tests include:

- The bovine corneal opacity test;
- The skin permeability assays;
- The EpiDerm™ model for dermal irritation/corrosivity;
- A neutral red uptake (NRU) assay for systemic toxicity;
- A primary rat hepatocyte assay for hepatic toxicity.

Five chemicals have been tested in this battery. The 14-day *in vivo* rodent study costs about $150,000, uses 120 animals, and takes about six months to perform. An accurate battery of *in vitro* tests would be less expensive in both time and cost.

4.1.4 *Initial Considerations*

The Breakout Group agreed for the purposes of this exercise to define acute toxicity as *"toxicity occurring within 14 days of a single exposure or multiple exposures within 24 hours"*. For evaluating chemicals for acute toxicity, the Breakout Group identified the following major organ systems as the ones that need to be considered:

- Liver;
- Central nervous system;
- Kidney;
- Heart;
- Hematopoietic system;
- Lung.

Damage significant enough to cause death can occur to these systems after a single acute exposure. The Breakout Group recognized that local effects of xenobiotics on the skin, gastrointestinal tract, and eye may also be important, but agreed to focus on systemic effects rather than local effects. The Breakout Group also recognized that the developing embryo may suffer serious, even lethal, consequences after a single acute exposure to a xenobiotic. However, the Breakout Group felt these effects are adequately evaluated by the standard battery of tests for reproductive and developmental effects and do not need to be included as part of an *in vitro* battery to replace the acute toxicity tests.

The Breakout Group discussed the use of rodent cell cultures as the basis of *in vitro* tests to predict acute toxicity. The work of Ekwall (Ekwall et al., 2000) indicates that for general cytotoxicity cells of human origin correlate best with human acute lethal blood concentrations. There are well recognized species differences in response to many classes of xenobiotics that must be taken into account as systems are developed to predict effects specific to individual organ systems. Considering the species differences currently recognized and other differences that might not yet be identified, the Breakout Group recommends that every effort should be made to use human-derived cells and tissues, preferably normal, as the basis for *in vitro* assays since data from the *in vitro* studies will ultimately be used to predict toxicity in humans.

4.2 Review of a Proposed Screen to Elucidate Mechanism of Injury

The Breakout Group examined specific endpoints or organ systems. Both *in vivo* and *in vitro* systems are used extensively in industry and academia to aid in the understanding and prediction of mechanisms of toxicity. The review attempted to highlight situations where *in vitro* studies provide information at least as useful and often more useful than *in vivo* studies and to identify areas where further research is needed before *in vitro* techniques will be able to replace whole animal studies.

The Breakout Group first reviewed a program using eight different normal, human epithelial cell lines or primary cells for initial toxicity screening to elucidate mechanisms of injury by measuring comparative tissue-specific cytotoxicity of cancer preventive agents (Elmore, 2000; Elmore, in press). Tissue-specific cytotoxicity was assessed using cell proliferation at three days and five days, mitochondrial function, and PCNA or albumin synthesis (hepatocytes only) as endpoints. The cells used were early passage cell lines following cryopreservation or were primary cultures (hepatocytes) and included liver, skin, prostate, renal, bronchial, oral mucosa, cervix, and mammary tissues.

The results suggest that different chemicals induced unique tissue-specific patterns of toxicity. Changes in toxicity following three and five day exposures provide additional information on both delayed toxicity and the potential for recovery. Confirmation of the predictive trends was confirmed with several agents in keratinocytes using 14-day cultures with multiple exposures. Ongoing studies will compare the *in vitro* data with blood levels from preclinical animal studies, and plasma levels and observed side effects from clinical trials.

4.3 *In Vitro* Methods for Determination of Acute Liver Toxicity

Adequate liver function is critical to the survival of an organism. The liver is at high risk for injury because it is actively involved in metabolizing xenobiotics, and because the liver is exposed first

to materials absorbed from the gastrointestinal tract. The liver also excretes many materials via the bile and this puts the biliary system at risk for toxicity as well. For these reasons, one of the highest priority needs is for a test system that can accurately evaluate the effects of xenobiotics on the liver. Test systems need to be able to assess both the potential for hepatic toxicity and whether the liver will be able to metabolize the test chemical either to a more or less toxic moiety. Xenobiotics may also affect the biliary tract, and an *in vitro* system to investigate these effects will also be needed.

4.3.1 *Available Non-Animal Models*

Available non-animal models include metabolically competent animal or human liver cells. Such cells have been cryopreserved and cryopreserved human cells are available commercially. The cells of human origin have a short life span, but they can be obtained with certain well-characterized metabolic profiles including specific active P450 systems. Immortalized human cell lines, some of which have been transfected to express specific recombinant phase I or II enzymes are also available, but most cell lines are limited to expressing only one enzyme.

Assessment of the potential for hepatic metabolism is possible using isolated hepatocytes (Cross and Bayliss, 2000; Guillouzo, 1997) and cell lines. Liver microsomes are used in high throughput screening assay systems to determine the extent of metabolism of a parent compound. Whole liver homogenates, subcellular fractions, and liver slices are also commonly used in basic research on hepatic function and toxicology (Guillouzo, 1998; Parrish, et al., 1995; Ulrich et al., 1995; Waring and Ulrich, 2000). A report on the ECVAM Workshop on the Use of Tissue Slices for Pharmacotoxicology Studies includes a comprehensive review of the use of liver slices in toxicology (Bach et al., 1996). These systems can be robust, but the supply of human liver tissue is limited and is decreasing as more donor liver is being used for transplantation

Recently, more complex systems have been developed in an attempt to better mimic hepatic function. Cell culture techniques that involve sandwiching liver cells between layers of collagen can be used to study induction of metabolic function, but it is difficult to examine the hepatocytes after treatment because of the collagen in the system. Liver cells can also be cultured as small compact spheres of cells. As these spheroids grow, they tend to become necrotic in the center so their usefulness in toxicology needs to be established.

There have been some attempts to develop *in vitro* systems to study effects on biliary function. A couplet system made up of two hepatocytes with bile canaliculi attached has been described. This system is very labor intensive and currently would not be viable as a routine test system but is useful as a way to study mechanisms of cholestasis. In addition, liver fibroblasts can be cultured for the study of mechanism of hepatic cirrhosis.

4.3.2 *Specific Endpoint Measurements*

As *in vitro* systems for hepatic function are developed to replace animals in acute toxicity studies, the specific endpoints which should be considered are changes in enzyme systems, membrane damage, changes in mitochondrial function, changes in albumin synthesis, and possibly cell detachment. It will be important to identify systems that express the most important metabolic systems present in normal human liver. The Breakout Group discussed the need for multiple cell lines to represent the known diversity of enzyme systems expressed by the human population. While such systems are very useful in drug development, the Breakout Group recognized that this degree of sophistication is not available with the current *in vivo* systems and should not be required for a replacement system for acute toxicity.

4.3.3 *Future Needs*

Future work in the area of hepatic toxicology will depend upon the development of more robust models that are as metabolically competent as mature human hepatocytes *in vivo*. Pharmaceutical companies are currently using *in vitro* assays of hepatic function for screening new drugs and as their methods become more readily

available, they may be useful in acute toxicity testing. An ILSI HESI Genomics Subcommittee is assessing changes in gene expression that occur in response to several prototypic chemicals, including hepatotoxicants, and will be attempting to correlate the gene expression changes with changes in various biological and toxicological parameters.

Two methodological issues need to be addressed as *in vitro* methods are developed and evaluated. First, when culturing liver cells, it is vital that the cells are constantly monitored to ensure they are still expressing the desired characteristics and this monitoring must be built into protocols. Second, there is considerable variability in enzyme function between cells from different individual donors, and for toxicity testing it will be necessary to agree upon the cell characteristics needed for an appropriate test system that will best represent the overall human population.

There is a high-priority need to develop a system for regulatory use that will be able to recognize which compounds the liver will metabolize to another compound or compounds. To replace whole animal, systems must be devised that can also determine the effect of the product or products of hepatic metabolism on other organ systems in a dose responsive manner.

There is a need for a worldwide database comparing human *in vitro* and *in vivo* data for hepatic toxicity. Scientists attempting to develop hepatic systems for toxicity testing are encouraged to share methodology and cell lines. Collaboration among laboratories would increase the pace of research and avoid development of multiple and competing test methods.

4.4 *In Vitro* Methods for the Determination of Acute Central Nervous System (CNS) Toxicity

Neurotoxic effects after a single dose are often expressed as either overall CNS depression resulting in sedation, or excitation, generating seizures or convulsions. The molecular mechanisms for these states may be related to very specific toxicant-target interaction, or the targets may be general for all cell types but are involved in critical functions in neurons. Because CNS effects can lead to acute lethality, a neurotoxicological screen should be performed when certain criteria in the tiered test battery, as described in Section 4.10.1, have been fulfilled. Briefly, the steps are physico-chemical or other information indicating that the toxicant can pass the BBB, low basal cytotoxicity (high EC20 or EC50 values) in non-neuronal cells, low hepatotoxicity, and no evidence of impaired energy metabolism at non-cytotoxic conditions. If these initial criteria are fulfilled, investigations of the neurotoxic potential of the test material must be carried out. The cellular targets can be either general or very specific functions.

4.4.1 *Important General Cellular Functions for CNS Toxicity*

Examples of important general cellular functions that upon impairment may cause severe brain damage after acute exposure are decreases in resting cell membrane potential, increases in intracellular free calcium concentration ($[Ca^{2+}]i$), and formation of free radicals and reactive oxygen species (ROS). Cytotoxicity may, eventually, occur as a result of severe insult to these cellular functions. In some cases, astrocytes are the immediate target and the toxic reaction may appear as astrocyte activation and formation of neurotoxic cytokines. An early marker for acute astrocyte activation is increased glial fibrillary acidic protein (GFAP) expression.

4.4.1.1 *General Endpoints*

Endpoints that can be assessed include cell membrane potential, increased $[Ca^{2+}]i$, and free radical formation that can easily be measured by fluorescent probes or by simple spectrophotometry. Cytokines and GFAP levels can be determined by immunochemical techniques, such as ELISA, or by mRNA quantification (e.g., *in situ* hybridization, RT-PCR, or gene array analysis). Most assays can be performed on adherent cells in microtitre plates, which make them useful for high throughput screening.

4.4.1.2 *Cell Models for General Functions*

Several cell models are available. General cell functions can be studied in cell types that possess a near normal cell membrane potential and

aerobic energy metabolism. Certain differentiated human neuroblastoma cell lines, such as SH-SY5Y, fulfill these criteria and are easy to obtain, culture, and differentiate. Human brain neural progenitor cell lines (e.g., NHNP and NT2) are now widely available. The NHNP cell line has the advantage that in culture it differentiates into a mixture of neurons and glia. It can be passed through numerous passages and forms spheroids in suspension (Svendsen et al., 1997). Glial cell lines are generally poorly differentiated even though there are reports of some GFAP-expressing human cell lines (Izumi et al., 1994; Matsumura and Kawamoto, 1994). Rat glioma 9L cells have been reported to manifest astrogliosis upon chemical exposure (Malhotra et al., 1997). Nevertheless, primary rat astrocyte cultures are used in most studies on astrocyte activation.

4.4.2 *Important Specific Functions for CNS Toxicity*

Specific functions can be measured by assessing neuronal targets that will cause acute CNS depression or excitation if their functions are impaired. These functions are voltage operated Na^+, K^+, and Ca^{2+} channels and the ionotropic glutamate NMDA, $GABA_A$, and nicotinergic acetylcholine (nACh) receptors. Furthermore, severe intoxication may occur after acute exposure to cholinesterase inhibitors. Besides the acute effect on cholinesterase function, delayed neuropathy may also be evident after a single dose.

4.4.2.1 ***Specific Endpoints***

Ion fluxes over the cellular membrane can be estimated by using various ion-selective fluorescent probes. However, upon stimulation, effects on ion channels or receptors change the net membrane potential. Eventually, this will result in altered Ca^{2+} -fluxes and $[Ca^{2+}]i$, which in turn will affect transmitter release. Therefore, effects of toxicants on receptor and ion channel functions may be detected as increased or decreased $[Ca^{2+}]i$ (Forsby et al., 1995) or neurotransmitter release (Andres et al., 1997; Nakamura et al., 2000; Smith and Hainsworth, 1998; Wade et al., 1998). The effects may be evident directly by the toxicant itself, but also after applied stimuli such as potassium-evoked cell membrane depolarization, possibly in the presence of receptor agonists. Acetylcholine esterase (AChE) activity in neuronal cells can be measured in differentiated cells such as SH-SY5Y cells. Evaluating changes in the ratio between AChE and neuropathy target esterase (NTE) has been proposed as a method for estimating the risk for delayed neuropathy (Ehrich et al., 1997).

4.4.2.2 ***Cell Models for Specific CNS Functions***

Cell models for studies on specific CNS functions should be of human origin, mainly because certain enzyme structures and receptor sub-unit expressions differ among different species. Furthermore, the level of cellular differentiation is crucial. The cell lines must, in most cases, be treated with differentiating agents such as retinoic acid to express features of normal, adult neurons. Cells that are transfected with genes expressing specific receptor and ion channel proteins can also be useful for studies on specific functions.

One example of non-primary neuronal cells is the human neuronal progenitor NT2 cells derived from a teratocarcinoma. The NT2 cells can be terminally differentiated to NT2-N cells after treatment with retinoic acid and mitosis-arresting agents after months in culture. NT2-N cells express functional NMDA and $GABA_A$ receptors (Younkin et al., 1993; Munir et al., 1996; Neelands et al., 1998). The previously cited NHNP neural human brain progenitor cell line could also serve as an important model system for neurotoxicity screening (Svendsen et al., 1997). It is not as well characterized as the NT2 line but deserves investigation. Alternatives to NT2-N may be native or differentiated human neuroblastoma cell lines (e.g., SH-SY5Y, IMR32 and CPH100). However, their receptor sub-unit expression and receptor function may vary from normal receptors present in adult brain tissue.

Co-cultures of neuronal and glial cells may be used for studies on interactions between neurons and glia cells. For instance, NT2 cells differentiate and establish functional synapses when they are cultured on astrocytes (Hartely et al., 1999). Upon differentiation, the NHNP cell line cultures contain a mixture of astrocytes and neurons varying in ratio from 1:9 to 2:3. In

suspension, the NHNP cells form spheroids (see Clonetics web site). Reaggregated embryonic brain cultures have been recommended for screening of neurotoxic compounds (Atterwill, 1994) but significant further work on this promising model is needed before it can be used as a standard test method.

4.4.3 *Future Needs*

Some endpoints, assays, and cell models for the more general endpoints have been studied and used extensively, which make them ready for formal validation. However, most assays and cell models determining effects on special functions still need significant basic research before they will be useful in screening systems.

4.5 *In Vitro* Methods to Assess Blood-Brain Barrier (BBB) Function

The CNS is dependent on a very stable internal environment. The BBB helps maintain this stable environment by regulating all uptake into and release from the brain of substances involved in CNS metabolism. The barrier acts as a functional interface between the blood and the brain, rather than as a true barrier, and this function is localized to the brain capillary endothelial cells. These cells differ from endothelial cells in other organs in that they form tight junctions. They have a higher turnover of energy and thus contain numerous mitochondria; they have a low endocytotic activity. Furthermore, they express specific transport proteins and enzymes. Water, gases, and lipid-soluble substances may pass the BBB by simple diffusion whereas glucose, monocarboxylic acids, neutral and basic amino acids, and choline are taken up from the blood by active processes. Ions pass the BBB very slowly and proteins generally not at all. Weak organic acids, halides, and potassium ions are actively transported out of the CNS.

From a toxicological viewpoint, three aspects of the BBB are of interest: (a) the BBB regulates uptake and release of endogenous substances and also xenobiotics, (b) toxic substances may interfere with the structural and functional properties of the BBB, and (c) certain parts of the CNS (e.g., areas in the hypothalamus and the choroid plexa), have poorly developed BBB functions. The latter is also true for all parts of the embryonic and juvenile brains.

Several authors and working parties have identified the need for a reliable *in vitro* model of BBB functions as being essential for the development of alternative methods for use in tests of acute systemic toxicity, neurotoxicity, and in drug development (Balls and Walum, 1999; Ekwall et al., 1999; Janigro et al., 1999; the ECVAM workshop on *In Vitro* Neurotoxicity [Atterwill et al., 1994], the ECVAM Neurotoxicity Task Force, [1996, unpublished], and the BTS Working Party Report on *In Vitro* Toxicology, [Combes and Earl, 1999]). ECVAM is currently supporting a prevalidation study of *in vitro* models for the BBB. The study largely follows the recommendations published by Garberg (1998).

4.5.1 *Endpoints for Acute Toxic Effects*

For acute toxic effects, there are two endpoints for toxic insult to the blood brain barrier: (a) partial or complete breakdown of the barrier function (i.e., effects on the ability of the BBB to exclude endogenous and exogenous substances) and (b) changes in the specific transport capacity of the BBB. There is a need to measure the ability of the normal BBB to transport toxicants into or out of the brain.

4.5.2 *Models*

Models currently being assessed in the ECVAM-sponsored prevalidation study include:

- Immortalized endothelial cell lines of both human and animal origin;
- Primary bovine endothelial cells co-cultured with glial cells;
- Barrier-forming continuous cell lines of non-endothelial origin.

Preliminary results from the ECVAM prevalidation study, as well as previously published results, show that the rate of penetration of compounds that pass the BBB by simple diffusion can be estimated by the determination of log P, or by the use of any cell system that forms a barrier (e.g., MDCK or

CaCo2 cells). This means that the distribution of lipophilic compounds over the BBB can be determined simply, and that the first aspect of acute toxic effects (i.e., impairment of the barrier function [see above]) can be studied in continuous cell lines, provided they are able to form tight junctions.

With respect to the second endpoint, impairment of the transporter functions and the transport-mediated brain uptake, the situation is different. The modeling of these features of the BBB ideally requires an *in vitro* system with a high degree of differentiation, including the significant expression of all transporter proteins representing species-specific properties. At present, this can only be achieved in primary cultures of brain endothelial cells co-cultured with brain glial cells.

A model presented by Stanness et al. (1997) shows development of a dynamic, tri-dimensional *in vitro* culture system (DIV-BBB) that mimics the *in vivo* BBB phenotype more closely than other models in use. In this system, cerebral endothelial cells are cultured in the presence of astrocytes using a hollow fiber technique. The fiber cartridge, representing artificial capillaries, is exposed to a luminal pulsatile flow of medium. Although a very good model for the *in vivo* situation, the DIV-BBB model may be too resource intensive to be of practical use in a screening situation.

4.6 *In Vitro* Systems to Study Kidney Toxicity

The major effect seen in the kidney after acute exposure to a nephrotoxin is acute tubular necrosis. In approximately 90% of the cases, the changes are seen in the proximal tubular cells (proximal to the convoluted tubules). These cells have high metabolic activity and a significant concentrating function, both of which put them at increased risk for damage. There are a much smaller number of substances that are toxic to the distal tubular cells. While acute toxicity in tubular cells is highly significant and can be fatal, it is important to recognize that these cells have great regenerative capacity and with adequate treatment and time will repopulate and replace the destroyed cells.

There are a few substances that cause direct glomerular damage which is more serious because glomerular damage is permanent resulting in the loss of the affected nephron. Although the kidney has a considerable reserve capacity of nephrons, it is important to understand the effects of a reduction of this reserve capacity particularly in individuals, such as the elderly, who may already have a reduced number of nephrons.

A comprehensive review of the use of *in vitro* systems to assess nephrotoxicity has been completed by ECVAM and was used as the basis for the discussion (Hawksworth et al., 1995). *In vitro* systems will need to utilize metabolically competent kidney tubular cells. This should not be as difficult as liver systems since much is known about the metabolic function of renal tubular cells, and there does not appear to be significant variability between individuals. In addition to direct cytotoxicity, *in vitro* systems must be able to evaluate the barrier function of the kidney. A system to assess this parameter is currently being studied in Europe, with support from ECVAM. In addition, *in vitro* systems may need to assess transport functions. At this time it is not clear how important these functions are in acute toxicity. It is also not known how much variability exists in these functions from one individual to another. The specific transport functions are not completely characterized and more basic research is needed before test systems can be developed.

It is possible to measure kidney function in a non-invasive fashion in humans who are exposed to low levels of xenobiotics, for instance, in occupational exposures. It would be valuable to evaluate the correlation of the results from *in vitro* toxicity tests with information from humans.

4.7 *In Vitro* Methods to Assess Cardiotoxicity

Cardiovascular toxicity can result from excessive accumulation of toxic chemicals within the tissue, cardiovascular-specific bioactivation of protoxicants, and/or chemical interference with specialized cellular functions. Because a cardiotoxic insult interferes with the ability of the

heart to pump blood through the vasculature, blood flow to major organs is often compromised. Vascular toxicities are often characterized by slow onsets and long latency periods and are not usually important in acute toxicity; however, changes in arterial pressure and blood flow control may be significant in acute effects.

The pathogenesis of cardiovascular injury often involves the elucidation of oxidative mechanisms and many cardiovascular disorders are characterized by loss of redox homeostasis. The central role for oxidant mechanisms is consistent with studies which show evidence of beneficial effects of antioxidants provided to patients with coronary heart disease (Napoli, 1997). The vascular production of reactive oxygen metabolites increases substantially in disease states (Harrison, 1997). Links between cardiovascular and cerebro-vascular disorders have also been established. During periods of emotional stress, adrenaline toxicity to vascular endothelial cells may involve its deamination by monoamine oxidase A to form methylamine, a product further deaminated by semicarbazide-sensitive amine oxidase to formaldehyde, hydrogen peroxide, and ammonia (Yu et al., 1997).

4.7.1 *Perfused Organ Preparations*

Perfused organ preparations are currently the most representative of the *in vivo* situation. Aortic preparations are most preferred; they can be readily excised, perfused, and super-perfused with appropriate buffers, (Crass et al., 1988). Perfused preparations are advantageous because they retain the level of structural organization found *in vivo.* Toxin-induced changes in physiologic/pharmacologic sensitivity and changes in excitability and/or contractility can be readily evaluated. The biological actions of nitric oxide, a soluble gas synthesized by the endothelium, was first discovered using perfused preparations. Because perfused organ preparations require harvesting fresh tissue, better methods are still needed. In addition, significant limitations of perfused preparations in toxicity testing include the small number of replicates that can be processed, the time required for isolation, and the provision that the system can only be used for short periods of time because of rapid loss of viability. Parameters measured include: (a) time to peak tension, (b) maximal rate of tension development, and (c) tension development. Oxygen concentration of the perfusate provides an index of myocardial oxygen consumption. Pin electrodes can be used to obtain electrocardiographic readings. Measurements of contractility and stress development can be used to evaluate effects of drugs and chemicals.

4.7.2 *Isolated Muscle Preparations*

Isolated muscle preparations consisting of strips of atrial, ventricular or papillary muscles (Foex, 1988), or segments from vascular beds (Hester and Ramos, 1991) can be super-perfused with oxygenated physiologic solutions for measurements of tension development. The pre-load and after-load placed on the tissue can be controlled accurately to evaluate isometric force development, isotonic force development, and quick-release contractions. Oxygenation of the tissue is a function of diffusion, and the thickness of the strips and oxygen concentration in the solution bath must be carefully monitored. The stability of these muscle strips is limited to short time periods. Because many preparations can be made from each animal, these systems use less numbers of animals than perfused organ preparations.

Isolated preparations have been used to examine the angiotoxic effects of ethanol (Rhee et al., 1995), acetaldehyde (Brown and Savage, 1996), palytoxin (Taylor et al., 1995), and cadmium (Ozdem and Ogutman, 1997). Regional differences in physiologic and pharmacologic responsiveness must be considered in developing strategies that examine vasculotoxic responses. Aortic rings exhibit higher sensitivity to norepinephrine than mesenteric artery rings, while the reverse effects are found with serotonin. However, no differences in sensitivity to KCl and $CaCl_2$ were observed (Adegunolye and Sofola, 1997). Differences between the two vessels appear dependent on agonist ability to mobilize calcium from intracellular stores.

4.7.3 *Organ Culture Preparations*

Organ culture preparations offer long-term stability as compared to other *in vitro* preparations. Whole fetal hearts from mice and chicks have allowed the study of processes associated with myocardial cell injury (Ingwall et al., 1975; Speralakis and Shigenoubu, 1974). Organ-cultured blood vessels have led to elucidation of structural/functional relationships of the vessel wall matrix (Koo and Gottlieb, 1992). However, organ culture of rat aortic rings results in significant loss of contractile responsiveness to different agonists within 24 hour (Wang et al., 1997).

4.7.4 *Tissue Slice Preparations*

Tissue slice preparations of cardiac tissue have been characterized as models to evaluate toxicity of xenobiotics (Gandolfi et al., 1995) and could be useful in toxicity testing applications (Parrish et al., 1995).

4.7.5 *Single-Cell Suspensions*

Single-cell suspensions of embryonic or neonatal cells that are derived from ventricular, atrial, or whole heart tissue can be easily prepared by enzymatic and/or mechanical dissociation of the tissues. Adult hearts can also be dissociated by a modified recirculating Langerdorff perfusion that yields a large proportion of cells which remain rod shaped and are quiescent in medium containing physiologic calcium levels (Piper et al., 1982). The anatomic distribution of cells within the walls of large and medium-sized mammalian vessels facilitates the isolation of relatively pure suspensions of fibroblastic, endothelial, or smooth muscle cells. In contrast to cardiac preparations, vascular cells from embryonic, neonatal, and adult vessels can be efficiently isolated in calcium- and magnesium-containing solutions.

Myocardial cell suspensions represent a heterogeneous population of muscle and non-muscle cells. Neonatal myocytes are remarkably resistant to injury and exhibit variable degrees of beating shortly after isolation. In contrast, spontaneous beating of adult cardiac myocytes is thought to be due to uncontrolled leakage of calcium through a permeable plasma membrane. Adult cardiac myocytes are mechanically at rest when properly isolated suggesting that functional differences in regulation exist between adult and neonatal cells. Isolated cells can be microinjected with fluorescent dyes for the assessment of multiple cellular functions following exposure to toxic chemicals. The viability of cells in suspension decreases rapidly as a function of time. Investigators rarely use these cell suspensions for more than four hours.

Changes in cell function or contractility can be assessed using these models. Because heart failure, in some instances, is characterized by contractile dysfunction of the myocardium and elevated sympathetic activity, cell function or contractility is of concern (Satoh et al., 2000). It has been demonstrated that adult rat ventricular myocytes in culture show signs of decreased contractility when exposed to adrenergic stimulation by norepinephrine + propanolol for 48 hours. This result seemed to be due to decreased Ca (2+)-ATPase. Consequently, sympathomimetic agents or other chemicals that decrease Ca (2+)-ATPase would have similar activity.

A number of anthracycline antineoplastic agents are known to cause cardiac cytotoxicity that can be severe and often irreversible. Doxorubicin and 4′-epirubicin significantly depress myocyte contractility in isolated neonatal and adult rat ventricular myocytes (Chan et al., 1996) but the etiology of the toxicity has not been determined definitively (Sawyer et al., 1999). The effect can be assessed by visualizing the beating of the myocytes (Jahangiri et al., 2000) or by measuring calcium flux using fluorescent dyes (Trollinger et al., 2000). Cultured fetal chick cardiac myocytes have also been used to study the toxicity of hydrogen peroxide and certain agents which can protect against such toxicity (Horwitz et al., 1996).

4.7.6 *Models Using Cell Lines*

Cardiac cell lines are generally preferred for the evaluation of chemical toxicity following prolonged exposures or following multiple challenges *in vitro.* Primary cultures can be established with relative ease from cell

suspensions of cardiac and vascular tissue. However, they must be characterized at the morphologic, ultrastructural, biochemical, and functional levels before being used in cytotoxicity testing applications because they undergo variable degrees of dedifferentiation, including loss of defined features and cell-specific functions. Vascular endothelial and smooth muscle cultures can also be established using explant methods, but the explant method selects cells with a growth advantage. Neonatal and embryonic cells of cardiac origin proliferate readily under appropriate *in vitro* conditions. Although adult cardiac myocytes do not divide in culture, the ability of cardiac myocytes to divide is only repressed and not completely lost (Barnes, 1988). A human fetal cardiac myocyte cell line was developed by transfection with the SV40 large T antigen to stimulate myocardial cell division, and many of the morphologic and functional features of human fetal cardiac myocytes were preserved (Wang et al., 1991).

4.7.7 *Endpoints That Can Be Assessed In Vitro*

Flow cytometry and computerized evaluation of cell images have added to toxicity evaluations of cardiac myocytes. Toxicity can also be evaluated based on the arrhythmogenic potential of chemicals (Aszalos et al., 1984). Ionic homeostasis can be used as an index of disturbances in the structural and functional integrity of the plasma membrane. Use of co-cultures of myocytes and endothelial cells or smooth muscle cells in the progression of the toxic response emphasizes the importance of cell-cell interactions (Saunders and D'Amore, 1992).

4.7.8 *Future Research Needs*

Vasculitis may need to be assessed by *in vitro* methods. It can be present in numerous forms such as lymphocytic vasculitis and leukocytoclastic vasculitis, the latter usually affecting the skin (Gupta et al., 2000). The most common type of vasculitis is Giant cell arteritis (Gonzalez-Gay et al, 2000), which generally involves large and medium-sized blood vessels. Further work will be needed to identify *in vitro* systems to assess this endpoint.

Certain drugs have the potential to alter the QT interval in the heart, producing ventricular arrhythmias and it will be necessary to develop systems to detect this effect. Halofantrine, an antimalarial drug, has been reported to produce such effects, and some drugs have been implicated in the sudden death of patients from ventricular arrhythmias (Champeroux et al., 2000). In a review by Champeroux (2000), different methodologies have been investigated as possible ways of examining this potential -- *in vitro* as well as *in vivo*. These include isolated cardiac tissues, Purkinje fibers, or papillary muscles. Wesche (2000) also used an isolated perfused heart model and isolated ventricular myocytes to determine potential cardiotoxicity associated with antimalarial drugs (Wesche et al., 2000).

A final important effect of acute exposure to xenobiotics is aseptic shock, which is associated with a fall in blood pressure. This is a systemic effect and no method of measuring or modeling this effect *in vitro* could be identified at this time. Further work to elucidate the exact causes of this effect may allow modeling of the change *in vitro*.

To the Breakout Group's knowledge, none of the cardiovascular toxicity models have been validated. After reviewing the literature, the likely candidate *in vitro* systems for an acute cardiotoxicity-testing scheme after chemical exposure could include the following:

- Short-term single-cell suspensions of adult rat myocytes to measure products of oxidation;
- Primary cultures of neonatal myocytes to measure changes in beating rates and plasma membrane potentials;
- Co-culture of smooth muscle cells or endothelial cells with macrophages, for example, to examine rate of wound healing (DNA synthesis);
- An immortalized cell line (e.g., the human fetal cardiac myocyte line) to measure classical cytotoxic endpoints.

It also may be important to include the perfused heart preparation, in spite of its limitations, for a comparison with the other *in vitro* models, because this system is the most representative of the *in vivo* situation.

4.8 *In Vitro* Methods to Study Hematopoietic Toxicity

Hematopoietic toxicity issues were recently reviewed by Gribaldo. [Progress in the Reduction, Refinement and Replacement of Animal Experiments, ed. M. Balls, A-M. van Zeller & M.E. Halder, pp. 671-677. Elsevier, Amsterdam, The Netherlands, 2000.] Xenobiotics can affect both the production and function of the various circulating cell populations, as well as the circulatory system that supports and helps maintain these cells. Acute effects on blood itself can also include the binding of materials to hemoglobin resulting in a loss of oxygen carrying capacity and cell lysis. Both of these latter endpoints should be easily modeled by *in vitro* systems if exposure conditions can be modeled.

During preclinical drug development it is often important to determine the following:

- Whether a new agent will be clinically toxic to the bone marrow cells;
- Whether the toxicity will be specific to one cell lineage (lymphocytes, neutrophils, megakaryocytes or erythrocytes);
- At what dose or plasma level the drug will be toxic;
- Which model best predicts the clinical situation, and
- When the onset and nadir of cytopenia and recovery will be likely to occur.

Validated *in vitro* tests using human cell systems are particularly important in this area as the prediction of human effects from animal systems are unreliable and necessitate the use of larger safety factors in human studies. *In vitro* colony-forming assays to study the growth and differentiation of various hematopoietic cell populations have been developed and perfected over the last twenty years, but none have yet been validated for use in regulatory toxicology testing. A validation study of the use of colony-forming assays to test for the possible development of neutropenia is being supported by ECVAM. Methods to assess effects on thrombocytopoiesis and erythropoiesis are also available and can be considered for validation.

Associated projects have been also been carried out, such as the optimization of a protocol for detecting apoptosis using FACS analysis with fluorescent antibodies against Annexin V (Vermes et al., 1995). Using this assay, the induction of apoptosis in established stromal cells (SR-4897) (Pessina et al., 1997) and in murine and human leukemia cells (WEHI-3B; HL-60), following exposure to anti-neoplastic agents, has been investigated in relation to the cell cycle. The relationship between these observations and chromosome damage during mitosis is under evaluation. The drug sensitivities of myeloid progenitors from fresh murine bone marrow and from long-term cultures have been investigated by many authors including (Gribaldo et al., 1998a) as well as the role of the microenvironment in the modulation of anti-cancer drug activity (Pessina et al., 1999; Gribaldo et al., 1999).

In the session on hematotoxicity at the *3rd World Congress on Alternatives and Animal Use in the Life Sciences*, results were described for possible new endpoints (Balls et al., 2000). For example, the toxic effects of drugs on the proliferation of erythroblastic progenitors were evaluated using human and murine progenitors from long-term bone marrow cultures. Two kinds of tests were employed: (a) continuous exposure of human cord blood cells (CBC) and murine bone marrow cells (BMC) during the assay, and (b) pretreatment of long-term murine bone marrow cultures (for 24 hours and 96 hours), with subsequent testing of the clonogenic capacity of progenitor cells collected in the absence of the drug. The classes of drugs of interest in the study were: antivirals (3'-azido-3'-deoxythymidine), antidiabetics (chlorpropamide), and heme-analogous compounds (protophorphirin IX/zinc [II]). The results indicate that all these drugs interfere with the normal hematopoietic process, causing a selective toxicity to the erythroid progenitors via

different mechanisms, and that human and murine progenitors have similar drug sensitivities. Moreover, the drugs exerted different toxicities based on the time of exposure.

Another aspect of hematotoxicology is in relation to the use of *in vitro* colony assays to support the risk assessment of industrial and food chemicals and pesticides. Some of these chemicals and formulations may interfere with the proliferative activity of the hematopoietic tissue and cause myelosuppression (Gribaldo et al., 1998b). One of the major difficulties in food toxicology is to establish the relationship between the consumption of a food contaminated by a toxin and the occurrence of a particular pathology. Clonogenic assays are a useful tool for establishing this relationship and for elucidating the mechanisms involved.

Three different clonogenic assays, with BFU-E (Burst-forming unit – erythrocytes), CFU-GM (Colony-forming unit granulocyte/macrophage), and CFU-MK (Colony-forming unit – megakaryocytes) cultures, have been used in toxicological investigations to detect or to confirm food-related hematotoxicity (Parent-Massin, 2000). By using these clonogenic assays, it has been possible to determine:

- The origin of neutropenia and hemorrhage induced by the consumption of trichothecene mycotoxin;
- The safety of a new process for manufacturing food additives;
- The mechanism of lead-induced hematotoxicity;
- The myelotoxicity of phycotoxins present in shellfish; and
- The risk to consumers and agricultural workers of hematological problems caused by pesticides (Parent-Massin and Thouvenot, 1995, 1993).

ECVAM is providing financial and organizational support to a new project on the development and prevalidation of *in vitro* assays for the prediction of thrombocytopenia. The continuous maintenance of an adequate supply of circulating platelets is essential for sustaining life. Since neither platelets nor megakaryocytes are capable of regeneration, their production is dependent on a continuous generative process from self-replicating precursors. The CFU-MK is the progenitor cell thought to be immediately responsible for the production of megakaryocytes and is therefore being evaluated for its ability to predict thrombocytopenia.

Drug effects are by far the most common cause of platelet suppression in the bone marrow (Miescher, 1980). In many instances, thrombocytopenia is the first evidence of drug-induced toxicity, and continued administration of the drug produces total aplasia. Cytotoxic agents, such as 5-fluorouracil, vincristine, and cytosine arabinoside, cause perturbation of the bone marrow, with changes within the proliferating compartments, as well as effects on the maturing cell pool. In contrast, the thiazide diuretics, estrogens, and alcohol appear to have specific effects on platelet production. In addition, solvents, including benzene, insecticides (DDT, chlordane, lindane), spot removers, and model airplane glue, have all been associated with marrow-related thrombocytopenia (Amess, 1993).

Following bone marrow transplantation, the restoration of a normal platelet count occurs as a result of a compensatory adjustment in megakaryocytopoiesis (Vainchenker, 1995). For these reasons, appropriate *in vitro* endpoints for megakaryocytopoiesis that correlate well with platelet levels *in vivo* should be identified. A preliminary study carried out in ECVAM's laboratories to optimize an *in vitro* CFU-MK permitted a comparison of the suitability and drug-sensitivities of human BMC and CBC. The percentage of enrichment in $CD34^{+}/CD38^{-}$ cells from both populations was measured by using a negative selection system, and their clonogenicity was evaluated. Furthermore, the effects on megakaryocyte colony formation of busulphan, a cytotoxic drug, and the non-cytotoxic drugs, quinidine-sulphate, D-penicillamine, sodium valproate, and indomethacin were investigated by using both the whole cell populations and selected cells from the two sources. The data analyses confirmed the usefulness of the *in vitro* test as a

potential tool for screening drug toxicity to megakaryocyte progenitors. The *in vitro* test showed that human CBC can be used as a human target source, was more suitable for this purpose, and provided a means of avoiding ethical problems that exist in some countries connected with the collection of human BMC.

Up until now, primary cells have been more-reliable and more-relevant targets for clonogenic assays than the immortalized cell lines, but in the future, attempts should be made to establish standardized cell populations for *in vitro* tests, and in particular, for screening purposes. This may help to avoid the technical problems related to the absence of primary cell repositories, and to avoid the problem of inter-individual variability of the donors, in terms of drug sensitivity. A future topic will be the automated scoring of colonies in the clonogenic assays, which will provide the opportunity to refine the performance of the assays in terms of accuracy and repeatability, and to reduce personnel costs.

4.9 *In Vitro* Methods to Study Respiratory System Toxicity

The lungs fulfill the vital function of exchanging oxygen and carbon dioxide and a secondary function of protecting the organism from noxious or irritating inhaled stimuli. As such, the nasal and pulmonary airways represent a crucial organ-system that is likely to debilitate the organism if injured or irritated. The airways are particularly difficult to evaluate in *in vitro* because of their complexity. The following is a discussion of relevant airway cells and target-specific endpoints that should be considered in an *in vitro* battery for target-specific acute toxicity.

4.9.1 *Cell Types*

The tracheal-bronchial epithelial lining consists of stratified epithelium and diverse populations of other cell types including ciliated, secretory (mucous, Clara, serous), and non-secretory cells. The cells lining the airways may be represented by various human cell lines such as CCL-30 (nasal septum) (Poliquin et al., 1985) and BEAS-2B (bronchial-tracheal epithelia/transformed) (Noah et al., 1991; Reddel et al., 1988). More distally, alveolar Type II epithelia (A549) function in conjunction with capillary endothelial cells for O_2:CO_2 exchange in the lower alveolar regions. This cell line can be used to show induction of P450 enzymes such as 1A1, 1B1, and 3A5 (Hukkanen et al., 2000), and to assess mucin production (Rose et al., 2000). The H441 cell line has been used in studies to evaluate toxicant effects on surfactant production *in vitro*. Various scavenger cells (alveolar macrophages) are present to engulf microbiological or foreign debris and destroy it. Several human alveolar macrophage cell lines exist which display the oxidative burst in response to irritants and biological debris (Marom et al., 1984). Neutrophils and eosinophils function as cellular sentinels of inflammation.

4.9.2 *Endpoint Markers*

A variety of endpoint markers valid for pulmonary cytotoxicity and irritation are available. ELISA-based assays can be used to quantitate many of these markers (e.g., cytokine, LDH), thus reducing the technical investment. The most useful markers will relate to the basic mechanisms by which airway epithelia respond to toxic exposure. LDH, a cytoplasmic enzyme released from damaged or lysed cells, is useful as a general marker of cytotoxicity. Mucous glycoprotein stain is a marker for alteration of mucous cells. Other possible endpoints include:

- Ciliary beat frequency (epithelial viability and function);
- Attachment (viability);
- Electrical resistance (to measure the integrity of the epithelial layer);
- Evans blue (to measure endothelial leakage);
- IL-8, IL-6, and TNFa (cytokine endpoints of inflammation).

As *in vitro* systems are developed and evaluated, biochemical markers of damage can be assayed in the lavaged fluid and directly compared to changes in similar markers in *in vitro* systems. Like the kidney, utilization of these comparisons will facilitate the development of predictive *in vitro* systems.

In vitro systems are available that can be used to indicate chemical-induced cell damage/death. The cells of the airways from animals or humans are relatively accessible to brushing, biopsy, and lavage, and therefore lend themselves for harvesting and use as primary cells (Larivee et al., 1990; Werle et al., 1994). Lung slices have been investigated for use in toxicology (Parrish, et al., 1995). The most useful markers are those that relate to the basic mechanisms by which airway epithelia respond to toxic exposure. However, most assays and cell models determining effects on special functions still need significant basic research before they will be useful as screening systems. The use of *in vitro* systems in respiratory toxicology was a subject of an ECVAM Workshop 18 (Lambre, et al. 1996).

4.10 Conclusions on the Use of *In Vitro* Systems for Assessing Organ-Specific Effects of Acute Exposure

There are significant ongoing advances in both technology and our understanding of biology that will have major effects on our ability to predict whole-animal (or human) toxic effects from non-whole animal model systems. For instance, toxicogenomics and proteonomics provide rapid identification of early changes in cells *in vitro* or from individual animals and humans. However, these systems are very early in development and significant work will be needed to understand how the changes seen relate to whole animal toxicity, and particularly which changes are the direct result of exposure and which are due to secondary effects as the cells and tissues react to the primary injury. Because these systems appear to be very sensitive, it will also be important to determine how the assays can be used in the prediction of dose-response information for toxicology.

In recognition of the possible importance of advances in toxicogenomics to toxicology, the Breakout Group recommends that some effort be put toward preserving samples from animal studies for future evaluation so as to avoid having to repeat these studies at a future time.

It is very important that the proper quality control procedures be built into any *in vitro* test system developed for use in screening such as:

- Stability of the test material;
- Reactions of the test material with plastic in culture dishes and laboratory ware;
- Measurement(s) of test material concentration in the test vehicle;
- Non-specific binding to proteins in the culture medium;
- Reactive compounds;
- Ensuring that the cells reliably express the necessary metabolic systems.

Each individual test system will need to have a complete, standardized protocol developed, evaluated, and validated. All test schemes that are developed will then build on these validated tests. The prediction model for the entire scheme may also need to be evaluated and validated.

4.10.1 *Proposed Scheme for Assessing Acute Toxicity Using Non-Whole Animal Methods*

For the assessment of acute systemic toxicity for the purposes of setting hazard and risk levels for chemicals and products, data on specific organ toxicity are usually not needed. The need is for a system to appropriately classify the hazard of materials that may cause death after acute exposure irrespective of the specific organ damage. For such a system, the routine use of *in vitro* models to evaluate all possible organ effects would be impractical from both a time and money standpoint and evaluation of the effects of xenobiotics on specific organ function is not included in the current assays for acute toxicity. Current acute toxicity assay systems utilize young adult animals, often of only one sex, and only recognize observable effects within 14 days. Currently standard assays do not evaluate effects in different sub-populations or the long-term effects of single acute exposures.

Acute toxicity assays are primarily used to predict the toxicity of materials to humans. For this reason, where species differences are known, the Breakout Group recommends that screening

systems be developed that will predict effects in humans.

Breakout Group 3 discussed what additional assay systems would be required, in addition to the basic cytotoxicity assay discussed by Breakout Group 1, in order to replace the current acute oral toxicity assays for regulatory purposes. Breakout Group 3 developed a stepwise approach to address those effects identified in the discussions of the specific organ systems that were highly relevant to the prediction of acute toxicity and would not be elucidated by a simple basal cytotoxicity test. This scheme is shown in Figure 4.1. The scheme includes a process for determining when additional specific effects need be evaluated, and gives some guidance on how to do so. The scheme includes steps proposed earlier by a expert workshop hosted by ECVAM and by Bjorn Ekwall in his series of papers.

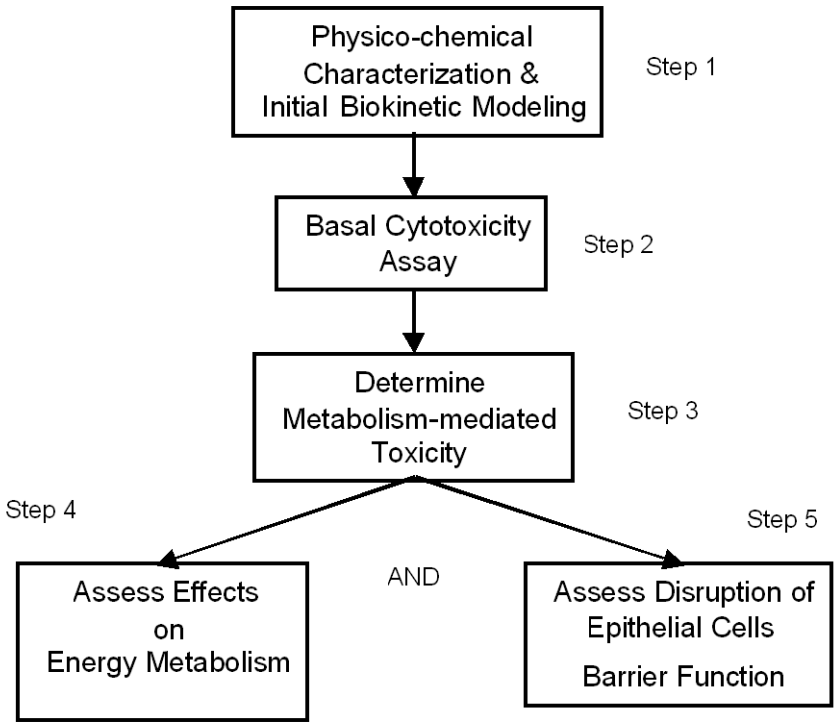


Figure 4.1 Proposed scheme for assessing acute toxicity using non-animal methods

(1) Step 1

- Perform physico-chemical characterization and initial biokinetic modeling (BG2 output). This information will be used for comparison with chemicals with similar structures or properties that have existing toxicity data. The information may also be useful in predicting organ distribution. It may be possible to accurately predict the toxicity effects of some chemicals from this step alone.

(2) Step 2

- Conduct a basal cytotoxicity assay (BG1 output).

(3) Step 3

Determine the potential that metabolism will mediate the effect seen in Step 2:

- Use HEPG2 cells transfected with major metabolizing enzymes – at this time at least four different cell lines, each containing one of the four major metabolic enzymes will be needed. A secondary, and perhaps more relevant, possibility would be to use metabolically competent, primary human hepatocytes, but cell lines would allow a more standardized approach for regulatory purposes.

- Both cytotoxicity and, ideally, some measure of metabolism of the test substance, must be determined, either by detecting a decrease in the parent compound or by some method that directly detects metabolites.

  A. If the material is more cytotoxic in the hepatocyte test system compared to that measured in Step 2, then assume the compound is metabolized to a toxic substance. In this case, the measure of cytotoxicity would use the value obtained from the metabolically active system instead of the value obtained in Step 2.

  B. If the material is less cytotoxic than seen in Step 2, then it is assumed there is detoxification, and in those exposure scenarios where it can be shown the materials will pass through the liver before the rest of the body is exposed (first pass effect) it may be possible to reduce the prediction of toxicity accordingly.

  C. If the cytotoxicity is similar to the basic cytotoxicity measured in Step 2, then the possibility of metabolite formation still must be assessed to assure the metabolite will not have an effect on some other cells that do not have the metabolic capabilities of hepatocytes.

     1. If there is no evidence of metabolism then the value used in Step 2 can be used.

     2. If there is evidence of metabolism, Step 2 must be repeated after exposure to the metabolite(s) either by directly identifying the metabolites and using them in the system, or by some other undetermined systems such as co-cultures or conditioned media; exact protocols will need to be determined. The system that is developed must be able quantitatively asses the effects of the initial toxicant. For instance, according to Breakout Group 2, co-cultures will not enable the biokinetic modelers to predict systemic toxicity in a quantitative manner.

(4) Step 4 (note: Steps 4 and 5 can be done in either order)

- Assess the test substance effect on energy metabolism by using a neuronal cell line that expresses good aerobic energy metabolism function. This system will help determine if the nervous or cardiovascular systems, both of which require high-energy metabolism, are likely target organs.

- The endpoints would be measurement of energy metabolism using a variety of specific probes of energy change, or oxygen consumption, or possibly mitochondrial function. The exact endpoint needs to be determined.

- If there is evidence of metabolism in Step 3, these tests must be done with both the parent compound and the metabolite(s).

(5) Step 5 (note: Steps 4 and 5 can be done in either order)

- Assess the ability of the compound to disrupt epithelial cell barrier function using a transepithelial resistance assay across a membrane, such as MDCK cells. The endpoint used could be dye leakage. This system will help in determining if organs dependent on epithelial barrier function for defense against toxic insult (e.g., brain, kidney) are likely target organs.

- If the compound causes disruption of barrier function at a value lower than the basal cytotoxicity, the endpoint used in determining the effect on the organism might need to be lowered to take this into consideration. [Note: Barrier disruption values will likely be lower than those that cause basal cytotoxicity.]

- If there is evidence of metabolism in Step 3, this test must be done with both the parent compound and the metabolite(s).

Next Steps

Before this system can be evaluated for implementation there is a need to:

- Identify the best cell culture systems to use based on accuracy, reproducibility, cost, and availability;

- Develop complete protocols for all the five steps and validate each assay;

- Develop prediction models for the prediction of relevant human toxic levels as required by regulatory agencies. Prediction of No Observed Adverse Effect Levels (NOAELs) would be addressed at this step;

- Evaluate the scheme with a number of test compounds covering all endpoints and then with enough compounds to develop a prediction model;

- Validate the entire scheme and prediction model.

The Breakout Group recommends that this work be done with the input and cooperation of the regulatory agencies and industries who have a need to use acute toxicity data in order to ensure the final result will meet everyone's needs.

4.11 References

Adegunloye, B.I., and O.A. Sofola. 1997. Differential Responses of Rat Aorta and Mesenteric Artery to Norepinephrine and Serotonin *In Vitro*. Pharmacology 55: 25-31.

Amess, J. 1993. Haematotoxicology. In: General and Applied Toxicology. (Ballantyne, B., T. Marrs, and P. Turner, eds). Volume 1. Basingstoke, UK: Macmillan Press. 839-867.

Andres, M.I., A. Forsby, and E. Walum . 1997. Polygodial-Induced Noradrenaline Release in Human Neuroblastoma SH-SY5Y Cells. Toxicol. *In Vitro* 11: 509-511.

Aszalos, A., J.A. Bradlaw, E.F. Reynaldo, G.C. Yang, and A.N. El-Hage. 1984. Studies on the Action of Nystatin on Cultured Rat Myocardial Cells and Cell Membranes. Biochem. Pharmacol. 33: 3779-3786.

Atterwill, C.K., A. Bruinink, J. Drejer, E. Duarte, P. McFarlene, E. Abdulla, C. Meredith, P. Nicotera, C. Regan, E. Rodriquez-Farre, M.G. Simpson, R. Smith, B. Veronesi, H. Vijverberg, E. Walum, and C. Williams. 1994. *In vitro* Neurotoxicity Testing. ATLA 22: 350-362.

Bach P.H., A.E.M. Vickers, R. Fisher, A. Baumann, E. Brittebo, D.J. Carlile, H.J. Koster, B.G. Lake, F. Salmon, T.W. Sawyer, G. Skibinski. 1996. The use of tissue slices for pharmacotoxicology studies - The report and recommendations of ECVAM workshop 20.
ATLA- 24: (6) 893-923.

Balls, M., and E. Walum. 1999. Toward the Acceptance of *In Vitro* Neurotoxicity Tests. In: Neurotoxicology *In Vitro*. (V.W. Pentreath, ed). Taylor & Francis, Philadelphia. pp. 269-283.

Balls, M., A.-M. van Zeller, and M.E. Halder, Eds. *Progress in the Reduction, Refinement, and Replacement of Animal Experimentation: Proceedings of the 3rd World Congress on Alternatives and Animal Use in the Life Sciences.* 2000. Elsevier Science, Amsterdam. pp.667-717.

Barnes, D.M. 1988. Joint Soviet-US. Attack on Heart Muscle Dogma. Science 242: 193-195.

Brown, R.A., and A.O. Savage. 1996. Effects of Acute Acetaldehyde, Chronic Ethanol and Pargyline Treatment on Agonist Responses of the Rat Aorta. Toxicol. Appl. Pharmacol. 136: 170-178.

Champeroux, P., E. Martel, C. Vannier, V. Blanc, J.Y. Liguennec, J. Fowler, and S. Richard. 2000. The Preclinical Assessment of the Risk for QT Interval Prolongation. Therapie Jan-Feb. 55 (1): 101-109.

Chan, E.M, M.J. Thomas, B. Bandy, and G.F. Tibbits. 1996. Effects of Doxorubicin, 4-Epirubicin, and Antioxidant Enzymes on the Contractility of Isolated Cardiomuyocytes. Can. J. Physiol. Pharmacol. Aug: 74 (8): 904-910.

Combes, R.D., and L.K. Earl. 1999. *In Vitro* Toxicology-Priorities for the Year 2000. Hum. and Exper. Toxicol.

Crass, M.F., S.M. Hulsey, and T.J. Bulkey. 1988. Use of a New Pulsatile Perfused Rat Aorta Preparation to Study the Characteristics of the Vasodilator Effect of Parathyroid Hormone. J. Pharmacol. Exp. Ther. 245: 723-734.

Cross, D.M., and M. K. Bayliss. 2000. A commentary on the Use of Hepatocytes in Drug Metabolism Studies during Drug Discovery and Development. Drug Metab. Rev. 32(2): 219-240.

Curren, R.D., J.A. Southee, H. Spielmann, M. Liebsch, J.H. Fentem, and M. Balls. 1995. The Role of the Prevalidation in the Development, Validation and Acceptance of Alternative Methods. ATLA 23: 211-217.

Ehrich, M., L. Correll, and B. Veronesi. 1997. Acetylcholinesterase and Neuropathy Target Esterase Inhibitors in Neuroblastoma Cells to Distinguish Organophosphorus Compounds Causing Acute and Delayed Neurotoxicity. Fund. Appl. Toxicol. 38: 55-63.

Ekwall B, B. Ekwall, M. Sjostrom. 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

Ekwall, B., C. Clemedson, B. 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.

Elmore, E., T-T.Luc, V.E. Steele, G.J. Kelloff, and J.L. Redpath. 2000. The Human Epithelial Cell Cytotoxicity Assay for Determining Tissue Specific Toxicity. Meth. Cell Sci. 22: 17-24.

Elmore, E., T-T.Luc, V.E. Steele, G.J. Kelloff, and J.L. Redpath. Comparative Tissue-Specific Toxicities of Twenty Cancer Preventive Agents Using Cultured Cells From Eight Different Normal Human Epithelia. (in press: *In Vitro* & Molecular Toxicology).

Foex, P. 1988. Experimental Models of Myocardial Ischemia. Br. J. Anaesth. 61: 44-55.

Forsby, A., F. Pilli, V. Bianchi, and E. Walum. 1995. Determinations of Critical Cellular Neurotoxic Concentrations in Human Neuroblastoma (SH-SY5Y) Cell Cultures. ATLA 23: 800-811.

Fuller, J., and D.D. Woodman. 1993. Hematology in Toxicology Studies. In: General and Applied Toxicology. (Ballantyne, B., T. Marrs, P. Turner, eds). Volume 1. Basingstoke, UK: Macmillan Press Ltd. 267-301.

Gandolfi, A.L., K. Brendel, R.L. Fisher, and J.P. Michaud. 1995. Use of Tissue Slices in Chemical Mixture Toxicology and Interspecies Investigations. Toxicology 105: 285-290.

Garberg, P. 1998. *In Vitro* Models of the Blood-Brain Barrier. ATLA 26: 821-847.

Gonzalez-Gay, M. A., C. Garcia-Porrua, J. Llorca, A. H. Hajeer, F. Branas, A. Dababneh, E. Gonzalez-Rodriguez-Gil, P. Rodriguiz-Ledo, and W. E. Ollier. 2000. Visula Manifestations of Giant Cell Arteritis. Trends and Clinical Spectrum in 1. Medicine (Baltimore) 79(5): 283-292.

Gribaldo, L., P. Catalani, and E. Marafante. 1999. Metabolism of Doxorubicin in Long-Term Bone Marrow Cultures and SR-4987 Stromal Established Cell Line. Drug Metabolism and Drug Interactions 15: 279-291.

Gribaldo, L., S. Casati, F.A. Castoldi, and A. Pessina. 1999. Comparison of *In Vitro* Drug-Sensitivity of Human Granulocyte-Macrophage Progenitors from Two Different Origins: Umbilical cord blood and bone marrow. Exp Hematology 27: 1593-1598.

Gribaldo, L., M. Piccirillo, S. Casati, A. Collotta, E. Mineo, and A. Pessina. 1998a. Drug Sensitivity of Granulocyte-Macrophage Precursors (GM-CFU) from Fresh Murine Bone Marrow and from Long-Term Bone Marrow Cultures. Toxicol. *In Vitro* 12: 39-45.

Gribaldo, L., S. Casati, L.E. Figliuzzi, and E. Marafante. 1998b. *In Vitro* Myelotoxicity of Environmental Contaminants. Env Toxicol. Pharmacol. 6: 135-141.

Gribaldo, L., J. Bueren, A. Deldar, P. Hokland, C. Meredith, D. Moneta, P. Mosesso, R. Parchment, D. Parent-Massin, A. Pessina, J. San Roman, and G. Schoeters. 1996. The Use of *In Vitro* Systems for Evaluating Haematotoxicity. ATLA 24: 211-231.

Guillouzo, A. 1997. Biotransformation of Drugs by Hepatocytes. In: *In Vitro* Methods in Pharmaceutical Research, Academic Press Ltd.

Guillouzo, A. 1998. Liver cell models in *in vitro* toxicology. Environ. Health Prespect. 106 Suppl. 2: 511-32.

Gupta, M. N., R. E. Sturrock and G. Gupta. 2000. Cutaneous Leucocytoclastic Vasculitis Caused by Cyclosporin A (Sandimmun). Ann. Rheum. Dis. 59(4): 319.

Harrison, D.G. 1997. Endothelial Function and Oxidant Stress (review). Clin. Cardiol. 20: 11-17.

Hartely, R.S., M. Margulis, P.S. Fishman, V.M. Lee, and C.M. Tang. 1999. Functional Synapses are Formed Between Human NTera2 (NT2N, hNT) Neurons Grown on Astrocytes. J. Comp. Neurol. 407: 1-10.

Hawksworth G.M., P.H. Bach, J.F. Nagelkerke, W. Dekant, J.E. Diezi, E. Harpur, E.A. Lock, C. Macdonald, J.P. Morin, W.Pfaller, Fajjle Rutten, M.P. Ryan, H.J. Toutain, A. Trevisan. 1995. Nephrotoxicity Testing In-Vitro - The Report And Recommendations Of Ecvam Workshop-10. ATLA 23: (5) 713-727.

Hester, R.K., and K. Ramos. 1991. Vessel Cylinders. In: Methods in Toxicology. (Tyson, C., and J. Frazier, eds). San Diego, CA, Academic Press.

Horwitz, L.D., J.S. Wallner, D.E. Decker, and S.E. Buxser. 1996. Efficacy of Lipid Soluble, Membrane-Protective Agents Against Hydrogen Peroxide Cytotoxicity in Cardiac Myocytes. Free Radic. Biol. Med. 21(6): 743-753.

Hukkanen, J., A. Lassila, K. Paivarinta, S. Valanne, S. Sarpo, J. Hakkola, O. Pelkonen, and H. Raunio. 2000. Induction and Regulation of Xenobiotic-Metabolizing Cytochrome P450s in the Human A549 Lung Adenocarcinoma Cell Line. Am. J. Respir. Cell Mol. Biol. 22: 360-366.

Ingwall, J.S., M. Deluca, H.D. Sybers, and K. Wildenthal. 1975. Fetal Mouse Hearts: A model for studying ischemia. Proc. Natl. Acad. Sci. USA 72: 2809-2813.

Izumi, I., K. Mineura, K. Watanabe, and M. Kowada. 1994. Establishment of the Two Glioma Cell Lines YH and AM. Hum. Cell. 7: 101-105.

Jahangiri, A., W. R. Leifert, G. S. Patten, and E. J. McMurchie. 2000. Termination of Asynchronous Contractile Activity in rat Atrial Myocytes by n-acids. Mol. Cell. Biochem. 206(1):33-41.

Janigro, D., S. Leaman , and K.A. Stanness. 1999. *In Vitro* Modeling of the BBB. Pharm. Sci. Technol. Today 2: 7-12.

Koo, E.W.Y., and A.I. Gottlieb. 1992. The Use of Organ Cultures to Study Vessel Wall Pathobiology. Scan. Micro. 6: 827-835.

Lambre, C. R., M. Aufderheide, R. E. Bolton, B. Fubini, H. P. Haagsman, P. M. Hext, M. Jorissen, Y. Landry, J-P. Morin, B. Nemery, P. Nettesheim, J. Pauluhn, R. J. Richards, A. Vickers, and R. Wu. 1996. *In Vitro* Tests for Respiratory Toxicity. The Report and Recommendations of ECVAM Workshop 18. ATLA 24:671-681.

Larivee, P., A. Cantin, A. Dufresne, and R. Begin. 1990. Enzyme Activities of Lung Lavage in Silicosis. Lung 168: 151-158.

Loegering, Djm, C.A. Richard, C.B. Davison, and G.A. Wirth. 1995. Diethyldithiocarbamate Ameliorates the Effect of Lipo-Polysaccharide on both Increased Nitrite Production by Vascular Smooth Muscle Cells and Decreased Contractile Response of Aortic Rings. Life Sci. 57: 169-176.

Malhotra, S.K., L.T. Loung, R. Bhatnagar, and T.K. Shnitka. 1997. Up-Regulation of Reactive Astrogliosis in the Rat Glioma 9L Cell Line by Combined Mechanical and Chemical Injuries. Cytobios. 89: 115-134.

Marom, Z., J.H. Shelhamer, and M. Kaliner. 1984. Human Pulmonary Macrophage-Derived Mucus Secretagogue. J. Exp. Med. 159: 844-860.

Matsumura, K., and K. Kawamoto. 1994. The Establishment and Charcteristics Lioma Cell Line (KMU100) with a Long Term Culture. Hum. Cell 7: 62-67.

Miescher, P.A. 1980. Drug-Induced Thrombocytopenia. Clin Haematol. 9: 505-519.

Munir, M., L. Lu, J. Luo, B.B. Wolfe, and P. McGonigle. 1996. Pharmacological and Immunological Characterization of N-methyl-D-aspartate Receptors in Human NT2-N Neurons. J. Pharmacol. Exp. Ther. 6: 819-829.

Nakamura, H., Y. Kawasaki, N. Arakawa, M. Saeki, S. Maeda, Y. Koyama, A. Baba, and T. Matsuda. 2000. The Na+-Ca2+ Exchange Inhibitor KB-R7943 Inhibits High K+-induced Increases in Intracellular Ca2+ Concentration and [3H]Noradrenaline Release in the Human Neuroblastoma SH-SY5Y. Neurochem. Res. 25: 385-387.

Naltner, A., M. Ghaffari, J. Whitsett, and C. Yan. 2000. Retinoic Acid Stimulation of the Human Surfactant Protein B Promoter Is Thyroid Transcription Factor 1 Site-Dependent. J. Biol. Chem. 275: 56-62.

Napoli, C. 1997. Low Density Lipoprotein Oxidation and Atherogenesis: From experimental models to clinical studies. Giornale Italiano di Cardiologia 27: 1302-1314.

Neelands, T.R., L.J. Greenfield Jr., J. Zhang, R.S. Turner, and R.L. Macdonald. 1998. GABAA Receptor Pharmacology and Subtype mRNA Expression in Human Neuronal NT2-N cells. J. Neurosci. 18: 4993-5007.

Noah, T.L., A.M. Paradiso, M.C. Madden, K.P. McKinnon, and R.B. Devlin. 1991. The Response of a Human Bronchial Epithelial Cell Line to Histamine: Intracellular calcium changes and extracellular release of inflammatory mediators. Am. J. Respir. Cell Mol.Biol. 5: 484-492.

Ozdem, S.S., and C. Ogutman. 1997. Responsiveness of Aortic Rings of Cadmium-Hypertensive Rats to Endothelin-1. Pharmacology 54: 328-332.

Parchment, R.E. 1998. Alternative Testing Systems for Evaluating Noncarcinogenic, Hematologic Toxicity. Env Hlth Persp. 106: 541-557.

Parchment, R.E., and M.J. Murphy. 1997. Human Hematopoietic Stem Cells: Laboartory assessment and response to toxic injury. In: Comprehensive Toxicology. (Sipes, G.I., C.A. McQueen, and A.J. Gandolfi, eds). Volume 4. New York: Pergamon. 335-362.

Parchment, R.E., D.A. Volpe, P.M. LoRusso, C.L. Erickson-Miller, M.J. Murphy, and C.K. Grieshaber. 1994. *In Vitro-In Vivo* Correlation of Myelotoxicity of 9-Methoxypyrazoloacridine to Myeloid and Erythroid Hematopoietic Progenitors from Human, Murine and Canine Marrow. J. Natl. Cancer Inst. 86: 273.

Parent-Massin, D. 2000. Relevance of Clonogenio Assays in Food Haematotoxicology. In: Progress in the Reduction, Refinement and Replacement of Animal Experimentation. (Balls, M., A-M van Zeller, M.E. Halder, eds). Amsterdam: Elsevier. 709-714.

Parent-Massin D, and D. Thouvenot. 1995. *In Vitro* Toxicity of Tricothecenes on Rat Haematopoietic Progenitors. Food Additives and Contaminants 12: 41-49.

Parent-Massin D, and D. Thouvenot. 1993. *In Vitro* Study of Pesticide Hematotoxicity in Human and Rat Progenitors. Jour. of Pharm. and Toxicol. Meth. 30: 203-207.

Parrish, A.R., A. J. Gandolfi and K. Brendel. 1995. Precision-cut Tissue Slices; Applications in Pharmacology and Toxicology. Life Sci. 57(21): 1887-901.

Pessina, A., B. Albella, J. Bueren, P. Bramtom, S. Casati, G. Corrao, L. Gribaldo, R. Parchment, D. Parent-Massin, M. Piccirillo, B. Rio, S. Sacchi, G. Schoeters, and R. van den Heuvel. 2000. Method Development for a Prevalidation Study of the *In Vitro* GM-CFU Assay for Predicting Myelotoxicity. In: Progress in the Reduction, Refinement and Replacement of Animal Experimentation. (Balls, M., A-M van Zeller, M.E. Halder, eds). Amsterdam: Elsevier. 679-692.

Pessina, A., M. Piccirillo, E. Mineo, P. Catalani, L. Gribaldo, E. Marafante, M.G. Neri, and A. Raimondi. 1999. Role of SR-4987 Stromal Cells in the Modulation of Doxorubicin Toxicity to *In Vitro* Granulocyte-Macrophage Progenitors (GM-CFU). Life Sci. 65: 513-523.

Pessina, A., M.G. Neri, E. Mineo, M. Piccirillo, L. Gribaldo, P. Brambilla, G. Zaleskis, and P. Ujhazy. 1997. Expression of B Cell Markers on SR-4987 Cells Derived from Murine Bone Marrow Stroma. Exp. Haematol. 25: 436-541.

Piacibello, W., D. Ferrero, F. Sanavio, R. Badoni, A. Stacchini, A. Sverino, and M. Aglietta. 1991. Responsiveness of Hghly Enriched CFU-GM Subpopulations from Bone Marrow, Peripheral Blood, and Cord Blood to Hemopoietic Growth Inhibitors. Exp. Hematol. 19: 1084.

Piper, H.M., I. Probst, P. Schwartz, F.J. Hutter, and P.G. Spieckermann. 1982. Culturing of Calcium Stable Adult Cardiac Myocytes. J. Mol. Cell. Cardiol. 14: 397-412.

Poliquin, J.F., and J. Crepeau. 1985. Immune Defence Mechanisms of the Nasal Mucosa. J. Otolaryngol. 14: 80-84.

Reddel, R.R., Y. Ke, M.E. Kaighn, L. Malan-Shibley, J.F. Lechner, J.S. Rhim, and C.C. Harris. 1988. Human Bronchial Epithelial Cells Neoplastically Transformed by v-Ki- ras: Altered response to inducers of terminal squamous differentiation. Oncogene Res. 3: 401-408.

Rhee, H.M., B.J. Song, S. Cushman, and S.E. Shoaf. 1995. Vascular Reactivity in Alcoholic Rat Aortas: *In vitro* reactions between catecholamines and alcohol. Neurotoxicology 16: 179-185.

Rose, M.C., F.M. Piazza, Y.A. Chen, M.Z. Alimam, M.V. Bautista, N. Letwin, and B. Rajput. 2000. Model Systems for Investigating Mucin Gene Expression in Airway Diseases. J. Aerosol Med. 13: 245-262.

Satoh, N., T.M Suter, R. Liao, and W.S. Colucci. 2000. Chronic Alpha-Adregenergic Receptor Stimulation Modulates the Contractile Phenotype of Cardiac Myocytes *In Vitro*. Circulation Oct. 31, (18): 2249-2254.

Saunders, K.B., and P.A. D'Amore. 1992. An *In Vitro* Model for Cell-Cell Interactions. *In Vitro* Cell. Dev. Biol. 28: 521-528.

Sawyer, D.B., R. Fukazawa, M.A. Arstall, and R.A. Kelly. 1999. Daunorubicin-Induced Apoptosis in Rat Cardiac Myocytes in Inhibited by Dexrazoxane. Circ. Res. Feb. 19, 84(3): 257-265.

Smith, E.L., and A.H. Hainsworth. 1998. Acute Effects of Interleukin-1 Beta on Noradrenaline Release from the Human Neuroblastoma Cell Line SH-SY5Y. Neurosci. Lett. 251: 89-92.

Speralakis, N., and R. Shigenoubu. 1974. Organ Cultured Chick Embryonic Heart Cells of Various Ages. Part I. Electrophysiology. J. Mol. Cell. Cardiol. 6: 449-471.

Spielmann, H., N.P. Bochkov, L. Costa, L. Gribaldo, A. Guillouzo, J.H. Heindel, M. Karol, R. Parchment, W. Pfaller, P. Prieto Peraita, and T. Zacharewski. 1998. Alternative Testing Methodologies for Organ Toxicity. Env Hlth Persp. 106 (suppl 2): 427-439.

Stanness, K., L. Westrum, P. Mascagni, E. Fornaciari, J. Nelson, and D. Janigro. 1997. Morphological and Functional Characterization of an *In Vitro* Blood-Brain Barrier Model. Brain Res. 771: 329-342.

Svendsen, C.N., M.A.Caldwell, J. Shen, M.G. ter Borg, A.E. Rosser, P. Tyers, S. Karmiol, and S.B. Dunnett. 1997. Long-Term Survival of Human Central Nervous System Progenitor Cells Transplanted into a Rat Model of Parkinson's Disease. Exp. Neurol. 148: 135-146.

Taylor, T.J., N.C. Smoth, M.J. Langford, and G.W. Parker Jr. 1995. Effect of Palytoxin on Endothelium-Dependent and -Independent Relaxation in Rat Aortic Rings. J. Appl. Toxicol. 15: 5-12.

Trollinger, D.R., W.E. Cascio, and J.J. Lemasters. 2000. Mitochondrial Calcium Transients in Adult Rabbit Cardiac Myocytes: Inhibition by ruthenium red and artifacts caused by lysosomal loading of Ca(2+)-indicating fluorophores. Biophys J Jul, 79:39-50

Ulrich, R.G., J. A. Bacon, C. T. Cramer, G. W. Peng, D. K. Petrella, R. P. Stryd and E. L. Sun. 1995. Toxicol. Lett. 82-83: 107-115.

Vainchenker, W. 1995. Megakaryocytopoiesis: Cellular aspects and regulation. Crit. Rev. Oncol. Hematol. 20: 165-192.

Vermes, I., C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger. 1995. A Novel Assay for Apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. Jour. Immunol. Meth. 184: 39-51.

Wade, J.A., P.F. Vaughan, and C. Peers. 1998. Hypoxia Enhances [3H]Noradrenaline Release Evoked by Nicotinic Receptor Activation from the Human Neuroblastoma SH-SY5Y. J. Neurochem. 71: 1482-1489.

Wang, S., G. Wright, W. Geng, and G.L. Wright. 1997. Retinol Influences Contractile Function and Exerts an Anti-Proliferative Effect on Vascular Smooth Muscle Cells through an Endothelium-Dependent Mechanism. Pflugers Archiv - Eur J. Physiol. 434: 669-677.

Wang, Y-C, N. Neckelmann, A. Mayne, A. Herskoqit, A. Srinivasan, K.W. Sell, and A. Ahmed Ansair. 1991. Establishment of a Human Fetal Cardiac Myocyte Cell Line. *In Vitro* Cell Dev. Biol. 27: 63-74.

Waring, J.F.and R.G. Ulrich. 2000. The impact of genomics-based technologies on drug safety evaluation. Annual Review of Pharmacology and Toxicology. 40: 335-352.

Werle, B., W. Ebert, W. Klein, and E. Spiess. 1994. Cathepsin B in Tumors, Normal Tissue and Isolated Cells from the Human Lung. Anticancer Res. 14: 1169-1176.

Wesche, D.L., B.G. Schuster, W.X. Wang, and R.L. Woosley. 2000. Mechanism of Cardiotoxicity of Halofantrine. Clin. Pharmacol. Ther. May, 67(5): 521-529.

Younkin, D.P., C-M.Tang, M. Hardy, U.R. Reddy, Q-Y. Shi, S.J. Pleasure, V. Lee, and D. Pleasure. 1993. Inducable Expression of Neuronal Glutamate Receptor Channels in the NT2 Human Cell Line. Proc. Natl. Acad. Sci. USA 90: 2174-2178

Yu, P.H., C.T. Lai, and D.M. Zuo. 1997. Formation of Formaldehyde from Adrenalin *In Vivo*: A potential risk factor for stress-related angiopathy. Neurochem. Res. 22: 615-20.

.

5.0 CHEMICAL DATA SETS FOR VALIDATION OF *IN VITRO* TOXICITY TESTS

5.1 Introduction

Breakout Group 4 discussed the selection of chemical data sets for validation of *in vitro* toxicity tests. The Breakout Group agreed that it would not develop specific lists of chemicals but would concentrate upon principles for the development of a database of chemicals that could be used in validation of individual tests or prediction models, and strategies for selection of the chemicals to be included in the database. Primary database development will most likely come from existing databases such as those available at the U.S. EPA, FDA, NCI, NTP, DOT, Galileo, Euclid, and others that are to be identified.

In addition to establishing criteria for primary database development, a set of criteria was developed for selecting chemicals for subset development. The chemicals in the subsets will be chosen from the primary database and will be used to validate individual tests or prediction models. The primary assumption in establishing criteria for subset development is that the purpose and proposed use of the test, the endpoint measured, the range of testable chemicals, and the prediction model must be clearly defined before chemical selection begins. Criteria that were considered important in selecting a set of reference chemicals were developed, as well as a set of fields considered relevant for the chemical reference database.

Lastly, the Breakout Group assembled a list of recommended actions that was divided into two parts: one that was database specific and one that was human toxicity specific.

5.2 Objectives

Before beginning a discussion of the primary database development, the Breakout Group defined some common points of reference and some points of agreement that would serve as the basis for discussions during the meeting. These are presented in the next sections.

5.2.1 *Points of Reference*

(1) The main function of the Breakout Group was to develop a set of general principles that would be useful for choosing test chemicals for validation.

(2) The Breakout Group would attempt to identify databases, and other sources that contain the information necessary to choose the test chemicals, and define their uses and limitations.

(3) The Breakout Group agreed that it would not identify specific chemicals or develop lists of chemicals at this time.

5.2.2 *Points of Agreement*

In addition to the three reference points, several items were set out by the Breakout Group to ensure that all members understood the exact aim of the discussion and their charge to the Breakout Group.

(1) It was agreed that the aim of the Breakout Group was to identify chemicals and supporting chemical information that can be used to validate replacement test(s) for acute toxicity tests.

(2) The chemicals used to validate a replacement test should cover the entire range of responses of the LD50 values. They should not be chosen to bracket just the range of classification used in the internationally agreed upon classification scheme(s).

(3) In addition to covering the entire range of responses, the chemicals chosen for use in a validation study should be uniformly distributed across that range, (i.e., there should not be a preponderance of either very toxic or non-toxic chemicals among those used).

(4) Identification of “chemical classes” is problematic. The basis for classification is the most significant issue. There was an unresolved discussion within the Breakout Group as to whether

classification should be done on the basis of chemical structure or mechanism of biological action. There was some discussion also about classifying according to use, such as "pesticide" or "food additive".

(5) The Breakout Group agreed that it is not necessary to be restricted to only one classification scheme. Chemicals could be classified by structure and by biological activity and/or use class. The classification approach would, by necessity, vary according to the type of test and its proposed uses.

(6) There are many public databases from which to draw information. These databases contain chemicals of concern to society. Investigators may not need, therefore, to use the proprietary databases such as the U.S. EPA OPP pesticides database or the FDA drug database to get the information and identify chemicals for use in tests for validation, but it would be helpful if information from those databases could be made available.

(7) There is a need for training sets of chemicals that can be used for method development, and validation sets of chemicals that can be used for confirming the predictive capacity of the tests.

(8) In selecting chemicals for use in validation studies, investigators need to consider the user community(ies) and assure that chemicals are chosen that meet their needs.

(9) The performance parameters of the *in vivo* tests must be clearly defined prior to chemical selection if the results of these tests are to serve as a baseline for judging success.

5.2.3 *Definition of Responsibility*

Breakout Group 4 defined its responsibility as follows:

- To define what chemical data sets are required for validation studies;
- To define the information to be included as part of the data set;
- To identify existing resources;
- To recommend approaches for using existing data sets;
- To recommend approaches for developing new data sets.

The Breakout Group explored the possible use of such databases as the HPV database, the U.S. EPA pesticides database, the NTP chemical database, the FDA database of drugs and food additive chemicals, and the use of QSAR to predict toxicity of chemicals.

5.3 Current Status: Discussions Regarding the Use of the NTP and HPV Databases, and the Use of QSAR

5.3.1 *The NTP Database*

The NTP chemicals were not tested for acute toxicity and therefore no LD50 data were developed. However, many were tested in 90-day studies, and some in 14-day studies, and these have associated target-organ toxicity data, as do the 2-year carcinogenicity studies. This information would be useful in validating *in vitro* tests for target-organ toxicity. The NTP database would be a useful component of any primary database of chemicals for validation.

Both the U.S. EPA pesticides database and the FDA drugs and food additive databases have associated LD50 data of good quality. However, there was some question about the ultimate accessibility of these data because of claims of confidentiality by the sponsors. Ease of access was a concern even where the data are not claimed to be confidential. Access through the Freedom of Information Act (FOIA) was discussed as a possibility, but this is a slow process and members of the Breakout Group expressed the desire that sources of unencumbered data should be used if they were available. Also, this approach may not provide the supporting information deemed necessary by the Breakout Group.

5.3.2 *The HPV Database*

There was a short presentation of the classification of the chemicals that are part of the HPV Program of the U.S. EPA OPPT. Using only

696 pure chemicals on the list and classifying them according to chemical structure, a list of 45 chemical classes with from 4 to 72 chemicals per class was developed. This classification is based solely upon chemical structure and each chemical is assigned to one class only. There is no indication of how many of these chemicals fall into more than one class. There is also no indication of which of these chemicals have LD50 data, the quality of these data where they exist, or the range of responses that is covered. Without this information, it is impossible to tell which of the HPV chemicals would be useful as validation chemicals. In addition, the chemicals on the HPV list are primarily industrial chemicals and their use as validation chemicals might not meet the needs of all user communities.

5.3.3 *QSAR Methods and Structure-Activity Methods for Toxicity*

QSAR methods can be applied to the problem of developing models to predict toxicity endpoints or toxic classes given sufficient quantity and quality of data.

The basis for the prediction of toxicity from chemical structure is that the properties of a chemical are implicit in its molecular structure. Biological activity can be expressed as a function of partition and reactivity. For a chemical to be able to express its toxicity, it must be transported from its site of administration to its site of action and then it must bind to or react with its receptor or target. This process may also involve metabolic transformation(s) of the chemical and its metabolites.

The application of QSAR principles to the prediction of the toxicity of new or untested chemicals has been achieved in a number of different ways and covers a wide range of complexity. The common feature of these approaches is that their starting point is a mechanistic hypothesis linking chemical structure and/or functionality with the toxicological endpoint of interest. A number of such "*in silico*" methodologies have also been applied with varying degrees of success to the evaluation of LD50 values and MTDs, and some are available commercially (e.g., DEREK, MCASE, and TOPKAT).

The prediction of toxicity from chemical structure and physical properties can make a valuable contribution to the reduction of animal usage in the screening out of potentially toxic chemicals at an early stage and in providing data for making positive classifications of toxicity. However, such methods should also be validated, using protocols similar to those described in these pages, so as to assess their potential effectiveness in assessing acute toxicity.

5.4 Identification of Needs

5.4.1 *Selection of Test Chemicals for Validation of In Vitro Tests*

In the context of using *in vitro* tests to replace or reduce animal usage, the performance of an *in vitro* test or an *in silico* test is assessed by its capability of correctly predicting the *in vivo* response. However, it is unreasonable to expect that the *in vitro* test will be able to predict the result of an *in vivo* test with any more accuracy than would a repeat *in vivo* test.

The assessment of any new test would be best accomplished by selecting a series of reference chemicals that cover the full range of responses, from negative, to weak, to intermediate, to strong. Selection of only strongly active chemicals will not provide information on the discriminating ability of a test, or its ability to detect the weakly active chemicals. The absence of chemicals known to be inactive will not allow a determination of the ability of the test to identify chemicals without activity, or of the false positive rate of the test.

5.4.2 *Evaluating the Quality of Data Used to Develop the Chemical Data Set*

A major challenge facing researchers developing either *in vitro* or *in silico* models is the sparse availability of high quality data derived from experiments with animals, or from human monitoring studies and clinical reports. Biological data which do not meet today's stringent requirements of acceptability,

particularly historical data generated prior to the advent of standardized test guidelines, but which are nevertheless of acceptable quality, can be used to validate newly developed test methods.

The Breakout Group discussed the establishment of a primary database from which sets of chemicals could be drawn for use as validation chemicals for specific tests or prediction models. In addition to the need to establish criteria for primary database development, a set of criteria for selecting chemicals for subset development should be developed.

5.5 Conclusions

5.5.1 *Primary Assumption for Data Set Development*

The primary assumption in establishing criteria for data set development is:

- The purpose and proposed use of the test, the endpoint measured, the range of testable chemicals, and the prediction model must be clearly defined before chemical selection begins.

Such information is used as the guide for choosing the most appropriate materials for evaluating whether or not the test method would satisfy its proposed uses.

5.5.2 *Criteria for Data Set Development*

The following criteria were established for data set development.

(1) The chemicals selected must be consistent with the test protocol and its prediction model.
   - The chemicals selected must be physically and chemically compatible with the test system.
   - The relevant chemical classes must be included.
     — The definition of chemical class is context-specific.
     — The developers of the test must specify the parameters that define the class.
     — The chemicals must be independently chosen.

(2) The toxicity must cover the range of response with uniform distribution.

(3) The number of chemicals used in the subset will depend on the nature of the test and the questions being asked, and should be determined with statistical advice.

5.5.3 *Primary Data Base Development*

Primary database development will most likely come from existing databases such as those available at the EPA, FDA, NCI, NTP, DOT, Galileo, Euclid, and others that are to be identified. As noted above, the more publicly available the database, the easier it will be to access the data. The problem, of course, is quality control of the data that goes into the database. The two most important considerations in assembling the primary set of reference chemicals are: (a) *in vivo* data must be of high quality, cover the range of response, and be uniformly distributed over that range and (b) the chemicals selected must be commercially available and their specifications (including purity) must be available.

The Breakout Group noted that there were some unresolved questions surrounding the issue of quality control. The first concerned protocol and, specifically, route of administration. There was some discussion about whether to accept tests done by all routes of administration or to limit the database to the oral route. It was decided that oral and inhalation routes were acceptable and that the dermal route while important for some purposes, was not of primary concern for most acute toxicity studies. However, the Breakout Group agreed, that if data were available from all routes, such data should be included in the database.

The Breakout Group agreed that, where possible, the data used should be derived from generally recognized test guidelines, such as those from the U.S. EPA, OECD, ICH, etc., because data from these guidelines carry a higher degree of assurance than data from an undefined or novel protocol. An issue that was not resolved was whether or not to require that the data used in the

database be from a study done according to Good Laboratory Practices (GLPs).

5.5.4 *Criteria for Choosing Reference Chemicals: Reference Test Data*

The following criteria were considered of prime importance in selecting a set of reference chemicals.

(1) The reference data for the endpoint predicted are available.

(2) The performance characteristics of the reference test must be defined.
- Variation will be introduced by protocol (including animal strain) differences.
- Different agencies use different protocols.
- The between-laboratory reproducibility of the test must be determined.
- The limitations of the reference test must be known.

(3) The reference test data must be of high quality.

(4) The protocol used must be available for review.

(5) Generally accepted methods (e.g., OECD, EPA, FDA, ICH guidelines) should have been used to generate the data.

(6) Details of the study should be available and ideally should satisfy ICCVAM and ECVAM Submission Guidelines.

(7) Study has sufficient supporting information. Ideally, GLPs should have been followed in study development.

(8) Other important considerations:
- The chemicals should be drawn from a wide range of structural and use classes.
- They should not be highly reactive, corrosive, or controlled substances.

5.5.5 *Database Fields*

The Breakout Group defined some of the information fields it considered relevant for the chemical reference database. These fields should include information about the identity, purities, and properties of the chemicals, and detailed reference test data.

(1) Chemical Information
- Name and Chemical Abstract Service (CAS) Number;
- Structure (coded, e.g., using Simplified Molecular Input Line Entry Specification [SMILES] nomenclature);
- Physical chemical characteristics (e.g., K_{ow}, pKa, water solubility, molecular weight., physical state);
- Purity;
- Chemical class (e.g., The International Union of Pure and Applied Chemistry [IUPAC] and use).

(2) Reference Test Data
- Specifications of chemical used in reference test;
- Information concerning the protocol used to generate the data;
- Endpoint value (e.g., LD50) and variance term (e.g., confidence interval), if available;
- Species, strain, sex;
- Route of exposure; duration of exposure;
- Information needed by Breakout Groups 2 and 3 should also be included.

5.6 Recommended Actions

5.6.1 *Rodent Toxicity Database*

(1) A study should be undertaken of existing databases to determine:
- The variation in the rodent LD50 introduced by differences in protocols;
- The within- and between-laboratory reproducibility of the rodent LD50 test and other acute toxicity tests that will be used as reference tests.

(2) An expert committee should be convened that will assemble a reference set of test chemicals from existing databases according to the criteria specified.

5.6.2 *Human Toxicity Database*

(1) There is a need to build upon the foundations of the MEIC and MEMO exercises.

(2) An expert panel should review the MEIC/MEMO approach for measuring acute toxicity parameters in humans.

(3) A consensus standard approach for measuring acute toxicity parameters is necessary.

(4) Existing sources of information need to be carefully searched in order to assure all relevant human data are obtained.

(5) A mechanism prospectively should be established to: (a) gather human toxicity data from hospital/Poison Control Center (PCC) sources; (b) retrieve existing human toxicity data; (c) collect and organize human toxicity data as accidents occur. Biomonitoring data should also be collected. Such information could define sub- or non-toxic levels, and be used to see if they overlap with the range of reported toxic levels.

6.0 GLOSSARY

Note: These definitions are based on (1) definitions used by one or more Breakout Groups at the *In vitro* Workshop or (2) a commonly used interpretation or definition.

Acute Toxic Class Method (ATC): An *in vivo* approach to assessing acute toxicity that tests animals in a step-wise fashion. Based on mortality and/or morbidity (or absence thereof), testing continues at the next highest (or lowest) fixed dose until an adequate assessment can be made. The method usually entails testing at two to four step-wise doses.

Acute Toxicity: The adverse effects occurring within a relatively short time after administration of a single dose of a substance or multiple doses within a 24-hour period. BG3 added: "*toxicity occurring within 14 days of a single exposure or multiple exposures within 24 hours"*.

Acute Systemic Toxicity: Acute effects that require absorption and distribution of the toxic agent from its entry point to a distant site at which adverse effects are produced vs. acute local toxicity.

ADAPT: (Automated Data Analysis by Pattern recognition Techniques); commercially available QSAR system for the evaluation of LD50s and MTDs; available from the laboratory of Peter Jurs, Penn State University.

ADME: biokinetic information on Absorption, Distribution, Metabolism, and Excretion.

Biotransformation: the series of chemical reactions of a compound in a biological system occurring within the body usually due to enzymatic metabolic reactions.

CASE: (Computer Automated Structure Evaluation); commercially available QSAR software

Cytotoxicity: The adverse effects of 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, metabolism, the synthesis and degradation or release of cellular constituents or products, ion regulation, and cell division.

Basal cytotoxicity: Involves one or more of the above mentioned structures or processes that would be expected to be intrinsic to all cell types. Sometimes called general cytotoxicity.

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, biotransformation, 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.

DEREK: (Deduction of Risk from Existing Knowledge); commercially available knowledge-based QSAR expert system.

EUCLID: (Electronically Useful Chemistry Laboratory Instructional Database); database of industrial chemicals tested in Europe maintained by the European Union.

Fixed Dose Procedure (FDP): An *in vivo* approach to assessing acute toxicity that avoids using death of animals as an endpoint, but instead uses the observation of clear signs of toxicity at one of a series of fixed dose levels. Instead of providing an LD50 value, this method estimates a range in which the LD50 of the test substance is estimated to occur.

Galileo: A publicly available database of chemicals that have been tested for toxicity (from alternative studies, mostly related to cosmetics testing).

Globally Harmonized System (GHS): Co-ordinating Group for the Harmonization of Chemical Classification Systems (CG/HCCS) was

established to promote and oversee the work to develop a GHS. The group would integrate the harmonized classification scheme with a harmonized hazard communication system to give an overall Globally Harmonized Classification and labeling System (GHS): OECD-sponsored.

IC50: (Inhibitory Concentration 50); the concentration of a material estimated to inhibit the biological endpoint of interest (e.g., cell growth, ATP levels) by 50%.

LD50: (Median Lethal Dose); a statistically derived single dose of a substance that can be expected to cause death in 50% of animals. This value is expressed in terms of the weight of the test substance per unit weight of the test animal.

LD50 Test, Conventional: An *in vivo* approach to assessing acute toxicity that tests several dose levels using groups of animals. Doses selected are often determined from a range-finding study. Observations of mortality and morbidity, as well as effects, are made for each dose group, and the LD50 is derived based on those observations.

MCASE: (Model-based Computer Automated Structure Evaluation); commercially available QSAR system for the evaluation of LD50s and MTDs available from Multicase, Inc.

Moribund: A clinical condition of a test animal that is indicative of impending death. Animals in the moribund state are humanely killed and are considered for acute toxicity testing purposes in the same way as animals that died.

MEIC: Multicenter Evaluation of In Vitro Cytotoxicity. Established by the Scandinavian Society for Cell Toxicology in 1989 to investigate the relevance of *in vitro* test results for predicting the acute toxic action of chemicals in humans directly rather than in rodents.

MEIC approach: The MEIC team collected case reports from human poisonings with the 50 reference chemicals to provide LC data with known times between ingestion and sampling/death. Constructed time-related LC curves for comparison with the IC50 values for different incubation times *in vitro (see.* 50 MEIC Monographs [MEMO]). Analyses of test results were based on *in vitro* cytotoxicity data presented as IC50 values. The predictability of *in vivo* acute toxicity from the *in vitro* IC50 data was assessed against human lethal blood concentrations compiled from three different data sets: clinically measured acute lethal serum concentrations, acute lethal blood concentrations measured post-mortem, and peak lethal concentrations derived from approximate LC50 curves over time. The analysis showed that *in vitro* assays that were among the most predictive generally used human cell lines. Human-derived cells appeared to be the most predictive for human acute toxicity. The most predictive and cost-effective test battery consisted 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. The test battery was found to be highly predictive of the peak human lethal blood concentrations of all 50 chemicals when incorporated into an algorithm developed by the team.

Mortality: Death of the test animals presumably due to the toxicity of the test material.

Predictive range: Range for various chemical properties over which the *in vitro* assay might be expected to provide reasonable LD50 estimates.

Quantitative Structure Activity Relationships (QSAR): The measurable biological activity of a series of similar compounds based on one or more physicochemical or structural properties of the compounds.

Registry of Cytotoxicity (RC): ZEBET database of acute oral LD50 data from rats and mice (taken from the NIOSH Registry of Toxic Effects of Chemical Substances [RTECS]) and IC50*x* values of chemicals and drugs from *in vitro* cytotoxicity assays. Currently contains data on 347 chemicals.

TOPKAT: (The Open Practical Knowledge Acquisition Toolkit); commercially available QSAR software.

Toxicokinetics: kinetics or biokinetics (BG2 definition).

Up-and-Down Procedure (UDP): An *in vivo* approach to assessing acute toxicity. Animals are dosed, one at a time, at 48-hour intervals. The first animal receives a dose at the investigator's best estimate of the LD50, and subsequent animals are given a higher or lower dose depending on the survival of the previous animal. After reaching the point where an increasing (or decreasing) dose pattern is reversed by giving a small (or higher dose), four additional animals are dosed following the same method, and the LD_{50} is calculated using the method of maximum likelihood.

ZEBET approach: Strategy to reduce the number of animals required for acute oral toxicity testing; Strategy involves using *in vitro* cytotoxicity data to determine the starting dose for *in vivo* testing. Researchers report the findings of an initial study conducted to assess the feasibility of applying the standard regression between mean IC50 values (i.e., IC50*x*, the mean concentration estimated to affect the endpoint in question by 50%) and acute oral LD50 data included in the Register of Cytotoxicity (RC) to estimate the LD50 value which can then be used to determine the *in vivo* starting dose.

ZEBET: Zentralstelle zur Erfassung und Bewertungvon Ersatz- und Ergänzungsmethoden zum Tierversuch (Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments)

7.0 REGISTRY OF CYTOTOXICITY (RC) DATA (ZEBET)

7.1 The ZEBET Database

ZEBET was established in Germany in 1989 at the Federal Institute for Consumer Health Protection and Veterinary Medicine (BgVV; http://www.bgvv.de). The ZEBET database contains evaluated information from the field of biomedicine and related fields on alternative methods that address the 3Rs concept of research that involves animals: refinement of animal use in experimentation, reduction of animal use, and replacement of animals. The database information was obtained from approximately 800 different documents (e.g., books, journals, monographs, etc.). The RC is part of the database and provides *in vitro* IC50 values as well as acute oral toxicity data (LD50) for rats and mice for 347 chemicals. The LD50 values come from the RTECS database at NIOSH. The ZEBET database also includes data for the 50 chemicals from the MEIC database. The German Institute for Medical Documentation and Information (DIMDI) provides access to the ZEBET database (http://www.dimdi.de).

7.1.1 *Tables*

The acute oral toxicity values are provided in mg/kg and mmol/l for rats and mice. Regression calculation values are in the last column of the data sheets. Rat LD50 values were used for the calculations if they were available; if not, then mouse LD50 values were used.

7.1.2 *Figures*

Regression calculations between cytotoxicity and acute oral toxicity are illustrated in the figures following the data.

7.1.3 *German Organizational Names*

ZEBET: Zentralstelle zur Erfassung und Bewertungvon Ersatz- und Ergänzungsmethoden zum Tierversuch
(German Centre for the Documentation and Validation of Alternative Methods [at BgVV])

DIMDI: Deutsches Institut für Medizinische Dokumentation und Information
(The German Institute for Medical Documentation and Information)

BgVV: Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin
(Federal Institute for Health Protection of Consumers and Veterinary Medicine)

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
1			Trenimon	68-76-8	0.00	0.0000033	NA	NA	NA	NA	231.28	
2			Actinomycin D	50-76-0	0.01	0.0000081	7.2	0.0057	12.6	0.01	1255.6	0.0057
3			Aminopterin	54-62-6	0.01	0.000012	NA	NA	3.0	0.0068	440.47	0.0068
4			Vincristine sulfate	2068-78-2	0.01	0.000015	NA	NA	NA	NA	923.14	
5		K-	Strophantin		0.03	0.000044	NA	NA	NA	NA	710.9	
132			Triphenyltin hydroxide	76-87-9	0.02	0.000049	44.0	0.12	245.9	0.67	367.03	0.12
6			Colchicine	64-86-8	0.02	0.000054	NA	NA	6.0	0.015	399.48	0.015
7			Ouabain	630-60-4	0.04	0.000072	NA	NA	NA	NA	584.73	
133			Cytochalasin D	22144-77-0	0.05	0.000092	NA	NA	36.0	0.071	507.68	0.071
8			Digitoxin	71-63-6	0.08	0.00011	55.8	0.073	NA	NA	765.05	0.073
134			Rotenone	83-79-4	0.05	0.00013	130.2	0.33	351.1	0.89	394.45	0.33
9			Amethopterin	59-05-2	0.06	0.00014	136.4	0.3	145.4	0.32	454.5	0.3
10			Emetine	483-18-1	0.08	0.00016	67.3	0.14	NA	NA	480.71	0.14
135		2,3,7,8-	Tetrachloro-dibenzo-p-dioxin	1746-01-6	0.06	0.0002	NA	NA	0.1	0.00035	321.96	0.00035
11			Doxorubicin * HCl	25316-40-9	0.19	0.00033	NA	NA	696.0	1.2	580.03	1.2
12			Puromycin	53-79-2	0.16	0.00033	NA	NA	674.4	1.43	471.58	1.43
136			Diethyldithiocarbamate sodium* 3H20	20624-25-3	0.09	0.00039	1500.7	6.66	1500.7	6.66	225.33	6.66
137			Triethyltin chloride	994-31-0	0.11	0.00046	5.1	0.021	NA	NA	241.35	0.021
138			Tributyltin chloride	1461-22-9	0.18	0.00054	120.4	0.37	NA	NA	325.53	0.37
139			Retinol	68-26-8	0.15	0.00054	1999.8	6.98	4011.0	14	286.5	6.98
140		6-	Thioguanine	154-42-7	0.10	0.00057	NA	NA	160.5	0.96	167.21	0.96
13			Cycloheximide	66-81-9	0.17	0.00059	2.0	0.0071	132.3	0.47	281.39	0.0071
141			Cytosine arabinoside	147-94-4	0.17	0.00068	NA	NA	3137.9	12.9	243.25	12.9
142			Methylmercury chloride	115-09-3	0.18	0.00071	NA	NA	57.7	0.23	251.08	0.23
143			Triethylene melamine	51-18-3	0.16	0.00078	1.0	0.005	14.9	0.073	204.27	0.005
14			Mitomycin C	50-07-7	0.28	0.00084	14.0	0.042	17.1	0.051	334.37	0.042
144			Sodium bichromate VI	10588-01-9	0.24	0.00093	49.8	0.19	NA	NA	261.98	0.19
15		8-	Azaguanine	134-58-7	0.20	0.0013	NA	NA	1500.1	9.86	152.14	9.86
145			Potassium chromate VI	7789-00-6	0.29	0.0015	NA	NA	180.6	0.93	194.2	0.93
16			Azaserine	115-02-6	0.35	0.002	169.7	0.98	150.6	0.87	173.15	0.98
146			Potassium bichromate VI		0.59	0.002	NA	NA	191.2	0.65	294.2	0.65
147			Mitoxantrone	65271-80-9	1.07	0.0024	586.8	1.32	NA	NA	444.54	1.32
148			Nitrogen mustard * HCl	55-86-7	0.50	0.0026	10.0	0.052	19.3	0.1	192.53	0.052
17		5-	Fluorouracil	51-21-8	0.34	0.0026	230.3	1.77	114.5	0.88	130.09	1.77
149			Chromium VI trioxide	1333-82-0	0.27	0.0027	80.0	0.8	127.0	1.27	100	0.8
150			Cis-platinum	15663-27-1	0.84	0.0028	25.8	0.086	33.0	0.11	300.07	0.086
151			Hexachlorocyclopentadiene	77-47-4	0.85	0.0031	111.8	0.41	NA	NA	272.75	0.41
152		8-	Hydroxyquinoline	148-24-3	0.48	0.0033	1200.6	8.27	NA	NA	145.17	8.27
18			Captan	133-06-2	1.17	0.0039	10009.6	33.3	7003.7	23.3	300.59	33.3

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
153	26		Arsenic III trioxide	1327-53-3	0.83	0.0042	19.8	0.1	45.5	0.23	197.84	0.1
154			Maneb	12427-38-2	1.12	0.0042	4500.6	16.9	3994.7	15	266.31	16.9
19			Cytochalasin B	14930-96-2	2.40	0.005	NA	NA	NA	NA	479.67	
155			Benzalkonium chloride	8001-54-5	1.90	0.0052	401.5	1.1	339.5	0.93	365	1.1
156			Stearyltrimethylammoniumchloride	112-03-8	2.09	0.006	NA	NA	536.1	1.54	348.13	1.54
20			Cadmium II chloride	10108-64-2	1.17	0.0064	88.0	0.48	174.1	0.95	183.3	0.48
157	38		Hexachlorophene	70-30-4	3.21	0.0079	61.0	0.15	65.1	0.16	406.89	0.15
21		6-	Mercaptopurine	50-44-2	1.22	0.008	NA	NA	280.0	1.84	152.19	1.84
158			Dichlorophene	97-23-4	2.23	0.0083	2691.3	10	1001.2	3.72	269.13	10
22	6		Digoxin	20830-75-5	6.64	0.0085	NA	NA	18.0	0.023	781.05	0.023
159			Hexadecyltrimethylammoniumbromide	57-09-0	3.24	0.0089	408.3	1.12	NA	NA	364.53	1.12
23			Daraprim	58-14-0	2.21	0.0089	NA	NA	126.9	0.51	248.74	0.51
24			Ethylenediamine-tetraacetic acid	60-00-4	2.92	0.01	NA	NA	NA	NA	292.28	
25			Thio-TEPA	52-24-4	2.08	0.011	NA	NA	37.8	0.2	189.24	0.2
160		N-	Methyl-N'-nitro-N-nitroso- guanidine	70-25-7	1.77	0.012	89.7	0.61	NA	NA	147.12	0.61
26			Kelthane	115-32-2	4.45	0.012	574.2	1.55	418.6	1.13	370.48	1.55
161			Silver I nitrate	7761-88-8	2.21	0.013	NA	NA	49.3	0.29	169.88	0.29
27			Chlorpromazine	50-53-3	4.46	0.014	140.3	0.44	261.5	0.82	318.89	0.44
28			Aldosterone	52-39-1	5.05	0.014	NA	NA	NA	NA	360.44	
29	28		Mercury II chloride	7487-94-7	4.07	0.015	1.0	0.0037	10.0	0.037	271.49	0.0037
162			Chlorhexidine	55-56-1	7.58	0.015	9200.5	18.2	9857.6	19.5	505.52	18.2
30			Sodium arsenate, dibasic	7778-43-0	2.79	0.015	NA	NA	NA	NA	185.91	
31	41		Chloroquine diphosphate	50-63-5	8.77	0.017	969.9	1.88	500.4	0.97	515.92	1.88
164			Oxatomide	60607-34-3	8.11	0.019	1412.1	3.31	9598.7	22.5	426.61	3.31
163			Cetyltrimethylammonium chloride	112-02-7	7.61	0.021	474.4	1.31	NA	NA	362.16	1.31
165			Isoproterenol * HCl	51-30-9	5.45	0.022	2219.8	8.96	NA	NA	247.75	8.96
32			Hydrocortisone	50-23-7	7.98	0.022	NA	NA	NA	NA	362.51	
166			Triisooctylamine	2757-28-0	8.14	0.023	1620.2	4.58	NA	NA	353.76	4.58
167		p,p'	DDD	72-54-8	7.68	0.024	112.0	0.35	NA	NA	320.04	0.35
33		p-	Chloromercuribenzoic acid	59-85-8	8.57	0.024	NA	NA	25.0	0.07	357.16	0.07
34			Diethylstilbestrol	56-53-1	6.71	0.025	NA	NA	NA	NA	268.38	
168			Dicoumarol	66-76-2	9.08	0.027	709.6	2.11	232.1	0.69	336.31	2.11
169			Epinephrine bitartrate	51-42-3	9.33	0.028	NA	NA	4.0	0.012	333.33	0.012
35			Flufenamic acid	530-78-9	8.16	0.029	272.8	0.97	714.4	2.54	281.25	0.97
170	29		Thioridazine * HCl	130-61-0	11.81	0.029	NA	NA	358.2	0.88	407.07	0.88
36			Progesterone	57-83-0	9.44	0.03	NA	NA	NA	NA	314.51	
171			Fumagillin	297-95-0	14.22	0.031	NA	NA	1999.5	4.36	458.6	4.36
37			Aflatoxin B1	1162-65-8	10.62	0.034	5.0	0.016	9.1	0.029	312.29	0.016
172			Nabam	142-59-6	8.97	0.035	394.8	1.54	579.3	2.26	256.34	1.54

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
173	39		Pentachlorophenol	87-86-5	9.59	0.036	50.6	0.19	NA	NA	266.32	0.19
174			Ambazone	539-21-9	9.02	0.038	749.9	3.16	999.1	4.21	237.32	3.16
175			Norepinephrine	51-41-2	6.60	0.039	NA	NA	20.3	0.12	169.2	0.12
46			Lead II chloride	7758-95-4	11.96	0.043	NA	NA	NA	NA	278.09	
176			Papaverine	58-74-2	15.27	0.045	325.8	0.96	230.8	0.68	339.42	0.96
177			Busulphan	55-98-1	11.33	0.046	1.9	0.0076	199.5	0.81	246.32	0.0076
178			Salicylanilide	87-17-2	9.81	0.046	NA	NA	2409.7	11.3	213.25	11.3
179			Acrolein	107-02-8	2.64	0.047	46.0	0.82	39.8	0.71	56.07	0.82
180		p-	Phenylenediamine	106-50-3	5.41	0.05	80.0	0.74	NA	NA	108.16	0.74
38			Imipramine * HCl	113-52-0	17.11	0.054	304.2	0.96	374.0	1.18	316.91	0.96
181	30		Thallium I sulfate	7446-18-6	27.26	0.054	NA	NA	28.8	0.057	504.8	0.057
39		2,4-	Dichlorophenol	120-83-2	8.97	0.055	580.3	3.56	1600.7	9.82	163	3.56
182			Triton X-100	9002-93-1	35.59	0.055	1798.7	2.78	NA	NA	647	2.78
183	5		Amitriptyline	50-48-6	15.54	0.056	319.1	1.15	147.0	0.53	277.44	1.15
184			Butylated hydroxytoluene	128-37-0	12.34	0.056	890.4	4.04	1040.2	4.72	220.39	4.04
185			Heptachlor	76-44-8	22.02	0.059	41.1	0.11	67.2	0.18	373.3	0.11
186			Zineb	12122-67-7	16.27	0.059	5211.3	18.9	7610.1	27.6	275.73	18.9
40			Chlordan	57-74-9	24.59	0.06	458.9	1.12	NA	NA	409.76	1.12
41			Chloroquine sulfate	132-73-0	25.08	0.06	1086.8	2.6	NA	NA	418	2.6
42		p-	Aminophenol	23-30-8	6.77	0.062	1658.9	15.2	NA	NA	109.14	15.2
187		4-	Hexylresorcinol	136-77-6	12.44	0.064	549.9	2.83	NA	NA	194.3	2.83
43			Aldrin	309-00-2	24.45	0.067	40.1	0.11	43.8	0.12	364.9	0.11
44			Hydroxyzine * HCl	1244-76-4	27.56	0.067	950.4	2.31	NA	NA	411.41	2.31
188		t-	Butyl hydroquinone	1948-33-0	11.47	0.069	799.6	4.81	1000.8	6.02	166.24	4.81
189			Antimycin	11118-72-2	17.52	0.07	NA	NA	112.6	0.45	250.27	0.45
45			Quinine * HCl	130-89-2	27.07	0.075	620.8	1.72	1158.6	3.21	360.92	1.72
190			Chlorambucil	305-03-3	23.12	0.076	76.1	0.25	100.4	0.33	304.24	0.25
191			Dimenhydrinate	523-87-5	35.72	0.076	1320.8	2.81	202.1	0.43	470.02	2.81
192		1,3-	Bis(2-chloroethyl)- 1-nitrosourea	154-93-8	16.70	0.078	19.9	0.093	19.1	0.089	214.07	0.093
193		5-	Azacytidine	320-67-2	19.29	0.079	NA	NA	571.5	2.34	244.24	2.34
47			Naftipramide	1505-95-9	25.07	0.084	1029.7	3.45	1086.4	3.64	298.47	3.45
48			Mefenamic acid	61-68-7	20.99	0.087	789.1	3.27	629.8	2.61	241.31	3.27
49			Parathion	56-38-2	27.09	0.093	2.0	0.0069	6.1	0.021	291.28	0.0069
194		p-	Toluylendiamine	95-70-5	11.49	0.094	101.4	0.83	NA	NA	122.19	0.83
50			Trypan blue	72-57-1	91.66	0.095	6204.2	6.43	NA	NA	964.88	6.43
195		p,p'	DDA	83-05-6	27.83	0.099	NA	NA	590.4	2.1	281.14	2.1
196	40		VerapamilHCl	152-11-4	49.11	0.1	108.0	0.22	162.1	0.33	491.13	0.22
197		p,p'	DDE	72-55-9	31.80	0.1	880.9	2.77	NA	NA	318.02	2.77
51			Disulfoton	298-04-4	30.19	0.11	2.0	0.0073	5.5	0.02	274.42	0.0073

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
198			Ioxynil	1689-83-4	40.80	0.11	111.3	0.3	NA	NA	370.91	0.3
199			Cupric chloride	7447-39-4	14.79	0.11	139.8	1.04	189.6	1.41	134.44	1.04
200			Dimethylaminoethyl methacrylate (polymer)	2867-47-2	17.30	0.11	1745.4	11.1	NA	NA	157.24	11.1
52			all-trans-Retinoic acid	302-79-4	33.05	0.11	2001.2	6.66	NA	NA	300.48	6.66
53	43		Quinidine sulfate	50-54-4	50.70	0.12	456.3	1.08	595.8	1.41	422.54	1.08
202			Formaldehyde	50-00-0	3.60	0.12	798.8	26.6	NA	NA	30.03	26.6
54	23		Propranolol * HCl	318-98-9	35.50	0.12	NA	NA	470.4	1.59	295.84	1.59
201		13-cis-	Retinoic acid	4759-48-2	36.06	0.12	NA	NA	3395.4	11.3	300.48	11.3
55			Zinc II chloride	7646-85-7	17.72	0.13	350.2	2.57	350.2	2.57	136.27	2.57
56			Manganese IIchloride *4 H2O	13446-34-9	25.73	0.13	1484.4	7.5	NA	NA	197.92	7.5
57		L-	Dopa	59-92-7	25.64	0.13	1780.8	9.03	2366.5	12	197.21	9.03
204			Azathioprine	446-86-6	38.82	0.14	535.2	1.93	1389.2	5.01	277.29	1.93
58			Dihydralazine sulfate	7327-87-9	40.36	0.14	818.8	2.84	400.8	1.39	288.32	2.84
59			Tetracycline * HCl	64-75-5	67.33	0.14	6444.6	13.4	NA	NA	480.94	13.4
203			Thallium I acetate	563-68-8	36.88	0.14	NA	NA	34.2	0.13	263.42	0.13
205			Versalide	88-29-9	38.77	0.15	315.3	1.22	NA	NA	258.44	1.22
60			Indomethacin	53-86-1	57.25	0.16	12.2	0.034	19.0	0.053	357.81	0.034
62			Cobalt II chloride	7646-79-9	20.77	0.16	80.5	0.62	80.5	0.62	129.83	0.62
61		p,p'	DDT	50-29-3	56.72	0.16	113.4	0.32	134.7	0.38	354.48	0.32
206			Diquat dibromide	85-00-7	55.05	0.16	230.5	0.67	234.0	0.68	344.08	0.67
63	4		Diazepam	439-14-5	45.56	0.16	709.1	2.49	535.3	1.88	284.76	2.49
207			Dieldrin	60-57-1	68.56	0.18	45.7	0.12	38.1	0.1	380.9	0.12
64			Bendiocarb	22781-23-3	40.19	0.18	178.6	0.8	NA	NA	223.25	0.8
208			Undecylenic acid	112-38-9	33.18	0.18	2506.6	13.6	8496.7	46.1	184.31	13.6
209			Propylparaben	94-13-3	32.44	0.18	NA	NA	6325.7	35.1	180.22	35.1
65			Oxyphenbutazone	129-20-4	61.64	0.19	999.2	3.08	480.1	1.48	324.41	3.08
66			Cortisone	53-06-5	68.49	0.19	NA	NA	NA	NA	360.49	
210		p-	Nitrophenol	100-02-7	27.82	0.2	350.6	2.52	467.4	3.36	139.12	2.52
67	15		Malathion	121-75-5	66.08	0.2	885.4	2.68	776.4	2.35	330.38	2.68
211			Catechol	120-80-9	22.02	0.2	3887.2	35.3	259.9	2.36	110.12	35.3
68		2,4-	Dinitrophenol	51-28-5	38.67	0.21	29.5	0.16	44.2	0.24	184.12	0.16
69			Secobarbital sodium	309-43-3	54.66	0.21	124.9	0.48	NA	NA	260.3	0.48
212		p-	Cresol	106-44-5	23.79	0.22	206.6	1.91	343.9	3.18	108.15	1.91
70	49		Atropine sulfate	55-48-1	148.92	0.22	622.7	0.92	764.9	1.13	676.9	0.92
213			Ammonium persulfate	7727-54-0	52.49	0.23	819.3	3.59	NA	NA	228.22	3.59
214			Thymol	89-83-8	34.56	0.23	979.6	6.52	1802.9	12	150.24	6.52
71			Diphenhydramine * HCl	147-24-0	70.04	0.24	855.1	2.93	113.8	0.39	291.85	2.93
72			Butylated hydoxyanisole	8003-24-5	43.26	0.24	2199.3	12.2	2001.0	11.1	180.27	12.2
215			Chlorotetracycline	57-62-5	114.94	0.24	NA	NA	2500.0	5.22	478.92	5.22

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
216			Refortan		78.28	0.25	3162.3	10.1	NA	NA	313.1	10.1
73			Carbaryl	63-25-2	52.32	0.26	249.5	1.24	438.7	2.18	201.24	1.24
74			Nickel II chloride	7718-54-9	34.99	0.27	105.0	0.81	NA	NA	129.61	0.81
75			Trichlorfon	52-68-6	69.51	0.27	450.5	1.75	298.6	1.16	257.44	1.75
76			Sodium dodecyl sulfate	151-21-3	78.15	0.27	1288.0	4.45	NA	NA	289.43	4.45
77			Cinchophen	132-60-5	67.31	0.27	NA	NA	NA	NA	249.28	
217			Amrinone	60719-84-8	52.42	0.28	101.1	0.54	288.3	1.54	187.22	0.54
218		o-	Phenylenediamine	95-54-5	33.53	0.31	1069.7	9.89	NA	NA	108.16	9.89
78		6-	Methylcoumarin	92-48-8	49.66	0.31	1681.9	10.5	NA	NA	160.18	10.5
79			Phenylbutazone	50-33-9	98.69	0.32	376.3	1.22	441.0	1.43	308.41	1.22
80		2-	Thiouracil	141-90-2	41.01	0.32	999.6	7.8	NA	NA	128.16	7.8
219			Hydralazine	86-54-4	52.87	0.33	89.7	0.56	121.8	0.76	160.2	0.56
81	27		Cupric sulfate * 5 H2O	7758-99-8	82.40	0.33	299.6	1.2	NA	NA	249.7	1.2
238			Imidazolidinyl urea	39236-46-9	100.17	0.36	2598.9	9.34	3700.9	13.3	278.26	9.34
220		m-	Dinitrobenzene	99-65-0	65.57	0.39	82.4	0.49	NA	NA	168.12	0.49
221		2-	Nitro-p-phenylene-diamine	5307-14-2	59.73	0.39	3078.5	20.1	NA	NA	153.16	20.1
82	44		Diphenylhydantoin	57-41-0	98.39	0.39	NA	NA	199.3	0.79	252.29	0.79
222			Glibenclamide	10238-21-8	197.62	0.4	NA	NA	3250.8	6.58	494.05	6.58
223	32		Lindane	58-89-9	119.24	0.41	75.6	0.26	87.2	0.3	290.82	0.26
224		n-	Butyl benzoate	136-60-7	73.08	0.41	5133.6	28.8	NA	NA	178.25	28.8
225			Ammonium sulfide	12135-76-1	21.47	0.42	168.2	3.29	NA	NA	51.12	3.29
226			Dodecylbenzene sodiumsulfonate	25155-30-0	146.38	0.42	1261.6	3.62	2000.5	5.74	348.52	3.62
227	46		Sodium oxalate	62-76-0	58.96	0.44	155.4	1.16	NA	NA	134	1.16
228		2,4,5-	Trichlorophen- oxyacetic acid	93-76-5	112.41	0.44	298.9	1.17	388.3	1.52	255.48	1.17
229	22		Dextropropoxyphene * HCl	1639-60-7	184.23	0.49	82.7	0.22	82.7	0.22	375.98	0.22
230	42		Orphenadrine * HCl	341-69-5	149.88	0.49	425.2	1.39	125.4	0.41	305.88	1.39
231			Tween 80	9005-65-6	641.90	0.49	NA	NA	25021.0	19.1	1310	19.1
232		o-	Cresol	95-48-7	56.24	0.52	121.1	1.12	343.9	3.18	108.15	1.12
233			Ibuprofen	15687-27-1	107.28	0.52	1008.9	4.89	980.0	4.75	206.31	4.89
234			Phenylthiourea	103-85-5	82.20	0.54	3.0	0.02	10.0	0.066	152.23	0.02
235	25		Paraquat	4685-14-7	100.58	0.54	57.7	0.31	195.6	1.05	186.25	0.31
83			Thiopental	76-75-5	133.30	0.55	NA	NA	601.1	2.48	242.37	2.48
84			Amobarbital	57-43-2	126.73	0.56	NA	NA	344.0	1.52	226.31	1.52
236			Hydrogen peroxide 90%	7722-84-1	19.05	0.56	NA	NA	2000.4	58.8	34.02	58.8
85			Metamizol	68-89-3	193.94	0.58	7189.2	21.5	NA	NA	334.38	21.5
237			Beryllium II sulfate	13510-49-1	64.09	0.61	82.0	0.78	79.9	0.76	105.07	0.78
239		m-	Cresol	108-39-4	71.38	0.66	242.3	2.24	828.4	7.66	108.15	2.24
240			Pentoxifylline	6493-05-6	183.71	0.66	NA	NA	1386.2	4.98	278.35	4.98
86	31		Warfarin	81-81-2	206.59	0.67	323.8	1.05	373.1	1.21	308.35	1.05

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
241			Sodium azide	26628-22-8	46.16	0.71	44.9	0.69	27.3	0.42	65.02	0.69
87			Pentobarbital sodium	57-33-0	176.29	0.71	201.1	0.81	280.6	1.13	248.29	0.81
242		1,2,4-	Trichlorobenzene	120-82-1	128.82	0.71	756.6	4.17	765.7	4.22	181.44	4.17
243		p-	Anisidine	104-94-9	89.91	0.73	1404.1	11.4	NA	NA	123.17	11.4
244			Doxylamine succinate	562-10-7	291.38	0.75	NA	NA	470.1	1.21	388.51	1.21
88			Dibutyl phthalate	84-74-2	211.57	0.76	11998.2	43.1	NA	NA	278.38	43.1
89	16	2,4-	Dichlorophenoxy- acetic acid	94-75-7	170.20	0.77	369.1	1.67	366.9	1.66	221.04	1.67
90			Iproniazid	54-92-2	141.61	0.79	365.7	2.04	681.2	3.8	179.25	2.04
91	45		Chloramphenicol	56-75-7	255.29	0.79	3393.1	10.5	2640.1	8.17	323.15	10.5
245			Resorcinol	108-46-3	88.10	0.8	300.6	2.73	NA	NA	110.12	2.73
246	37		Barium II nitrate	10022-31-8	211.70	0.81	355.4	1.36	NA	NA	261.36	1.36
247		(+)-	Thalidomide	731-40-8	209.18	0.81	NA	NA	400.3	1.55	258.25	1.55
92			Di(2-ethylhexyl)phthalate	117-81-7	328.12	0.84	31015.2	79.4	29999.6	76.8	390.62	79.4
93			Sulfisoxazole	127-69-5	227.23	0.85	NA	NA	6790.2	25.4	267.33	25.4
248		m-	Aminophenol	591-27-5	93.86	0.86	1658.9	15.2	NA	NA	109.14	15.2
94			Menthol	89-78-1	148.49	0.95	3172.9	20.3	NA	NA	156.3	20.3
249		3-	Cyano-2-morpholino-5-(pyrid-4-yl)-pyridine (Chemical 122)		255.66	0.96	346.2	1.3	NA	NA	266.31	1.3
250			Valproate sodium	1069-66-5	166.22	1	NA	NA	1695.4	10.2	166.22	10.2
251			Scopolamine * HBr	6533-68-2	415.05	1.08	1268.2	3.3	1879.3	4.89	384.31	3.3
95			Salicylamide	65-45-2	148.12	1.08	1892.7	13.8	1398.9	10.2	137.15	13.8
252	19		Potassium cyanide	151-50-8	72.93	1.12	9.8	0.15	8.5	0.13	65.12	0.15
96			Cygon	60-51-5	284.29	1.24	151.3	0.66	59.6	0.26	229.27	0.66
97			Phenacetin	62-44-2	227.63	1.27	1650.8	9.21	1220.6	6.81	179.24	9.21
253			Isoxepac	55453-87-7	356.81	1.33	198.5	0.74	NA	NA	268.28	0.74
254			Buflomedil	55837-25-7	415.03	1.35	365.8	1.19	NA	NA	307.43	1.19
98			Methylparaben	99-76-3	216.07	1.42	NA	NA	1749.8	11.5	152.16	11.5
255			Sodium monochloroacetate	3926-62-3	168.90	1.45	75.7	0.65	NA	NA	116.48	0.65
99			Nalidixic acid	389-08-2	348.39	1.5	1349.4	5.81	571.4	2.46	232.26	5.81
256			Tin II chloride	7772-99-8	286.28	1.51	699.6	3.69	1200.1	6.33	189.59	3.69
257			Isononylaldehyde	5435-64-3	216.25	1.52	3243.8	22.8	NA	NA	142.27	22.8
100		L-	Ascorbic acid	50-81-7	267.73	1.52	11907.1	67.6	3364.3	19.1	176.14	67.6
101			Glutethimide	77-21-4	338.97	1.56	599.7	2.76	360.7	1.66	217.29	2.76
102			Acrylamide	79-06-1	114.45	1.61	169.9	2.39	169.9	2.39	71.09	2.39
258			Diethyl sebacate	110-40-7	421.19	1.63	14470.4	56	NA	NA	258.4	56
259			Methyl salicylate	119-36-8	258.67	1.7	887.1	5.83	NA	NA	152.16	5.83
260			Coumarin	91-64-5	249.92	1.71	292.3	2	195.8	1.34	146.15	2
103	18		Nicotine	54-11-5	290.45	1.79	50.3	0.31	24.3	0.15	162.26	0.31
104			Tolbutamide	64-77-7	489.39	1.81	NA	NA	2601.1	9.62	270.38	9.62
105	21		Theophylline	58-55-9	329.75	1.83	NA	NA	600.0	3.33	180.19	3.33

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
106	14		Sodium I fluoride	7681-49-4	77.68	1.85	180.1	4.29	NA	NA	41.99	4.29
261	3		Ferrous sulfate	7720-78-7	281.03	1.85	319.0	2.1	978.3	6.44	151.91	2.1
262	47		Amphetamine sulfate	60-13-9	726.02	1.97	55.3	0.15	24.0	0.065	368.54	0.15
107	2		Acetylsalicylic acid	50-78-2	408.99	2.27	999.9	5.55	814.4	4.52	180.17	5.55
108			Gibberellic acid	77-06-5	796.74	2.3	6304.7	18.2	NA	NA	346.41	18.2
109			Frusemide	54-31-9	770.67	2.33	2599.8	7.86	4597.6	13.9	330.76	7.86
110			Acrylonitrile	107-13-1	128.43	2.42	81.7	1.54	27.1	0.51	53.07	1.54
263			Acetaldehyde	75-07-0	107.95	2.45	1929.8	43.8	NA	NA	44.06	43.8
111			Clofibric acid	882-09-7	560.26	2.61	1249.3	5.82	1169.9	5.45	214.66	5.82
112	48		Caffeine	58-08-2	512.74	2.64	192.3	0.99	619.6	3.19	194.22	0.99
264			Chloral hydrate	302-17-0	438.31	2.65	479.7	2.9	1101.6	6.66	165.4	2.9
113	1		Acetaminophen	103-90-2	409.70	2.71	2403.8	15.9	338.6	2.24	151.18	15.9
265			Streptomycin sulfate	298-39-5	3979.25	2.73	NA	NA	495.6	0.34	1457.6	0.34
114			Natulan * HCl	366-70-1	706.37	2.74	783.7	3.04	NA	NA	257.8	3.04
266			Potassium hexacyanoferrate III	13746-66-2	928.54	2.82	NA	NA	2970.0	9.02	329.27	9.02
267		p-	Hydroxybenzoic acid	99-96-7	403.34	2.92	NA	NA	2196.3	15.9	138.13	15.9
115	12		Phenol	108-95-2	283.30	3.01	414.1	4.4	300.2	3.19	94.12	4.4
268		1-	Octanol	111-87-5	398.60	3.06	NA	NA	1784.6	13.7	130.26	13.7
116			Cyclophosphamide * H2O	6055-19-2	870.89	3.12	94.9	0.34	136.8	0.49	279.13	0.34
269			Potassium I fluoride	7789-23-3	181.85	3.13	245.2	4.22	NA	NA	58.1	4.22
117			Di(2-ethylhexyl)adipate	103-23-1	1167.52	3.15	9117.7	24.6	NA	NA	370.64	24.6
270			Propionaldehyde	123-38-6	188.79	3.25	1411.6	24.3	NA	NA	58.09	24.3
271			Styrene	100-42-5	343.73	3.3	4999.7	48	315.6	3.03	104.16	48
272			Salicylic acid	69-72-7	466.88	3.38	890.9	6.45	479.3	3.47	138.13	6.45
273			Bromobenzene	108-86-1	543.29	3.46	2700.7	17.2	NA	NA	157.02	17.2
274		L-	Cysteine	52-90-4	431.37	3.56	NA	NA	660.4	5.45	121.17	5.45
275			Nitrilotriacetic acid	139-13-9	690.09	3.61	1470.0	7.69	3154.1	16.5	191.16	7.69
276			Ambuphylline	5634-34-4	988.51	3.67	NA	NA	600.7	2.23	269.35	2.23
118	24		Phenobarbital	50-06-6	884.91	3.81	162.6	0.7	167.2	0.72	232.26	0.7
277			Potassium cyanate	590-28-3	335.84	4.14	NA	NA	843.6	10.4	81.12	10.4
278			Phenylephrine * HCl	939-38-8	847.35	4.16	350.3	1.72	120.2	0.59	203.69	1.72
279			Thioacetamide	62-55-5	313.33	4.17	301.3	4.01	NA	NA	75.14	4.01
280			Theophylline sodium acetate	8002-89-9	1098.74	4.19	582.2	2.22	NA	NA	262.23	2.22
281		1,2-	Dibromomethane	106-93-4	730.17	4.2	107.8	0.62	NA	NA	173.85	0.62
119			Sodium salicylate	54-21-7	693.28	4.33	1599.5	9.99	899.8	5.62	160.11	9.99
282		(-)-	Phenylephrine	59-42-7	744.17	4.45	349.5	2.09	NA	NA	167.23	2.09
283			Milrinone	78415-72-2	1007.61	4.77	90.8	0.43	137.3	0.65	211.24	0.43
120		5-	Aminosalicylic acid	89-57-6	776.47	5.07	NA	NA	7749.4	50.6	153.15	50.6
121			Aminophenazone	58-15-1	1246.87	5.39	999.3	4.32	358.6	1.55	231.33	4.32

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
284			Ammonium chloride	12125-02-9	295.32	5.52	1647.8	30.8	NA	NA	53.5	30.8
122			Diethyl phthalate	84-66-2	1226.88	5.52	8601.5	38.7	6178.8	27.8	222.26	38.7
285			Caffeine sodium benzoate	8000-95-1	1918.33	5.67	859.4	2.54	798.5	2.36	338.33	2.54
286			Benzylpenicillin sodium	69-57-8	2042.17	5.73	6914.2	19.4	NA	NA	356.4	19.4
287			Benzylalcohol	100-51-6	628.35	5.81	1232.9	11.4	1579.0	14.6	108.15	11.4
288		1-	Heptanol	111-70-6	726.44	6.25	3254.4	28	1499.4	12.9	116.23	28
289			Tetrachloroethene	127-18-4	1084.46	6.54	8854.8	53.4	8092.0	48.8	165.82	53.4
290			Sodium sulfite	7757-83-7	854.55	6.78	NA	NA	820.5	6.51	126.04	6.51
291			Aniline	62-53-3	642.67	6.9	439.6	4.72	439.6	4.72	93.14	4.72
292			Allylalcohol	107-18-6	403.14	6.94	63.9	1.1	95.8	1.65	58.09	1.1
293			Diisopropylamine dichloroacetate	660-27-5	1611.12	7	NA	NA	1700.9	7.39	230.16	7.39
123	35		Isoniazid	54-85-3	1027.33	7.49	650.1	4.74	NA	NA	137.16	4.74
294			Trichloroacetic acid	76-03-9	1338.08	8.19	4999.4	30.6	5636.6	34.5	163.38	30.6
295		2,5-	Hexanedione	110-13-4	964.65	8.45	2705.6	23.7	NA	NA	114.16	23.7
124			Acetazolamide	59-66-5	1886.99	8.49	NA	NA	4289.6	19.3	222.26	19.3
125	34		Carbon tetrachloride	56-23-5	1308.92	8.51	2799.3	18.2	12797.0	83.2	153.81	18.2
296			Homatropine methylbromide	80-49-9	3332.97	9	1199.9	3.24	1399.8	3.78	370.33	3.24
297	11	1,1,1-	Trichloroethane	71-55-6	1374.02	10.3	10298.5	77.2	11245.6	84.3	133.4	77.2
298			Dichloroacetic acid	79-43-6	1482.81	11.5	2823.8	21.9	5518.6	42.8	128.94	21.9
299			Imidazole	288-32-4	783.04	11.5	NA	NA	1879.3	27.6	68.09	27.6
300			Antipyrine	60-80-0	2183.70	11.6	1799.7	9.56	1699.9	9.03	188.25	9.56
301	17		Xylene	1330-20-7	1274.16	12	4300.3	40.5	NA	NA	106.18	40.5
302			Nitrobenzene	98-95-3	1502.06	12.2	640.2	5.2	NA	NA	123.12	5.2
304			Calcium II chloride	10043-52-4	1376.15	12.4	999.9	9.01	NA	NA	110.98	9.01
303			Theophylline sodium	3485-82-3	2519.43	12.4	NA	NA	445.0	2.19	203.18	2.19
305		n-	Butanal	123-72-8	923.14	12.8	2488.1	34.5	NA	NA	72.12	34.5
306			Anisole	100-66-3	1427.58	13.2	3698.7	34.2	NA	NA	108.15	34.2
307		2-	Ethylbutanal	97-96-1	1322.38	13.2	3977.1	39.7	NA	NA	100.18	39.7
308	33		Chloroform	67-66-3	1599.56	13.4	908.4	7.61	35.8	0.3	119.37	7.61
309			Isobutanal	78-84-2	973.62	13.5	2812.7	39	NA	NA	72.12	39
126			Triethyl citrate	77-93-0	4061.90	14.7	6990.9	25.3	NA	NA	276.32	25.3
310			Tributylamine	102-82-9	2855.16	15.4	539.5	2.91	NA	NA	185.4	2.91
311		1-	Hexanol	111-27-3	1573.88	15.4	719.5	7.04	1952.0	19.1	102.2	7.04
312			Benzoic acid	65-85-0	1917.44	15.7	2528.1	20.7	2369.3	19.4	122.13	20.7
313			Xanthinol nicotinate	437-74-1	6865.26	15.8	14121.6	32.5	17336.9	39.9	434.51	32.5
314			Saccharin	81-07-2	3004.32	16.4	NA	NA	17000.0	92.8	183.19	92.8
315			Isobenzoic furano dione		2518.04	17	4014.1	27.1	1999.6	13.5	148.12	27.1
316			Toluene	108-88-3	1575.77	17.1	5003.7	54.3	NA	NA	92.15	54.3
317			Barbital sodium	144-02-5	3835.32	18.6	NA	NA	800.1	3.88	206.2	3.88

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
318			Trifluoroacetic acid	76-05-1	2337.62	20.5	199.6	1.75	NA	NA	114.03	1.75
127			Dimethyl phthalate	131-11-3	4544.28	23.4	6894.1	35.5	7204.8	37.1	194.2	35.5
319			Methylpentinol	77-75-8	2336.21	23.8	NA	NA	525.2	5.35	98.16	5.35
320		N,N-	Dimethylacetamide	127-19-5	2108.79	24.2	5089.0	58.4	4618.4	53	87.14	58.4
321			Acetic acid	64-19-7	1459.46	24.3	3309.3	55.1	4961.0	82.6	60.06	55.1
322		1-	Pentanol	71-41-0	2195.43	24.9	3033.0	34.4	200.1	2.27	88.17	34.4
323			Urethan	51-79-6	2307.95	25.9	NA	NA	2504.0	28.1	89.11	28.1
324		2-	Butoxyethanol	111-76-2	3073.20	26	1477.5	12.5	1229.3	10.4	118.2	12.5
325			Cyclohexanol	108-93-0	2634.73	26.3	2063.7	20.6	NA	NA	100.18	20.6
326			Halothane	151-67-7	6138.83	31.1	5684.8	28.8	NA	NA	197.39	28.8
327	20		Lithium I sulfate	10377-48-7	3704.98	33.7	NA	NA	1187.4	10.8	109.94	10.8
328	36		Dichloromethane	75-09-2	2964.06	34.9	1596.7	18.8	NA	NA	84.93	18.8
329			Sodium cyclamate	139-05-9	7123.90	35.4	15254.0	75.8	17004.8	84.5	201.24	75.8
330			Sulfuric acid	7664-93-9	3530.88	36	2138.1	21.8	NA	NA	98.08	21.8
331			Strontium II chloride	10476-85-4	5770.13	36.4	2251.0	14.2	3107.0	19.6	158.52	14.2
332		1,4-	Dioxane	123-91-1	3357.37	38.1	4203.3	47.7	5701.4	64.7	88.12	47.7
333			Lithium I chloride	7447-41-8	1636.25	38.6	758.8	17.9	1165.7	27.5	42.39	17.9
334			Isobutanol	78-83-1	2973.01	40.1	2461.4	33.2	NA	NA	74.14	33.2
335			Potassium hexacyano- ferrate II	13943-58-3	15582.05	42.3	6409.6	17.4	5009.8	13.6	368.37	17.4
336			Nicotinamide	98-92-0	5423.02	44.4	3505.4	28.7	NA	NA	122.14	28.7
337			Pyridine	110-86-1	3710.26	46.9	893.9	11.3	NA	NA	79.11	11.3
338		1-	Butanol	71-36-3	3892.35	52.5	793.3	10.7	NA	NA	74.14	10.7
339		1-	Nitropropane	79-46-9	5159.47	57.9	455.4	5.11	NA	NA	89.11	5.11
340			Diethylene glycol	111-46-6	6591.29	62.1	14753.5	139	23669.2	223	106.14	139
341			Lactic acid	598-82-3	5945.94	66	3729.7	41.4	4873.9	54.1	90.09	41.4
342			Piperazine	110-85-0	5789.95	67.2	1904.1	22.1	1438.9	16.7	86.16	22.1
343			Magnesium II chloride * 6 H2O	7791-18-6	14314.43	70.4	8092.5	39.8	NA	NA	203.33	39.8
344	13		Sodium chloride	7647-14-5	4435.60	75.9	2998.0	51.3	3997.3	68.4	58.44	51.3
345			Sodium I bromide	7647-15-6	8120.81	77.4	3504.3	33.4	6998.2	66.7	104.92	33.4
346	50		Potassium I chloride	7447-40-7	6113.10	82	2601.8	34.9	1498.5	20.1	74.55	34.9
347			Thiourea	62-56-6	6547.18	86	124.9	1.64	8526.6	112	76.13	1.64
348		1-	Propanol	71-23-8	5800.62	96.5	5397.9	89.8	NA	NA	60.11	89.8
349			Ethyl methyl ketone	78-93-3	7500.48	104	3396.9	47.1	NA	NA	72.12	47.1
350			Tetrahydrofurfuryl alcohol	97-99-4	11338.65	111	2502.7	24.5	2298.4	22.5	102.15	24.5
351			Dimethylformamide	68-12-2	8334.54	114	2800.1	38.3	3750.5	51.3	73.11	38.3
352		1,2,6-	Hexanetriol	106-69-4	16506.60	123	15969.8	119	NA	NA	134.2	119
353			Ethyl acetate	141-78-6	11279.36	128	11015.0	125	NA	NA	88.12	125
128	10	2-	Propanol	67-63-0	10038.37	167	5842.7	97.2	NA	NA	60.11	97.2
354		1,3,5-	Trioxane	110-88-3	19189.17	213	800.0	8.88	NA	NA	90.09	8.88

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
355			D-Glucose	50-99-7	40720.68	226	25765.7	143	NA	NA	180.18	143
356		2-	Methoxyethanol	109-86-4	19103.61	251	2458.4	32.3	NA	NA	76.11	32.3
129			Dimethyl sulfoxide	75-18-3	19691.28	252	19691.3	252	16487.5	211	78.14	252
357			Propylene glycol	57-55-6	26029.62	342	20016.9	263	23974.7	315	76.11	263
358			Acetonitrile	75-05-8	15110.08	368	3798.1	92.5	NA	NA	41.06	92.5
130	9		Ethanol	64-17-5	17464.32	379	14008.3	304	7787.5	169	46.08	304
359			Acetone	67-64-1	25791.96	444	9759.1	168	NA	NA	58.09	168
360	7		Ethylene glycol	107-21-1	34454.40	555	8567.0	138	7511.7	121	62.08	138
131			Glycerol	56-81-5	57476.64	624	12619.1	137	25975.0	282	92.11	137
361	8		Methanol	67-56-1	29806.50	930	13012.3	406	NA	NA	32.05	406

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
29	28		Mercury II chloride	7487-94-7	4.07	0.015	1.0	0.0037	10.0	0.037	271.49	0.0037
143			Triethylene melamine	51-18-3	0.16	0.00078	1.0	0.005	14.9	0.073	204.27	0.005
177			Busulphan	55-98-1	11.33	0.046	1.9	0.0076	199.5	0.81	246.32	0.0076
13			Cycloheximide	66-81-9	0.17	0.00059	2.0	0.0071	132.3	0.47	281.39	0.0071
51			Disulfoton	298-04-4	30.19	0.11	2.0	0.0073	5.5	0.02	274.42	0.0073
49			Parathion	56-38-2	27.09	0.093	2.0	0.0069	6.1	0.021	291.28	0.0069
234			Phenylthiourea	103-85-5	82.20	0.54	3.0	0.02	10.0	0.066	152.23	0.02
37			Aflatoxin B1	1162-65-8	10.62	0.034	5.0	0.016	9.1	0.029	312.29	0.016
137			Triethyltin chloride	994-31-0	0.11	0.00046	5.1	0.021	NA	NA	241.35	0.021
2			Actinomycin D	50-76-0	0.01	0.0000081	7.2	0.0057	12.6	0.01	1255.6	0.0057
252	19		Potassium cyanide	151-50-8	72.93	1.12	9.8	0.15	8.5	0.13	65.12	0.15
148			Nitrogen mustard * HCl	55-86-7	0.50	0.0026	10.0	0.052	19.3	0.1	192.53	0.052
60			Indomethacin	53-86-1	57.25	0.16	12.2	0.034	19.0	0.053	357.81	0.034
14			Mitomycin C	50-07-7	0.28	0.00084	14.0	0.042	17.1	0.051	334.37	0.042
153	26		Arsenic III trioxide	1327-53-3	0.83	0.0042	19.8	0.1	45.5	0.23	197.84	0.1
192		1,3-	Bis(2-chloroethyl)- 1-nitrosourea	154-93-8	16.70	0.078	19.9	0.093	19.1	0.089	214.07	0.093
150			Cis-platinum	15663-27-1	0.84	0.0028	25.8	0.086	33.0	0.11	300.07	0.086
68		2,4-	Dinitrophenol	51-28-5	38.67	0.21	29.5	0.16	44.2	0.24	184.12	0.16
43			Aldrin	309-00-2	24.45	0.067	40.1	0.11	43.8	0.12	364.9	0.11
185			Heptachlor	76-44-8	22.02	0.059	41.1	0.11	67.2	0.18	373.3	0.11
132			Triphenyltin hydroxide	76-87-9	0.02	0.000049	44.0	0.12	245.9	0.67	367.03	0.12
241			Sodium azide	26628-22-8	46.16	0.71	44.9	0.69	27.3	0.42	65.02	0.69
207			Dieldrin	60-57-1	68.56	0.18	45.7	0.12	38.1	0.1	380.9	0.12
179			Acrolein	107-02-8	2.64	0.047	46.0	0.82	39.8	0.71	56.07	0.82
144			Sodium bichromate VI	10588-01-9	0.24	0.00093	49.8	0.19	NA	NA	261.98	0.19
103	18		Nicotine	54-11-5	290.45	1.79	50.3	0.31	24.3	0.15	162.26	0.31
173	39		Pentachlorophenol	87-86-5	9.59	0.036	50.6	0.19	NA	NA	266.32	0.19
262	47		Amphetamine sulfate	60-13-9	726.02	1.97	55.3	0.15	24.0	0.065	368.54	0.15
8			Digitoxin	71-63-6	0.08	0.00011	55.8	0.073	NA	NA	765.05	0.073
235	25		Paraquat	4685-14-7	100.58	0.54	57.7	0.31	195.6	1.05	186.25	0.31
157	38		Hexachlorophene	70-30-4	3.21	0.0079	61.0	0.15	65.1	0.16	406.89	0.15
292			Allylalcohol	107-18-6	403.14	6.94	63.9	1.1	95.8	1.65	58.09	1.1
10			Emetine	483-18-1	0.08	0.00016	67.3	0.14	NA	NA	480.71	0.14
223	32		Lindane	58-89-9	119.24	0.41	75.6	0.26	87.2	0.3	290.82	0.26
255			Sodium monochloroacetate	3926-62-3	168.90	1.45	75.7	0.65	NA	NA	116.48	0.65
190			Chlorambucil	305-03-3	23.12	0.076	76.1	0.25	100.4	0.33	304.24	0.25
149			Chromium VI trioxide	1333-82-0	0.27	0.0027	80.0	0.8	127.0	1.27	100	0.8
180		p-	Phenylenediamine	106-50-3	5.41	0.05	80.0	0.74	NA	NA	108.16	0.74
62			Cobalt II chloride	7646-79-9	20.77	0.16	80.5	0.62	80.5	0.62	129.83	0.62

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
110			Acrylonitrile	107-13-1	128.43	2.42	81.7	1.54	27.1	0.51	53.07	1.54
237			Beryllium II sulfate	13510-49-1	64.09	0.61	82.0	0.78	79.9	0.76	105.07	0.78
220		m-	Dinitrobenzene	99-65-0	65.57	0.39	82.4	0.49	NA	NA	168.12	0.49
229	22		Dextropropoxyphene * HCl	1639-60-7	184.23	0.49	82.7	0.22	82.7	0.22	375.98	0.22
20			Cadmium II chloride	10108-64-2	1.17	0.0064	88.0	0.48	174.1	0.95	183.3	0.48
219			Hydralazine	86-54-4	52.87	0.33	89.7	0.56	121.8	0.76	160.2	0.56
160		N-	Methyl-N'-nitro-N-nitroso- guanidine	70-25-7	1.77	0.012	89.7	0.61	NA	NA	147.12	0.61
283			Milrinone	78415-72-2	1007.61	4.77	90.8	0.43	137.3	0.65	211.24	0.43
116			Cyclophosphamide * H2O	6055-19-2	870.89	3.12	94.9	0.34	136.8	0.49	279.13	0.34
217			Amrinone	60719-84-8	52.42	0.28	101.1	0.54	288.3	1.54	187.22	0.54
194		p-	Toluylendiamine	95-70-5	11.49	0.094	101.4	0.83	NA	NA	122.19	0.83
74			Nickel II chloride	7718-54-9	34.99	0.27	105.0	0.81	NA	NA	129.61	0.81
281		1,2-	Dibromomethane	106-93-4	730.17	4.2	107.8	0.62	NA	NA	173.85	0.62
196	40		VerapamilHCl	152-11-4	49.11	0.1	108.0	0.22	162.1	0.33	491.13	0.22
198			Ioxynil	1689-83-4	40.80	0.11	111.3	0.3	NA	NA	370.91	0.3
151			Hexachlorocyclopentadiene	77-47-4	0.85	0.0031	111.8	0.41	NA	NA	272.75	0.41
167		p,p'	DDD	72-54-8	7.68	0.024	112.0	0.35	NA	NA	320.04	0.35
61		p,p'	DDT	50-29-3	56.72	0.16	113.4	0.32	134.7	0.38	354.48	0.32
138			Tributyltin chloride	1461-22-9	0.18	0.00054	120.4	0.37	NA	NA	325.53	0.37
232		o-	Cresol	95-48-7	56.24	0.52	121.1	1.12	343.9	3.18	108.15	1.12
347			Thiourea	62-56-6	6547.18	86	124.9	1.64	8526.6	112	76.13	1.64
69			Secobarbital sodium	309-43-3	54.66	0.21	124.9	0.48	NA	NA	260.3	0.48
134			Rotenone	83-79-4	0.05	0.00013	130.2	0.33	351.1	0.89	394.45	0.33
9			Amethopterin	59-05-2	0.06	0.00014	136.4	0.3	145.4	0.32	454.5	0.3
199			Cupric chloride	7447-39-4	14.79	0.11	139.8	1.04	189.6	1.41	134.44	1.04
27			Chlorpromazine	50-53-3	4.46	0.014	140.3	0.44	261.5	0.82	318.89	0.44
96			Cygon	60-51-5	284.29	1.24	151.3	0.66	59.6	0.26	229.27	0.66
227	46		Sodium oxalate	62-76-0	58.96	0.44	155.4	1.16	NA	NA	134	1.16
118	24		Phenobarbital	50-06-6	884.91	3.81	162.6	0.7	167.2	0.72	232.26	0.7
225			Ammonium sulfide	12135-76-1	21.47	0.42	168.2	3.29	NA	NA	51.12	3.29
16			Azaserine	115-02-6	0.35	0.002	169.7	0.98	150.6	0.87	173.15	0.98
102			Acrylamide	79-06-1	114.45	1.61	169.9	2.39	169.9	2.39	71.09	2.39
64			Bendiocarb	22781-23-3	40.19	0.18	178.6	0.8	NA	NA	223.25	0.8
106	14		Sodium I fluoride	7681-49-4	77.68	1.85	180.1	4.29	NA	NA	41.99	4.29
112	48		Caffeine	58-08-2	512.74	2.64	192.3	0.99	619.6	3.19	194.22	0.99
253			Isoxepac	55453-87-7	356.81	1.33	198.5	0.74	NA	NA	268.28	0.74
318			Trifluoroacetic acid	76-05-1	2337.62	20.5	199.6	1.75	NA	NA	114.03	1.75
87			Pentobarbital sodium	57-33-0	176.29	0.71	201.1	0.81	280.6	1.13	248.29	0.81
212		p-	Cresol	106-44-5	23.79	0.22	206.6	1.91	343.9	3.18	108.15	1.91

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
17		5-	Fluorouracil	51-21-8	0.34	0.0026	230.3	1.77	114.5	0.88	130.09	1.77
206			Diquat dibromide	85-00-7	55.05	0.16	230.5	0.67	234.0	0.68	344.08	0.67
239		m-	Cresol	108-39-4	71.38	0.66	242.3	2.24	828.4	7.66	108.15	2.24
269			Potassium I fluoride	7789-23-3	181.85	3.13	245.2	4.22	NA	NA	58.1	4.22
73			Carbaryl	63-25-2	52.32	0.26	249.5	1.24	438.7	2.18	201.24	1.24
35			Flufenamic acid	530-78-9	8.16	0.029	272.8	0.97	714.4	2.54	281.25	0.97
260			Coumarin	91-64-5	249.92	1.71	292.3	2	195.8	1.34	146.15	2
228		2,4,5-	Trichlorophen- oxyacetic acid	93-76-5	112.41	0.44	298.9	1.17	388.3	1.52	255.48	1.17
81	27		Cupric sulfate * 5 H2O	7758-99-8	82.40	0.33	299.6	1.2	NA	NA	249.7	1.2
245			Resorcinol	108-46-3	88.10	0.8	300.6	2.73	NA	NA	110.12	2.73
279			Thioacetamide	62-55-5	313.33	4.17	301.3	4.01	NA	NA	75.14	4.01
38			Imipramine * HCl	113-52-0	17.11	0.054	304.2	0.96	374.0	1.18	316.91	0.96
205			Versalide	88-29-9	38.77	0.15	315.3	1.22	NA	NA	258.44	1.22
261	3		Ferrous sulfate	7720-78-7	281.03	1.85	319.0	2.1	978.3	6.44	151.91	2.1
183	5		Amitriptyline	50-48-6	15.54	0.056	319.1	1.15	147.0	0.53	277.44	1.15
86	31		Warfarin	81-81-2	206.59	0.67	323.8	1.05	373.1	1.21	308.35	1.05
176			Papaverine	58-74-2	15.27	0.045	325.8	0.96	230.8	0.68	339.42	0.96
249		3-	Cyano-2-morpholino-5-(pyrid-4-yl)-pyridine (Chemical 122)		255.66	0.96	346.2	1.3	NA	NA	266.31	1.3
282		(-)-	Phenylephrine	59-42-7	744.17	4.45	349.5	2.09	NA	NA	167.23	2.09
55			Zinc II chloride	7646-85-7	17.72	0.13	350.2	2.57	350.2	2.57	136.27	2.57
278			Phenylephrine * HCl	939-38-8	847.35	4.16	350.3	1.72	120.2	0.59	203.69	1.72
210		p-	Nitrophenol	100-02-7	27.82	0.2	350.6	2.52	467.4	3.36	139.12	2.52
246	37		Barium II nitrate	10022-31-8	211.70	0.81	355.4	1.36	NA	NA	261.36	1.36
90			Iproniazid	54-92-2	141.61	0.79	365.7	2.04	681.2	3.8	179.25	2.04
254			Buflomedil	55837-25-7	415.03	1.35	365.8	1.19	NA	NA	307.43	1.19
89	16	2,4-	Dichlorophenoxy- acetic acid	94-75-7	170.20	0.77	369.1	1.67	366.9	1.66	221.04	1.67
79			Phenylbutazone	50-33-9	98.69	0.32	376.3	1.22	441.0	1.43	308.41	1.22
172			Nabam	142-59-6	8.97	0.035	394.8	1.54	579.3	2.26	256.34	1.54
155			Benzalkonium chloride	8001-54-5	1.90	0.0052	401.5	1.1	339.5	0.93	365	1.1
159			Hexadecyltrimethylammoniumbromide	57-09-0	3.24	0.0089	408.3	1.12	NA	NA	364.53	1.12
115	12		Phenol	108-95-2	283.30	3.01	414.1	4.4	300.2	3.19	94.12	4.4
230	42		Orphenadrine * HCl	341-69-5	149.88	0.49	425.2	1.39	125.4	0.41	305.88	1.39
291			Aniline	62-53-3	642.67	6.9	439.6	4.72	439.6	4.72	93.14	4.72
75			Trichlorfon	52-68-6	69.51	0.27	450.5	1.75	298.6	1.16	257.44	1.75
339		1-	Nitropropane	79-46-9	5159.47	57.9	455.4	5.11	NA	NA	89.11	5.11
53	43		Quinidine sulfate	50-54-4	50.70	0.12	456.3	1.08	595.8	1.41	422.54	1.08
40			Chlordan	57-74-9	24.59	0.06	458.9	1.12	NA	NA	409.76	1.12
163			Cetyltrimethylammonium chloride	112-02-7	7.61	0.021	474.4	1.31	NA	NA	362.16	1.31
264			Chloral hydrate	302-17-0	438.31	2.65	479.7	2.9	1101.6	6.66	165.4	2.9

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
204			Azathioprine	446-86-6	38.82	0.14	535.2	1.93	1389.2	5.01	277.29	1.93
310			Tributylamine	102-82-9	2855.16	15.4	539.5	2.91	NA	NA	185.4	2.91
187		4-	Hexylresorcinol	136-77-6	12.44	0.064	549.9	2.83	NA	NA	194.3	2.83
26			Kelthane	115-32-2	4.45	0.012	574.2	1.55	418.6	1.13	370.48	1.55
39		2,4-	Dichlorophenol	120-83-2	8.97	0.055	580.3	3.56	1600.7	9.82	163	3.56
280			Theophylline sodium acetate	8002-89-9	1098.74	4.19	582.2	2.22	NA	NA	262.23	2.22
147			Mitoxantrone	65271-80-9	1.07	0.0024	586.8	1.32	NA	NA	444.54	1.32
101			Glutethimide	77-21-4	338.97	1.56	599.7	2.76	360.7	1.66	217.29	2.76
45			Quinine * HCl	130-89-2	27.07	0.075	620.8	1.72	1158.6	3.21	360.92	1.72
70	49		Atropine sulfate	55-48-1	148.92	0.22	622.7	0.92	764.9	1.13	676.9	0.92
302			Nitrobenzene	98-95-3	1502.06	12.2	640.2	5.2	NA	NA	123.12	5.2
123	35		Isoniazid	54-85-3	1027.33	7.49	650.1	4.74	NA	NA	137.16	4.74
256			Tin II chloride	7772-99-8	286.28	1.51	699.6	3.69	1200.1	6.33	189.59	3.69
63	4		Diazepam	439-14-5	45.56	0.16	709.1	2.49	535.3	1.88	284.76	2.49
168			Dicoumarol	66-76-2	9.08	0.027	709.6	2.11	232.1	0.69	336.31	2.11
311		1-	Hexanol	111-27-3	1573.88	15.4	719.5	7.04	1952.0	19.1	102.2	7.04
174			Ambazone	539-21-9	9.02	0.038	749.9	3.16	999.1	4.21	237.32	3.16
242		1,2,4-	Trichlorobenzene	120-82-1	128.82	0.71	756.6	4.17	765.7	4.22	181.44	4.17
333			Lithium I chloride	7447-41-8	1636.25	38.6	758.8	17.9	1165.7	27.5	42.39	17.9
114			Natulan * HCl	366-70-1	706.37	2.74	783.7	3.04	NA	NA	257.8	3.04
48			Mefenamic acid	61-68-7	20.99	0.087	789.1	3.27	629.8	2.61	241.31	3.27
338		1-	Butanol	71-36-3	3892.35	52.5	793.3	10.7	NA	NA	74.14	10.7
202			Formaldehyde	50-00-0	3.60	0.12	798.8	26.6	NA	NA	30.03	26.6
188		t-	Butyl hydroquinone	1948-33-0	11.47	0.069	799.6	4.81	1000.8	6.02	166.24	4.81
354		1,3,5-	Trioxane	110-88-3	19189.17	213	800.0	8.88	NA	NA	90.09	8.88
58			Dihydralazine sulfate	7327-87-9	40.36	0.14	818.8	2.84	400.8	1.39	288.32	2.84
213			Ammonium persulfate	7727-54-0	52.49	0.23	819.3	3.59	NA	NA	228.22	3.59
71			Diphenhydramine * HCl	147-24-0	70.04	0.24	855.1	2.93	113.8	0.39	291.85	2.93
285			Caffeine sodium benzoate	8000-95-1	1918.33	5.67	859.4	2.54	798.5	2.36	338.33	2.54
197		p,p'	DDE	72-55-9	31.80	0.1	880.9	2.77	NA	NA	318.02	2.77
67	15		Malathion	121-75-5	66.08	0.2	885.4	2.68	776.4	2.35	330.38	2.68
259			Methyl salicylate	119-36-8	258.67	1.7	887.1	5.83	NA	NA	152.16	5.83
184			Butylated hydroxytoluene	128-37-0	12.34	0.056	890.4	4.04	1040.2	4.72	220.39	4.04
272			Salicylic acid	69-72-7	466.88	3.38	890.9	6.45	479.3	3.47	138.13	6.45
337			Pyridine	110-86-1	3710.26	46.9	893.9	11.3	NA	NA	79.11	11.3
308	33		Chloroform	67-66-3	1599.56	13.4	908.4	7.61	35.8	0.3	119.37	7.61
44			Hydroxyzine * HCl	1244-76-4	27.56	0.067	950.4	2.31	NA	NA	411.41	2.31
31	41		Chloroquine diphosphate	50-63-5	8.77	0.017	969.9	1.88	500.4	0.97	515.92	1.88
214			Thymol	89-83-8	34.56	0.23	979.6	6.52	1802.9	12	150.24	6.52

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
65			Oxyphenbutazone	129-20-4	61.64	0.19	999.2	3.08	480.1	1.48	324.41	3.08
121			Aminophenazone	58-15-1	1246.87	5.39	999.3	4.32	358.6	1.55	231.33	4.32
80		2-	Thiouracil	141-90-2	41.01	0.32	999.6	7.8	NA	NA	128.16	7.8
304			Calcium II chloride	10043-52-4	1376.15	12.4	999.9	9.01	NA	NA	110.98	9.01
107	2		Acetylsalicylic acid	50-78-2	408.99	2.27	999.9	5.55	814.4	4.52	180.17	5.55
233			Ibuprofen	15687-27-1	107.28	0.52	1008.9	4.89	980.0	4.75	206.31	4.89
47			Naftipramide	1505-95-9	25.07	0.084	1029.7	3.45	1086.4	3.64	298.47	3.45
218		o-	Phenylenediamine	95-54-5	33.53	0.31	1069.7	9.89	NA	NA	108.16	9.89
41			Chloroquine sulfate	132-73-0	25.08	0.06	1086.8	2.6	NA	NA	418	2.6
296			Homatropine methylbromide	80-49-9	3332.97	9	1199.9	3.24	1399.8	3.78	370.33	3.24
152		8-	Hydroxyquinoline	148-24-3	0.48	0.0033	1200.6	8.27	NA	NA	145.17	8.27
287			Benzylalcohol	100-51-6	628.35	5.81	1232.9	11.4	1579.0	14.6	108.15	11.4
111			Clofibric acid	882-09-7	560.26	2.61	1249.3	5.82	1169.9	5.45	214.66	5.82
226			Dodecylbenzene sodiumsulfonate	25155-30-0	146.38	0.42	1261.6	3.62	2000.5	5.74	348.52	3.62
251			Scopolamine * HBr	6533-68-2	415.05	1.08	1268.2	3.3	1879.3	4.89	384.31	3.3
76			Sodium dodecyl sulfate	151-21-3	78.15	0.27	1288.0	4.45	NA	NA	289.43	4.45
191			Dimenhydrinate	523-87-5	35.72	0.076	1320.8	2.81	202.1	0.43	470.02	2.81
99			Nalidixic acid	389-08-2	348.39	1.5	1349.4	5.81	571.4	2.46	232.26	5.81
243		p-	Anisidine	104-94-9	89.91	0.73	1404.1	11.4	NA	NA	123.17	11.4
270			Propionaldehyde	123-38-6	188.79	3.25	1411.6	24.3	NA	NA	58.09	24.3
164			Oxatomide	60607-34-3	8.11	0.019	1412.1	3.31	9598.7	22.5	426.61	3.31
275			Nitrilotriacetic acid	139-13-9	690.09	3.61	1470.0	7.69	3154.1	16.5	191.16	7.69
324		2-	Butoxyethanol	111-76-2	3073.20	26	1477.5	12.5	1229.3	10.4	118.2	12.5
56			Manganese IIchloride *4 H2O	13446-34-9	25.73	0.13	1484.4	7.5	NA	NA	197.92	7.5
136			Diethyldithiocarbamate sodium* 3H20	20624-25-3	0.09	0.00039	1500.7	6.66	1500.7	6.66	225.33	6.66
328	36		Dichloromethane	75-09-2	2964.06	34.9	1596.7	18.8	NA	NA	84.93	18.8
119			Sodium salicylate	54-21-7	693.28	4.33	1599.5	9.99	899.8	5.62	160.11	9.99
166			Triisooctylamine	2757-28-0	8.14	0.023	1620.2	4.58	NA	NA	353.76	4.58
284			Ammonium chloride	12125-02-9	295.32	5.52	1647.8	30.8	NA	NA	53.5	30.8
97			Phenacetin	62-44-2	227.63	1.27	1650.8	9.21	1220.6	6.81	179.24	9.21
248		m-	Aminophenol	591-27-5	93.86	0.86	1658.9	15.2	NA	NA	109.14	15.2
42		p-	Aminophenol	23-30-8	6.77	0.062	1658.9	15.2	NA	NA	109.14	15.2
78		6-	Methylcoumarin	92-48-8	49.66	0.31	1681.9	10.5	NA	NA	160.18	10.5
200			Dimethylaminoethyl methacrylate (polymer)	2867-47-2	17.30	0.11	1745.4	11.1	NA	NA	157.24	11.1
57		L-	Dopa	59-92-7	25.64	0.13	1780.8	9.03	2366.5	12	197.21	9.03
182			Triton X-100	9002-93-1	35.59	0.055	1798.7	2.78	NA	NA	647	2.78
300			Antipyrine	60-80-0	2183.70	11.6	1799.7	9.56	1699.9	9.03	188.25	9.56
95			Salicylamide	65-45-2	148.12	1.08	1892.7	13.8	1398.9	10.2	137.15	13.8
342			Piperazine	110-85-0	5789.95	67.2	1904.1	22.1	1438.9	16.7	86.16	22.1

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
263			Acetaldehyde	75-07-0	107.95	2.45	1929.8	43.8	NA	NA	44.06	43.8
139			Retinol	68-26-8	0.15	0.00054	1999.8	6.98	4011.0	14	286.5	6.98
52			all-trans-Retinoic acid	302-79-4	33.05	0.11	2001.2	6.66	NA	NA	300.48	6.66
325			Cyclohexanol	108-93-0	2634.73	26.3	2063.7	20.6	NA	NA	100.18	20.6
330			Sulfuric acid	7664-93-9	3530.88	36	2138.1	21.8	NA	NA	98.08	21.8
72			Butylated hydoxyanisole	8003-24-5	43.26	0.24	2199.3	12.2	2001.0	11.1	180.27	12.2
165			Isoproterenol * HCl	51-30-9	5.45	0.022	2219.8	8.96	NA	NA	247.75	8.96
331			Strontium II chloride	10476-85-4	5770.13	36.4	2251.0	14.2	3107.0	19.6	158.52	14.2
113	1		Acetaminophen	103-90-2	409.70	2.71	2403.8	15.9	338.6	2.24	151.18	15.9
356		2-	Methoxyethanol	109-86-4	19103.61	251	2458.4	32.3	NA	NA	76.11	32.3
334			Isobutanol	78-83-1	2973.01	40.1	2461.4	33.2	NA	NA	74.14	33.2
305		n-	Butanal	123-72-8	923.14	12.8	2488.1	34.5	NA	NA	72.12	34.5
350			Tetrahydrofurfuryl alcohol	97-99-4	11338.65	111	2502.7	24.5	2298.4	22.5	102.15	24.5
208			Undecylenic acid	112-38-9	33.18	0.18	2506.6	13.6	8496.7	46.1	184.31	13.6
312			Benzoic acid	65-85-0	1917.44	15.7	2528.1	20.7	2369.3	19.4	122.13	20.7
238			Imidazolidinyl urea	39236-46-9	100.17	0.36	2598.9	9.34	3700.9	13.3	278.26	9.34
109			Frusemide	54-31-9	770.67	2.33	2599.8	7.86	4597.6	13.9	330.76	7.86
346	50		Potassium I chloride	7447-40-7	6113.10	82	2601.8	34.9	1498.5	20.1	74.55	34.9
158			Dichlorophene	97-23-4	2.23	0.0083	2691.3	10	1001.2	3.72	269.13	10
273			Bromobenzene	108-86-1	543.29	3.46	2700.7	17.2	NA	NA	157.02	17.2
295		2,5-	Hexanedione	110-13-4	964.65	8.45	2705.6	23.7	NA	NA	114.16	23.7
125	34		Carbon tetrachloride	56-23-5	1308.92	8.51	2799.3	18.2	12797.0	83.2	153.81	18.2
351			Dimethylformamide	68-12-2	8334.54	114	2800.1	38.3	3750.5	51.3	73.11	38.3
309			Isobutanal	78-84-2	973.62	13.5	2812.7	39	NA	NA	72.12	39
298			Dichloroacetic acid	79-43-6	1482.81	11.5	2823.8	21.9	5518.6	42.8	128.94	21.9
344	13		Sodium chloride	7647-14-5	4435.60	75.9	2998.0	51.3	3997.3	68.4	58.44	51.3
322		1-	Pentanol	71-41-0	2195.43	24.9	3033.0	34.4	200.1	2.27	88.17	34.4
221		2-	Nitro-p-phenylene-diamine	5307-14-2	59.73	0.39	3078.5	20.1	NA	NA	153.16	20.1
216			Refortan		78.28	0.25	3162.3	10.1	NA	NA	313.1	10.1
94			Menthol	89-78-1	148.49	0.95	3172.9	20.3	NA	NA	156.3	20.3
257			Isononylaldehyde	5435-64-3	216.25	1.52	3243.8	22.8	NA	NA	142.27	22.8
288		1-	Heptanol	111-70-6	726.44	6.25	3254.4	28	1499.4	12.9	116.23	28
321			Acetic acid	64-19-7	1459.46	24.3	3309.3	55.1	4961.0	82.6	60.06	55.1
91	45		Chloramphenicol	56-75-7	255.29	0.79	3393.1	10.5	2640.1	8.17	323.15	10.5
349			Ethyl methyl ketone	78-93-3	7500.48	104	3396.9	47.1	NA	NA	72.12	47.1
345			Sodium I bromide	7647-15-6	8120.81	77.4	3504.3	33.4	6998.2	66.7	104.92	33.4
336			Nicotinamide	98-92-0	5423.02	44.4	3505.4	28.7	NA	NA	122.14	28.7
306			Anisole	100-66-3	1427.58	13.2	3698.7	34.2	NA	NA	108.15	34.2
341			Lactic acid	598-82-3	5945.94	66	3729.7	41.4	4873.9	54.1	90.09	41.4

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
358			Acetonitrile	75-05-8	15110.08	368	3798.1	92.5	NA	NA	41.06	92.5
211			Catechol	120-80-9	22.02	0.2	3887.2	35.3	259.9	2.36	110.12	35.3
307		2-	Ethylbutanal	97-96-1	1322.38	13.2	3977.1	39.7	NA	NA	100.18	39.7
315			Isobenzoic furano dione		2518.04	17	4014.1	27.1	1999.6	13.5	148.12	27.1
332		1,4-	Dioxane	123-91-1	3357.37	38.1	4203.3	47.7	5701.4	64.7	88.12	47.7
301	17		Xylene	1330-20-7	1274.16	12	4300.3	40.5	NA	NA	106.18	40.5
154			Maneb	12427-38-2	1.12	0.0042	4500.6	16.9	3994.7	15	266.31	16.9
294			Trichloroacetic acid	76-03-9	1338.08	8.19	4999.4	30.6	5636.6	34.5	163.38	30.6
271			Styrene	100-42-5	343.73	3.3	4999.7	48	315.6	3.03	104.16	48
316			Toluene	108-88-3	1575.77	17.1	5003.7	54.3	NA	NA	92.15	54.3
320		N,N-	Dimethylacetamide	127-19-5	2108.79	24.2	5089.0	58.4	4618.4	53	87.14	58.4
224		n-	Butyl benzoate	136-60-7	73.08	0.41	5133.6	28.8	NA	NA	178.25	28.8
186			Zineb	12122-67-7	16.27	0.059	5211.3	18.9	7610.1	27.6	275.73	18.9
348		1-	Propanol	71-23-8	5800.62	96.5	5397.9	89.8	NA	NA	60.11	89.8
326			Halothane	151-67-7	6138.83	31.1	5684.8	28.8	NA	NA	197.39	28.8
128	10	2-	Propanol	67-63-0	10038.37	167	5842.7	97.2	NA	NA	60.11	97.2
50			Trypan blue	72-57-1	91.66	0.095	6204.2	6.43	NA	NA	964.88	6.43
108			Gibberellic acid	77-06-5	796.74	2.3	6304.7	18.2	NA	NA	346.41	18.2
335			Potassium hexacyano- ferrate II	13943-58-3	15582.05	42.3	6409.6	17.4	5009.8	13.6	368.37	17.4
59			Tetracycline * HCl	64-75-5	67.33	0.14	6444.6	13.4	NA	NA	480.94	13.4
127			Dimethyl phthalate	131-11-3	4544.28	23.4	6894.1	35.5	7204.8	37.1	194.2	35.5
286			Benzylpenicillin sodium	69-57-8	2042.17	5.73	6914.2	19.4	NA	NA	356.4	19.4
126			Triethyl citrate	77-93-0	4061.90	14.7	6990.9	25.3	NA	NA	276.32	25.3
85			Metamizol	68-89-3	193.94	0.58	7189.2	21.5	NA	NA	334.38	21.5
343			Magnesium II chloride * 6 H2O	7791-18-6	14314.43	70.4	8092.5	39.8	NA	NA	203.33	39.8
360	7		Ethylene glycol	107-21-1	34454.40	555	8567.0	138	7511.7	121	62.08	138
122			Diethyl phthalate	84-66-2	1226.88	5.52	8601.5	38.7	6178.8	27.8	222.26	38.7
289			Tetrachloroethene	127-18-4	1084.46	6.54	8854.8	53.4	8092.0	48.8	165.82	53.4
117			Di(2-ethylhexyl)adipate	103-23-1	1167.52	3.15	9117.7	24.6	NA	NA	370.64	24.6
162			Chlorhexidine	55-56-1	7.58	0.015	9200.5	18.2	9857.6	19.5	505.52	18.2
359			Acetone	67-64-1	25791.96	444	9759.1	168	NA	NA	58.09	168
18			Captan	133-06-2	1.17	0.0039	10009.6	33.3	7003.7	23.3	300.59	33.3
297	11	1,1,1-	Trichloroethane	71-55-6	1374.02	10.3	10298.5	77.2	11245.6	84.3	133.4	77.2
353			Ethyl acetate	141-78-6	11279.36	128	11015.0	125	NA	NA	88.12	125
100		L-	Ascorbic acid	50-81-7	267.73	1.52	11907.1	67.6	3364.3	19.1	176.14	67.6
88			Dibutyl phthalate	84-74-2	211.57	0.76	11998.2	43.1	NA	NA	278.38	43.1
131			Glycerol	56-81-5	57476.64	624	12619.1	137	25975.0	282	92.11	137
361	8		Methanol	67-56-1	29806.50	930	13012.3	406	NA	NA	32.05	406
130	9		Ethanol	64-17-5	17464.32	379	14008.3	304	7787.5	169	46.08	304

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
313			Xanthinol nicotinate	437-74-1	6865.26	15.8	14121.6	32.5	17336.9	39.9	434.51	32.5
258			Diethyl sebacate	110-40-7	421.19	1.63	14470.4	56	NA	NA	258.4	56
340			Diethylene glycol	111-46-6	6591.29	62.1	14753.5	139	23669.2	223	106.14	139
329			Sodium cyclamate	139-05-9	7123.90	35.4	15254.0	75.8	17004.8	84.5	201.24	75.8
352		1,2,6-	Hexanetriol	106-69-4	16506.60	123	15969.8	119	NA	NA	134.2	119
129			Dimethyl sulfoxide	75-18-3	19691.28	252	19691.3	252	16487.5	211	78.14	252
357			Propylene glycol	57-55-6	26029.62	342	20016.9	263	23974.7	315	76.11	263
355			D-Glucose	50-99-7	40720.68	226	25765.7	143	NA	NA	180.18	143
92			Di(2-ethylhexyl)phthalate	117-81-7	328.12	0.84	31015.2	79.4	29999.6	76.8	390.62	79.4
124			Acetazolamide	59-66-5	1886.99	8.49	NA	NA	4289.6	19.3	222.26	19.3
28			Aldosterone	52-39-1	5.05	0.014	NA	NA	NA	NA	360.44	
276			Ambuphylline	5634-34-4	988.51	3.67	NA	NA	600.7	2.23	269.35	2.23
3			Aminopterin	54-62-6	0.01	0.000012	NA	NA	3.0	0.0068	440.47	0.0068
120		5-	Aminosalicylic acid	89-57-6	776.47	5.07	NA	NA	7749.4	50.6	153.15	50.6
84			Amobarbital	57-43-2	126.73	0.56	NA	NA	344.0	1.52	226.31	1.52
189			Antimycin	11118-72-2	17.52	0.07	NA	NA	112.6	0.45	250.27	0.45
193		5-	Azacytidine	320-67-2	19.29	0.079	NA	NA	571.5	2.34	244.24	2.34
15		8-	Azaguanine	134-58-7	0.20	0.0013	NA	NA	1500.1	9.86	152.14	9.86
317			Barbital sodium	144-02-5	3835.32	18.6	NA	NA	800.1	3.88	206.2	3.88
33		p-	Chloromercuribenzoic acid	59-85-8	8.57	0.024	NA	NA	25.0	0.07	357.16	0.07
215			Chlorotetracycline	57-62-5	114.94	0.24	NA	NA	2500.0	5.22	478.92	5.22
77			Cinchophen	132-60-5	67.31	0.27	NA	NA	NA	NA	249.28	
6			Colchicine	64-86-8	0.02	0.000054	NA	NA	6.0	0.015	399.48	0.015
66			Cortisone	53-06-5	68.49	0.19	NA	NA	NA	NA	360.49	
274		L-	Cysteine	52-90-4	431.37	3.56	NA	NA	660.4	5.45	121.17	5.45
19			Cytochalasin B	14930-96-2	2.40	0.005	NA	NA	NA	NA	479.67	
133			Cytochalasin D	22144-77-0	0.05	0.000092	NA	NA	36.0	0.071	507.68	0.071
141			Cytosine arabinoside	147-94-4	0.17	0.00068	NA	NA	3137.9	12.9	243.25	12.9
23			Daraprim	58-14-0	2.21	0.0089	NA	NA	126.9	0.51	248.74	0.51
195		p,p'	DDA	83-05-6	27.83	0.099	NA	NA	590.4	2.1	281.14	2.1
34			Diethylstilbestrol	56-53-1	6.71	0.025	NA	NA	NA	NA	268.38	
22	6		Digoxin	20830-75-5	6.64	0.0085	NA	NA	18.0	0.023	781.05	0.023
293			Diisopropylamine dichloroacetate	660-27-5	1611.12	7	NA	NA	1700.9	7.39	230.16	7.39
82	44		Diphenylhydantoin	57-41-0	98.39	0.39	NA	NA	199.3	0.79	252.29	0.79
11			Doxorubicin * HCl	25316-40-9	0.19	0.00033	NA	NA	696.0	1.2	580.03	1.2
244			Doxylamine succinate	562-10-7	291.38	0.75	NA	NA	470.1	1.21	388.51	1.21
169			Epinephrine bitartrate	51-42-3	9.33	0.028	NA	NA	4.0	0.012	333.33	0.012
24			Ethylenediamine-tetraacetic acid	60-00-4	2.92	0.01	NA	NA	NA	NA	292.28	
171			Fumagillin	297-95-0	14.22	0.031	NA	NA	1999.5	4.36	458.6	4.36

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
222			Glibenclamide	10238-21-8	197.62	0.4	NA	NA	3250.8	6.58	494.05	6.58
32			Hydrocortisone	50-23-7	7.98	0.022	NA	NA	NA	NA	362.51	
236			Hydrogen peroxide 90%	7722-84-1	19.05	0.56	NA	NA	2000.4	58.8	34.02	58.8
267		p-	Hydroxybenzoic acid	99-96-7	403.34	2.92	NA	NA	2196.3	15.9	138.13	15.9
299			Imidazole	288-32-4	783.04	11.5	NA	NA	1879.3	27.6	68.09	27.6
46			Lead II chloride	7758-95-4	11.96	0.043	NA	NA	NA	NA	278.09	
327	20		Lithium I sulfate	10377-48-7	3704.98	33.7	NA	NA	1187.4	10.8	109.94	10.8
21		6-	Mercaptopurine	50-44-2	1.22	0.008	NA	NA	280.0	1.84	152.19	1.84
142			Methylmercury chloride	115-09-3	0.18	0.00071	NA	NA	57.7	0.23	251.08	0.23
98			Methylparaben	99-76-3	216.07	1.42	NA	NA	1749.8	11.5	152.16	11.5
319			Methylpentinol	77-75-8	2336.21	23.8	NA	NA	525.2	5.35	98.16	5.35
175			Norepinephrine	51-41-2	6.60	0.039	NA	NA	20.3	0.12	169.2	0.12
268		1-	Octanol	111-87-5	398.60	3.06	NA	NA	1784.6	13.7	130.26	13.7
7			Ouabain	630-60-4	0.04	0.000072	NA	NA	NA	NA	584.73	
240			Pentoxifylline	6493-05-6	183.71	0.66	NA	NA	1386.2	4.98	278.35	4.98
146			Potassium bichromate VI	7778-50-9	0.59	0.002	NA	NA	191.2	0.65	294.2	0.65
145			Potassium chromate VI	7789-00-6	0.29	0.0015	NA	NA	180.6	0.93	194.2	0.93
277			Potassium cyanate	590-28-3	335.84	4.14	NA	NA	843.6	10.4	81.12	10.4
266			Potassium hexacyanoferrate III	13746-66-2	928.54	2.82	NA	NA	2970.0	9.02	329.27	9.02
36			Progesterone	57-83-0	9.44	0.03	NA	NA	NA	NA	314.51	
54	23		Propranolol * HCl	318-98-9	35.50	0.12	NA	NA	470.4	1.59	295.84	1.59
209			Propylparaben	94-13-3	32.44	0.18	NA	NA	6325.7	35.1	180.22	35.1
12			Puromycin	53-79-2	0.16	0.00033	NA	NA	674.4	1.43	471.58	1.43
201		13-cis-	Retinoic acid	4759-48-2	36.06	0.12	NA	NA	3395.4	11.3	300.48	11.3
314			Saccharin	81-07-2	3004.32	16.4	NA	NA	17000.0	92.8	183.19	92.8
178			Salicylanilide	87-17-2	9.81	0.046	NA	NA	2409.7	11.3	213.25	11.3
161			Silver I nitrate	7761-88-8	2.21	0.013	NA	NA	49.3	0.29	169.88	0.29
30			Sodium arsenate, dibasic	7778-43-0	2.79	0.015	NA	NA	NA	NA	185.91	
290			Sodium sulfite	7757-83-7	854.55	6.78	NA	NA	820.5	6.51	126.04	6.51
156			Stearyltrimethylammoniumchloride	112-03-8	2.09	0.006	NA	NA	536.1	1.54	348.13	1.54
265			Streptomycin sulfate	298-39-5	3979.25	2.73	NA	NA	495.6	0.34	1457.6	0.34
5		K-	Strophantin		0.03	0.000044	NA	NA	NA	NA	710.9	
93			Sulfisoxazole	127-69-5	227.23	0.85	NA	NA	6790.2	25.4	267.33	25.4
135		2,3,7,8-	Tetrachloro-dibenzo-p-dioxin	1746-01-6	0.06	0.0002	NA	NA	0.1	0.00035	321.96	0.00035
247		(+)-	Thalidomide	731-40-8	209.18	0.81	NA	NA	400.3	1.55	258.25	1.55
203			Thallium I acetate	563-68-8	36.88	0.14	NA	NA	34.2	0.13	263.42	0.13
181	30		Thallium I sulfate	7446-18-6	27.26	0.054	NA	NA	28.8	0.057	504.8	0.057
105	21		Theophylline	58-55-9	329.75	1.83	NA	NA	600.0	3.33	180.19	3.33
303			Theophylline sodium	3485-82-3	2519.43	12.4	NA	NA	445.0	2.19	203.18	2.19

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
25			Thio-TEPA	52-24-4	2.08	0.011	NA	NA	37.8	0.2	189.24	0.2
140		6-	Thioguanine	154-42-7	0.10	0.00057	NA	NA	160.5	0.96	167.21	0.96
83			Thiopental	76-75-5	133.30	0.55	NA	NA	601.1	2.48	242.37	2.48
170	29		Thioridazine * HCl	130-61-0	11.81	0.029	NA	NA	358.2	0.88	407.07	0.88
104			Tolbutamide	64-77-7	489.39	1.81	NA	NA	2601.1	9.62	270.38	9.62
1			Trenimon	68-76-8	0.00	0.0000033	NA	NA	NA	NA	231.28	
231			Tween 80	9005-65-6	641.90	0.49	NA	NA	25021.0	19.1	1310	19.1
323			Urethan	51-79-6	2307.95	25.9	NA	NA	2504.0	28.1	89.11	28.1
250			Valproate sodium	1069-66-5	166.22	1	NA	NA	1695.4	10.2	166.22	10.2
4			Vincristine sulfate	2068-78-2	0.01	0.000015	NA	NA	NA	NA	923.14	

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
263			Acetaldehyde	75-07-0	107.95	2.45	1929.8	43.8	NA	NA	44.06	43.8
113	1		Acetaminophen	103-90-2	409.70	2.71	2403.8	15.9	338.6	2.24	151.18	15.9
124			Acetazolamide	59-66-5	1886.99	8.49	NA	NA	4289.6	19.3	222.26	19.3
321			Acetic acid	64-19-7	1459.46	24.3	3309.3	55.1	4961.0	82.6	60.06	55.1
359			Acetone	67-64-1	25791.96	444	9759.1	168	NA	NA	58.09	168
358			Acetonitrile	75-05-8	15110.08	368	3798.1	92.5	NA	NA	41.06	92.5
107	2		Acetylsalicylic acid	50-78-2	408.99	2.27	999.9	5.55	814.4	4.52	180.17	5.55
179			Acrolein	107-02-8	2.64	0.047	46.0	0.82	39.8	0.71	56.07	0.82
102			Acrylamide	79-06-1	114.45	1.61	169.9	2.39	169.9	2.39	71.09	2.39
110			Acrylonitrile	107-13-1	128.43	2.42	81.7	1.54	27.1	0.51	53.07	1.54
2			Actinomycin D	50-76-0	0.01	0.0000081	7.2	0.0057	12.6	0.01	1255.6	0.0057
37			Aflatoxin B1	1162-65-8	10.62	0.034	5.0	0.016	9.1	0.029	312.29	0.016
28			Aldosterone	52-39-1	5.05	0.014	NA	NA	NA	NA	360.44	
43			Aldrin	309-00-2	24.45	0.067	40.1	0.11	43.8	0.12	364.9	0.11
52			all-trans-Retinoic acid	302-79-4	33.05	0.11	2001.2	6.66	NA	NA	300.48	6.66
292			Allylalcohol	107-18-6	403.14	6.94	63.9	1.1	95.8	1.65	58.09	1.1
174			Ambazone	539-21-9	9.02	0.038	749.9	3.16	999.1	4.21	237.32	3.16
276			Ambuphylline	5634-34-4	988.51	3.67	NA	NA	600.7	2.23	269.35	2.23
9			Amethopterin	59-05-2	0.06	0.00014	136.4	0.3	145.4	0.32	454.5	0.3
121			Aminophenazone	58-15-1	1246.87	5.39	999.3	4.32	358.6	1.55	231.33	4.32
248		m-	Aminophenol	591-27-5	93.86	0.86	1658.9	15.2	NA	NA	109.14	15.2
42		p-	Aminophenol	23-30-8	6.77	0.062	1658.9	15.2	NA	NA	109.14	15.2
3			Aminopterin	54-62-6	0.01	0.000012	NA	NA	3.0	0.0068	440.47	0.0068
120		5-	Aminosalicylic acid	89-57-6	776.47	5.07	NA	NA	7749.4	50.6	153.15	50.6
183	5		Amitriptyline	50-48-6	15.54	0.056	319.1	1.15	147.0	0.53	277.44	1.15
284			Ammonium chloride	12125-02-9	295.32	5.52	1647.8	30.8	NA	NA	53.5	30.8
213			Ammonium persulfate	7727-54-0	52.49	0.23	819.3	3.59	NA	NA	228.22	3.59
225			Ammonium sulfide	12135-76-1	21.47	0.42	168.2	3.29	NA	NA	51.12	3.29
84			Amobarbital	57-43-2	126.73	0.56	NA	NA	344.0	1.52	226.31	1.52
262	47		Amphetamine sulfate	60-13-9	726.02	1.97	55.3	0.15	24.0	0.065	368.54	0.15
217			Amrinone	60719-84-8	52.42	0.28	101.1	0.54	288.3	1.54	187.22	0.54
291			Aniline	62-53-3	642.67	6.9	439.6	4.72	439.6	4.72	93.14	4.72
243		p-	Anisidine	104-94-9	89.91	0.73	1404.1	11.4	NA	NA	123.17	11.4
306			Anisole	100-66-3	1427.58	13.2	3698.7	34.2	NA	NA	108.15	34.2
189			Antimycin	11118-72-2	17.52	0.07	NA	NA	112.6	0.45	250.27	0.45
300			Antipyrine	60-80-0	2183.70	11.6	1799.7	9.56	1699.9	9.03	188.25	9.56
153	26		Arsenic III trioxide	1327-53-3	0.83	0.0042	19.8	0.1	45.5	0.23	197.84	0.1
100		L-	Ascorbic acid	50-81-7	267.73	1.52	11907.1	67.6	3364.3	19.1	176.14	67.6
70	49		Atropine sulfate	55-48-1	148.92	0.22	622.7	0.92	764.9	1.13	676.9	0.92

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
193		5-	Azacytidine	320-67-2	19.29	0.079	NA	NA	571.5	2.34	244.24	2.34
15		8-	Azaguanine	134-58-7	0.20	0.0013	NA	NA	1500.1	9.86	152.14	9.86
16			Azaserine	115-02-6	0.35	0.002	169.7	0.98	150.6	0.87	173.15	0.98
204			Azathioprine	446-86-6	38.82	0.14	535.2	1.93	1389.2	5.01	277.29	1.93
317			Barbital sodium	144-02-5	3835.32	18.6	NA	NA	800.1	3.88	206.2	3.88
246	37		Barium II nitrate	10022-31-8	211.70	0.81	355.4	1.36	NA	NA	261.36	1.36
64			Bendiocarb	22781-23-3	40.19	0.18	178.6	0.8	NA	NA	223.25	0.8
155			Benzalkonium chloride	8001-54-5	1.90	0.0052	401.5	1.1	339.5	0.93	365	1.1
312			Benzoic acid	65-85-0	1917.44	15.7	2528.1	20.7	2369.3	19.4	122.13	20.7
287			Benzylalcohol	100-51-6	628.35	5.81	1232.9	11.4	1579.0	14.6	108.15	11.4
286			Benzylpenicillin sodium	69-57-8	2042.17	5.73	6914.2	19.4	NA	NA	356.4	19.4
237			Beryllium II sulfate	13510-49-1	64.09	0.61	82.0	0.78	79.9	0.76	105.07	0.78
192		1,3-	Bis(2-chloroethyl)- 1-nitrosourea	154-93-8	16.70	0.078	19.9	0.093	19.1	0.089	214.07	0.093
273			Bromobenzene	108-86-1	543.29	3.46	2700.7	17.2	NA	NA	157.02	17.2
254			Buflomedil	55837-25-7	415.03	1.35	365.8	1.19	NA	NA	307.43	1.19
177			Busulphan	55-98-1	11.33	0.046	1.9	0.0076	199.5	0.81	246.32	0.0076
305		n-	Butanal	123-72-8	923.14	12.8	2488.1	34.5	NA	NA	72.12	34.5
338		1-	Butanol	71-36-3	3892.35	52.5	793.3	10.7	NA	NA	74.14	10.7
324		2-	Butoxyethanol	111-76-2	3073.20	26	1477.5	12.5	1229.3	10.4	118.2	12.5
224		n-	Butyl benzoate	136-60-7	73.08	0.41	5133.6	28.8	NA	NA	178.25	28.8
188		t-	Butyl hydroquinone	1948-33-0	11.47	0.069	799.6	4.81	1000.8	6.02	166.24	4.81
72			Butylated hydoxyanisole	8003-24-5	43.26	0.24	2199.3	12.2	2001.0	11.1	180.27	12.2
184			Butylated hydroxytoluene	128-37-0	12.34	0.056	890.4	4.04	1040.2	4.72	220.39	4.04
20			Cadmium II chloride	10108-64-2	1.17	0.0064	88.0	0.48	174.1	0.95	183.3	0.48
112	48		Caffeine	58-08-2	512.74	2.64	192.3	0.99	619.6	3.19	194.22	0.99
285			Caffeine sodium benzoate	8000-95-1	1918.33	5.67	859.4	2.54	798.5	2.36	338.33	2.54
304			Calcium II chloride	10043-52-4	1376.15	12.4	999.9	9.01	NA	NA	110.98	9.01
18			Captan	133-06-2	1.17	0.0039	10009.6	33.3	7003.7	23.3	300.59	33.3
73			Carbaryl	63-25-2	52.32	0.26	249.5	1.24	438.7	2.18	201.24	1.24
125	34		Carbon tetrachloride	56-23-5	1308.92	8.51	2799.3	18.2	12797.0	83.2	153.81	18.2
211			Catechol	120-80-9	22.02	0.2	3887.2	35.3	259.9	2.36	110.12	35.3
163			Cetyltrimethylammonium chloride	112-02-7	7.61	0.021	474.4	1.31	NA	NA	362.16	1.31
264			Chloral hydrate	302-17-0	438.31	2.65	479.7	2.9	1101.6	6.66	165.4	2.9
190			Chlorambucil	305-03-3	23.12	0.076	76.1	0.25	100.4	0.33	304.24	0.25
91	45		Chloramphenicol	56-75-7	255.29	0.79	3393.1	10.5	2640.1	8.17	323.15	10.5
40			Chlordan	57-74-9	24.59	0.06	458.9	1.12	NA	NA	409.76	1.12
162			Chlorhexidine	55-56-1	7.58	0.015	9200.5	18.2	9857.6	19.5	505.52	18.2
308	33		Chloroform	67-66-3	1599.56	13.4	908.4	7.61	35.8	0.3	119.37	7.61
33		p-	Chloromercuribenzoic acid	59-85-8	8.57	0.024	NA	NA	25.0	0.07	357.16	0.07

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
31	41		Chloroquine diphosphate	50-63-5	8.77	0.017	969.9	1.88	500.4	0.97	515.92	1.88
41			Chloroquine sulfate	132-73-0	25.08	0.06	1086.8	2.6	NA	NA	418	2.6
215			Chlorotetracycline	57-62-5	114.94	0.24	NA	NA	2500.0	5.22	478.92	5.22
27			Chlorpromazine	50-53-3	4.46	0.014	140.3	0.44	261.5	0.82	318.89	0.44
149			Chromium VI trioxide	1333-82-0	0.27	0.0027	80.0	0.8	127.0	1.27	100	0.8
77			Cinchophen	132-60-5	67.31	0.27	NA	NA	NA	NA	249.28	
150			Cis-platinum	15663-27-1	0.84	0.0028	25.8	0.086	33.0	0.11	300.07	0.086
111			Clofibric acid	882-09-7	560.26	2.61	1249.3	5.82	1169.9	5.45	214.66	5.82
62			Cobalt II chloride	7646-79-9	20.77	0.16	80.5	0.62	80.5	0.62	129.83	0.62
6			Colchicine	64-86-8	0.02	0.000054	NA	NA	6.0	0.015	399.48	0.015
66			Cortisone	53-06-5	68.49	0.19	NA	NA	NA	NA	360.49	
260			Coumarin	91-64-5	249.92	1.71	292.3	2	195.8	1.34	146.15	2
239		m-	Cresol	108-39-4	71.38	0.66	242.3	2.24	828.4	7.66	108.15	2.24
232		o-	Cresol	95-48-7	56.24	0.52	121.1	1.12	343.9	3.18	108.15	1.12
212		p-	Cresol	106-44-5	23.79	0.22	206.6	1.91	343.9	3.18	108.15	1.91
199			Cupric chloride	7447-39-4	14.79	0.11	139.8	1.04	189.6	1.41	134.44	1.04
81	27		Cupric sulfate * 5 H2O	7758-99-8	82.40	0.33	299.6	1.2	NA	NA	249.7	1.2
249		3-	Cyano-2-morpholino-5-(pyrid-4-yl)-pyridine (Chemical 122)		255.66	0.96	346.2	1.3	NA	NA	266.31	1.3
325			Cyclohexanol	108-93-0	2634.73	26.3	2063.7	20.6	NA	NA	100.18	20.6
13			Cycloheximide	66-81-9	0.17	0.00059	2.0	0.0071	132.3	0.47	281.39	0.0071
116			Cyclophosphamide * H2O	6055-19-2	870.89	3.12	94.9	0.34	136.8	0.49	279.13	0.34
96			Cygon	60-51-5	284.29	1.24	151.3	0.66	59.6	0.26	229.27	0.66
274		L-	Cysteine	52-90-4	431.37	3.56	NA	NA	660.4	5.45	121.17	5.45
19			Cytochalasin B	14930-96-2	2.40	0.005	NA	NA	NA	NA	479.67	
133			Cytochalasin D	22144-77-0	0.05	0.000092	NA	NA	36.0	0.071	507.68	0.071
141			Cytosine arabinoside	147-94-4	0.17	0.00068	NA	NA	3137.9	12.9	243.25	12.9
355			D-Glucose	50-99-7	40720.68	226	25765.7	143	NA	NA	180.18	143
23			Daraprim	58-14-0	2.21	0.0089	NA	NA	126.9	0.51	248.74	0.51
195		p,p'	DDA	83-05-6	27.83	0.099	NA	NA	590.4	2.1	281.14	2.1
167		p,p'	DDD	72-54-8	7.68	0.024	112.0	0.35	NA	NA	320.04	0.35
197		p,p'	DDE	72-55-9	31.80	0.1	880.9	2.77	NA	NA	318.02	2.77
61		p,p'	DDT	50-29-3	56.72	0.16	113.4	0.32	134.7	0.38	354.48	0.32
229	22		Dextropropoxyphene * HCl	1639-60-7	184.23	0.49	82.7	0.22	82.7	0.22	375.98	0.22
117			Di(2-ethylhexyl)adipate	103-23-1	1167.52	3.15	9117.7	24.6	NA	NA	370.64	24.6
92			Di(2-ethylhexyl)phthalate	117-81-7	328.12	0.84	31015.2	79.4	29999.6	76.8	390.62	79.4
63	4		Diazepam	439-14-5	45.56	0.16	709.1	2.49	535.3	1.88	284.76	2.49
281		1,2-	Dibromomethane	106-93-4	730.17	4.2	107.8	0.62	NA	NA	173.85	0.62
88			Dibutyl phthalate	84-74-2	211.57	0.76	11998.2	43.1	NA	NA	278.38	43.1
298			Dichloroacetic acid	79-43-6	1482.81	11.5	2823.8	21.9	5518.6	42.8	128.94	21.9

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
328	36		Dichloromethane	75-09-2	2964.06	34.9	1596.7	18.8	NA	NA	84.93	18.8
158			Dichlorophene	97-23-4	2.23	0.0083	2691.3	10	1001.2	3.72	269.13	10
39		2,4-	Dichlorophenol	120-83-2	8.97	0.055	580.3	3.56	1600.7	9.82	163	3.56
89	16	2,4-	Dichlorophenoxy- acetic acid	94-75-7	170.20	0.77	369.1	1.67	366.9	1.66	221.04	1.67
168			Dicoumarol	66-76-2	9.08	0.027	709.6	2.11	232.1	0.69	336.31	2.11
207			Dieldrin	60-57-1	68.56	0.18	45.7	0.12	38.1	0.1	380.9	0.12
122			Diethyl phthalate	84-66-2	1226.88	5.52	8601.5	38.7	6178.8	27.8	222.26	38.7
258			Diethyl sebacate	110-40-7	421.19	1.63	14470.4	56	NA	NA	258.4	56
136			Diethyldithiocarbamate sodium* 3H20	20624-25-3	0.09	0.00039	1500.7	6.66	1500.7	6.66	225.33	6.66
340			Diethylene glycol	111-46-6	6591.29	62.1	14753.5	139	23669.2	223	106.14	139
34			Diethylstilbestrol	56-53-1	6.71	0.025	NA	NA	NA	NA	268.38	
8			Digitoxin	71-63-6	0.08	0.00011	55.8	0.073	NA	NA	765.05	0.073
22	6		Digoxin	20830-75-5	6.64	0.0085	NA	NA	18.0	0.023	781.05	0.023
58			Dihydralazine sulfate	7327-87-9	40.36	0.14	818.8	2.84	400.8	1.39	288.32	2.84
293			Diisopropylamine dichloroacetate	660-27-5	1611.12	7	NA	NA	1700.9	7.39	230.16	7.39
191			Dimenhydrinate	523-87-5	35.72	0.076	1320.8	2.81	202.1	0.43	470.02	2.81
127			Dimethyl phthalate	131-11-3	4544.28	23.4	6894.1	35.5	7204.8	37.1	194.2	35.5
129			Dimethyl sulfoxide	75-18-3	19691.28	252	19691.3	252	16487.5	211	78.14	252
320		N,N-	Dimethylacetamide	127-19-5	2108.79	24.2	5089.0	58.4	4618.4	53	87.14	58.4
200			Dimethylaminoethyl methacrylate (polymer)	2867-47-2	17.30	0.11	1745.4	11.1	NA	NA	157.24	11.1
351			Dimethylformamide	68-12-2	8334.54	114	2800.1	38.3	3750.5	51.3	73.11	38.3
220		m-	Dinitrobenzene	99-65-0	65.57	0.39	82.4	0.49	NA	NA	168.12	0.49
68		2,4-	Dinitrophenol	51-28-5	38.67	0.21	29.5	0.16	44.2	0.24	184.12	0.16
332		1,4-	Dioxane	123-91-1	3357.37	38.1	4203.3	47.7	5701.4	64.7	88.12	47.7
71			Diphenhydramine * HCl	147-24-0	70.04	0.24	855.1	2.93	113.8	0.39	291.85	2.93
82	44		Diphenylhydantoin	57-41-0	98.39	0.39	NA	NA	199.3	0.79	252.29	0.79
206			Diquat dibromide	85-00-7	55.05	0.16	230.5	0.67	234.0	0.68	344.08	0.67
51			Disulfoton	298-04-4	30.19	0.11	2.0	0.0073	5.5	0.02	274.42	0.0073
226			Dodecylbenzene sodiumsulfonate	25155-30-0	146.38	0.42	1261.6	3.62	2000.5	5.74	348.52	3.62
57		L-	Dopa	59-92-7	25.64	0.13	1780.8	9.03	2366.5	12	197.21	9.03
11			Doxorubicin * HCl	25316-40-9	0.19	0.00033	NA	NA	696.0	1.2	580.03	1.2
244			Doxylamine succinate	562-10-7	291.38	0.75	NA	NA	470.1	1.21	388.51	1.21
10			Emetine	483-18-1	0.08	0.00016	67.3	0.14	NA	NA	480.71	0.14
169			Epinephrine bitartrate	51-42-3	9.33	0.028	NA	NA	4.0	0.012	333.33	0.012
130	9		Ethanol	64-17-5	17464.32	379	14008.3	304	7787.5	169	46.08	304
353			Ethyl acetate	141-78-6	11279.36	128	11015.0	125	NA	NA	88.12	125
349			Ethyl methyl ketone	78-93-3	7500.48	104	3396.9	47.1	NA	NA	72.12	47.1
307		2-	Ethylbutanal	97-96-1	1322.38	13.2	3977.1	39.7	NA	NA	100.18	39.7
360	7		Ethylene glycol	107-21-1	34454.40	555	8567.0	138	7511.7	121	62.08	138

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
24			Ethylenediamine-tetraacetic acid	60-00-4	2.92	0.01	NA	NA	NA	NA	292.28	
261	3		Ferrous sulfate	7720-78-7	281.03	1.85	319.0	2.1	978.3	6.44	151.91	2.1
35			Flufenamic acid	530-78-9	8.16	0.029	272.8	0.97	714.4	2.54	281.25	0.97
17		5-	Fluorouracil	51-21-8	0.34	0.0026	230.3	1.77	114.5	0.88	130.09	1.77
202			Formaldehyde	50-00-0	3.60	0.12	798.8	26.6	NA	NA	30.03	26.6
109			Frusemide	54-31-9	770.67	2.33	2599.8	7.86	4597.6	13.9	330.76	7.86
171			Fumagillin	297-95-0	14.22	0.031	NA	NA	1999.5	4.36	458.6	4.36
108			Gibberellic acid	77-06-5	796.74	2.3	6304.7	18.2	NA	NA	346.41	18.2
222			Glibenclamide	10238-21-8	197.62	0.4	NA	NA	3250.8	6.58	494.05	6.58
101			Glutethimide	77-21-4	338.97	1.56	599.7	2.76	360.7	1.66	217.29	2.76
131			Glycerol	56-81-5	57476.64	624	12619.1	137	25975.0	282	92.11	137
326			Halothane	151-67-7	6138.83	31.1	5684.8	28.8	NA	NA	197.39	28.8
185			Heptachlor	76-44-8	22.02	0.059	41.1	0.11	67.2	0.18	373.3	0.11
288		1-	Heptanol	111-70-6	726.44	6.25	3254.4	28	1499.4	12.9	116.23	28
151			Hexachlorocyclopentadiene	77-47-4	0.85	0.0031	111.8	0.41	NA	NA	272.75	0.41
157	38		Hexachlorophene	70-30-4	3.21	0.0079	61.0	0.15	65.1	0.16	406.89	0.15
159			Hexadecyltrimethylammoniumbromide	57-09-0	3.24	0.0089	408.3	1.12	NA	NA	364.53	1.12
295		2,5-	Hexanedione	110-13-4	964.65	8.45	2705.6	23.7	NA	NA	114.16	23.7
352		1,2,6-	Hexanetriol	106-69-4	16506.60	123	15969.8	119	NA	NA	134.2	119
311		1-	Hexanol	111-27-3	1573.88	15.4	719.5	7.04	1952.0	19.1	102.2	7.04
187		4-	Hexylresorcinol	136-77-6	12.44	0.064	549.9	2.83	NA	NA	194.3	2.83
296			Homatropine methylbromide	80-49-9	3332.97	9	1199.9	3.24	1399.8	3.78	370.33	3.24
219			Hydralazine	86-54-4	52.87	0.33	89.7	0.56	121.8	0.76	160.2	0.56
32			Hydrocortisone	50-23-7	7.98	0.022	NA	NA	NA	NA	362.51	
236			Hydrogen peroxide 90%	7722-84-1	19.05	0.56	NA	NA	2000.4	58.8	34.02	58.8
267		p-	Hydroxybenzoic acid	99-96-7	403.34	2.92	NA	NA	2196.3	15.9	138.13	15.9
152		8-	Hydroxyquinoline	148-24-3	0.48	0.0033	1200.6	8.27	NA	NA	145.17	8.27
44			Hydroxyzine * HCl	1244-76-4	27.56	0.067	950.4	2.31	NA	NA	411.41	2.31
233			Ibuprofen	15687-27-1	107.28	0.52	1008.9	4.89	980.0	4.75	206.31	4.89
299			Imidazole	288-32-4	783.04	11.5	NA	NA	1879.3	27.6	68.09	27.6
238			Imidazolidinyl urea	39236-46-9	100.17	0.36	2598.9	9.34	3700.9	13.3	278.26	9.34
38			Imipramine * HCl	113-52-0	17.11	0.054	304.2	0.96	374.0	1.18	316.91	0.96
60			Indomethacin	53-86-1	57.25	0.16	12.2	0.034	19.0	0.053	357.81	0.034
198			Ioxynil	1689-83-4	40.80	0.11	111.3	0.3	NA	NA	370.91	0.3
90			Iproniazid	54-92-2	141.61	0.79	365.7	2.04	681.2	3.8	179.25	2.04
315			Isobenzoic furano dione		2518.04	17	4014.1	27.1	1999.6	13.5	148.12	27.1
309			Isobutanal	78-84-2	973.62	13.5	2812.7	39	NA	NA	72.12	39
334			Isobutanol	78-83-1	2973.01	40.1	2461.4	33.2	NA	NA	74.14	33.2
123	35		Isoniazid	54-85-3	1027.33	7.49	650.1	4.74	NA	NA	137.16	4.74

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
257			Isononylaldehyde	5435-64-3	216.25	1.52	3243.8	22.8	NA	NA	142.27	22.8
165			Isoproterenol * HCl	51-30-9	5.45	0.022	2219.8	8.96	NA	NA	247.75	8.96
253			Isoxepac	55453-87-7	356.81	1.33	198.5	0.74	NA	NA	268.28	0.74
26			Kelthane	115-32-2	4.45	0.012	574.2	1.55	418.6	1.13	370.48	1.55
341			Lactic acid	598-82-3	5945.94	66	3729.7	41.4	4873.9	54.1	90.09	41.4
46			Lead II chloride	7758-95-4	11.96	0.043	NA	NA	NA	NA	278.09	
223	32		Lindane	58-89-9	119.24	0.41	75.6	0.26	87.2	0.3	290.82	0.26
333			Lithium I chloride	7447-41-8	1636.25	38.6	758.8	17.9	1165.7	27.5	42.39	17.9
327	20		Lithium I sulfate	10377-48-7	3704.98	33.7	NA	NA	1187.4	10.8	109.94	10.8
343			Magnesium II chloride * 6 H2O	7791-18-6	14314.43	70.4	8092.5	39.8	NA	NA	203.33	39.8
67	15		Malathion	121-75-5	66.08	0.2	885.4	2.68	776.4	2.35	330.38	2.68
154			Maneb	12427-38-2	1.12	0.0042	4500.6	16.9	3994.7	15	266.31	16.9
56			Manganese IIchloride *4 H2O	13446-34-9	25.73	0.13	1484.4	7.5	NA	NA	197.92	7.5
48			Mefenamic acid	61-68-7	20.99	0.087	789.1	3.27	629.8	2.61	241.31	3.27
94			Menthol	89-78-1	148.49	0.95	3172.9	20.3	NA	NA	156.3	20.3
21		6-	Mercaptopurine	50-44-2	1.22	0.008	NA	NA	280.0	1.84	152.19	1.84
29	28		Mercury II chloride	7487-94-7	4.07	0.015	1.0	0.0037	10.0	0.037	271.49	0.0037
85			Metamizol	68-89-3	193.94	0.58	7189.2	21.5	NA	NA	334.38	21.5
361	8		Methanol	67-56-1	29806.50	930	13012.3	406	NA	NA	32.05	406
356		2-	Methoxyethanol	109-86-4	19103.61	251	2458.4	32.3	NA	NA	76.11	32.3
259			Methyl salicylate	119-36-8	258.67	1.7	887.1	5.83	NA	NA	152.16	5.83
160		N-	Methyl-N'-nitro-N-nitroso- guanidine	70-25-7	1.77	0.012	89.7	0.61	NA	NA	147.12	0.61
78		6-	Methylcoumarin	92-48-8	49.66	0.31	1681.9	10.5	NA	NA	160.18	10.5
142			Methylmercury chloride	115-09-3	0.18	0.00071	NA	NA	57.7	0.23	251.08	0.23
98			Methylparaben	99-76-3	216.07	1.42	NA	NA	1749.8	11.5	152.16	11.5
319			Methylpentinol	77-75-8	2336.21	23.8	NA	NA	525.2	5.35	98.16	5.35
283			Milrinone	78415-72-2	1007.61	4.77	90.8	0.43	137.3	0.65	211.24	0.43
14			Mitomycin C	50-07-7	0.28	0.00084	14.0	0.042	17.1	0.051	334.37	0.042
147			Mitoxantrone	65271-80-9	1.07	0.0024	586.8	1.32	NA	NA	444.54	1.32
172			Nabam	142-59-6	8.97	0.035	394.8	1.54	579.3	2.26	256.34	1.54
47			Naftipramide	1505-95-9	25.07	0.084	1029.7	3.45	1086.4	3.64	298.47	3.45
99			Nalidixic acid	389-08-2	348.39	1.5	1349.4	5.81	571.4	2.46	232.26	5.81
114			Natulan * HCl	366-70-1	706.37	2.74	783.7	3.04	NA	NA	257.8	3.04
74			Nickel II chloride	7718-54-9	34.99	0.27	105.0	0.81	NA	NA	129.61	0.81
336			Nicotinamide	98-92-0	5423.02	44.4	3505.4	28.7	NA	NA	122.14	28.7
103	18		Nicotine	54-11-5	290.45	1.79	50.3	0.31	24.3	0.15	162.26	0.31
275			Nitrilotriacetic acid	139-13-9	690.09	3.61	1470.0	7.69	3154.1	16.5	191.16	7.69
221		2-	Nitro-p-phenylene-diamine	5307-14-2	59.73	0.39	3078.5	20.1	NA	NA	153.16	20.1
302			Nitrobenzene	98-95-3	1502.06	12.2	640.2	5.2	NA	NA	123.12	5.2

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
148			Nitrogen mustard * HCl	55-86-7	0.50	0.0026	10.0	0.052	19.3	0.1	192.53	0.052
210		p-	Nitrophenol	100-02-7	27.82	0.2	350.6	2.52	467.4	3.36	139.12	2.52
339		1-	Nitropropane	79-46-9	5159.47	57.9	455.4	5.11	NA	NA	89.11	5.11
175			Norepinephrine	51-41-2	6.60	0.039	NA	NA	20.3	0.12	169.2	0.12
268		1-	Octanol	111-87-5	398.60	3.06	NA	NA	1784.6	13.7	130.26	13.7
230	42		Orphenadrine * HCl	341-69-5	149.88	0.49	425.2	1.39	125.4	0.41	305.88	1.39
7			Ouabain	630-60-4	0.04	0.000072	NA	NA	NA	NA	584.73	
164			Oxatomide	60607-34-3	8.11	0.019	1412.1	3.31	9598.7	22.5	426.61	3.31
65			Oxyphenbutazone	129-20-4	61.64	0.19	999.2	3.08	480.1	1.48	324.41	3.08
176			Papaverine	58-74-2	15.27	0.045	325.8	0.96	230.8	0.68	339.42	0.96
235	25		Paraquat	4685-14-7	100.58	0.54	57.7	0.31	195.6	1.05	186.25	0.31
49			Parathion	56-38-2	27.09	0.093	2.0	0.0069	6.1	0.021	291.28	0.0069
173	39		Pentachlorophenol	87-86-5	9.59	0.036	50.6	0.19	NA	NA	266.32	0.19
322		1-	Pentanol	71-41-0	2195.43	24.9	3033.0	34.4	200.1	2.27	88.17	34.4
87			Pentobarbital sodium	57-33-0	176.29	0.71	201.1	0.81	280.6	1.13	248.29	0.81
240			Pentoxifylline	6493-05-6	183.71	0.66	NA	NA	1386.2	4.98	278.35	4.98
97			Phenacetin	62-44-2	227.63	1.27	1650.8	9.21	1220.6	6.81	179.24	9.21
118	24		Phenobarbital	50-06-6	884.91	3.81	162.6	0.7	167.2	0.72	232.26	0.7
115	12		Phenol	108-95-2	283.30	3.01	414.1	4.4	300.2	3.19	94.12	4.4
79			Phenylbutazone	50-33-9	98.69	0.32	376.3	1.22	441.0	1.43	308.41	1.22
218		o-	Phenylenediamine	95-54-5	33.53	0.31	1069.7	9.89	NA	NA	108.16	9.89
180		p-	Phenylenediamine	106-50-3	5.41	0.05	80.0	0.74	NA	NA	108.16	0.74
282		(-)-	Phenylephrine	59-42-7	744.17	4.45	349.5	2.09	NA	NA	167.23	2.09
278			Phenylephrine * HCl	939-38-8	847.35	4.16	350.3	1.72	120.2	0.59	203.69	1.72
234			Phenylthiourea	103-85-5	82.20	0.54	3.0	0.02	10.0	0.066	152.23	0.02
342			Piperazine	110-85-0	5789.95	67.2	1904.1	22.1	1438.9	16.7	86.16	22.1
146			Potassium bichromate VI	7778-50-9	0.59	0.002	NA	NA	191.2	0.65	294.2	0.65
145			Potassium chromate VI	7789-00-6	0.29	0.0015	NA	NA	180.6	0.93	194.2	0.93
277			Potassium cyanate	590-28-3	335.84	4.14	NA	NA	843.6	10.4	81.12	10.4
252	19		Potassium cyanide	151-50-8	72.93	1.12	9.8	0.15	8.5	0.13	65.12	0.15
335			Potassium hexacyano- ferrate II	13943-58-3	15582.05	42.3	6409.6	17.4	5009.8	13.6	368.37	17.4
266			Potassium hexacyanoferrate III	13746-66-2	928.54	2.82	NA	NA	2970.0	9.02	329.27	9.02
346	50		Potassium I chloride	7447-40-7	6113.10	82	2601.8	34.9	1498.5	20.1	74.55	34.9
269			Potassium I fluoride	7789-23-3	181.85	3.13	245.2	4.22	NA	NA	58.1	4.22
36			Progesterone	57-83-0	9.44	0.03	NA	NA	NA	NA	314.51	
348		1-	Propanol	71-23-8	5800.62	96.5	5397.9	89.8	NA	NA	60.11	89.8
128	10	2-	Propanol	67-63-0	10038.37	167	5842.7	97.2	NA	NA	60.11	97.2
270			Propionaldehyde	123-38-6	188.79	3.25	1411.6	24.3	NA	NA	58.09	24.3
54	23		Propranolol * HCl	318-98-9	35.50	0.12	NA	NA	470.4	1.59	295.84	1.59

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
357			Propylene glycol	57-55-6	26029.62	342	20016.9	263	23974.7	315	76.11	263
209			Propylparaben	94-13-3	32.44	0.18	NA	NA	6325.7	35.1	180.22	35.1
12			Puromycin	53-79-2	0.16	0.00033	NA	NA	674.4	1.43	471.58	1.43
337			Pyridine	110-86-1	3710.26	46.9	893.9	11.3	NA	NA	79.11	11.3
53	43		Quinidine sulfate	50-54-4	50.70	0.12	456.3	1.08	595.8	1.41	422.54	1.08
45			Quinine * HCl	130-89-2	27.07	0.075	620.8	1.72	1158.6	3.21	360.92	1.72
216			Refortan		78.28	0.25	3162.3	10.1	NA	NA	313.1	10.1
245			Resorcinol	108-46-3	88.10	0.8	300.6	2.73	NA	NA	110.12	2.73
201		13-cis-	Retinoic acid	4759-48-2	36.06	0.12	NA	NA	3395.4	11.3	300.48	11.3
139			Retinol	68-26-8	0.15	0.00054	1999.8	6.98	4011.0	14	286.5	6.98
134			Rotenone	83-79-4	0.05	0.00013	130.2	0.33	351.1	0.89	394.45	0.33
314			Saccharin	81-07-2	3004.32	16.4	NA	NA	17000.0	92.8	183.19	92.8
95			Salicylamide	65-45-2	148.12	1.08	1892.7	13.8	1398.9	10.2	137.15	13.8
178			Salicylanilide	87-17-2	9.81	0.046	NA	NA	2409.7	11.3	213.25	11.3
272			Salicylic acid	69-72-7	466.88	3.38	890.9	6.45	479.3	3.47	138.13	6.45
251			Scopolamine * HBr	6533-68-2	415.05	1.08	1268.2	3.3	1879.3	4.89	384.31	3.3
69			Secobarbital sodium	309-43-3	54.66	0.21	124.9	0.48	NA	NA	260.3	0.48
161			Silver I nitrate	7761-88-8	2.21	0.013	NA	NA	49.3	0.29	169.88	0.29
30			Sodium arsenate, dibasic	7778-43-0	2.79	0.015	NA	NA	NA	NA	185.91	
241			Sodium azide	26628-22-8	46.16	0.71	44.9	0.69	27.3	0.42	65.02	0.69
144			Sodium bichromate VI	10588-01-9	0.24	0.00093	49.8	0.19	NA	NA	261.98	0.19
344	13		Sodium chloride	7647-14-5	4435.60	75.9	2998.0	51.3	3997.3	68.4	58.44	51.3
329			Sodium cyclamate	139-05-9	7123.90	35.4	15254.0	75.8	17004.8	84.5	201.24	75.8
76			Sodium dodecyl sulfate	151-21-3	78.15	0.27	1288.0	4.45	NA	NA	289.43	4.45
345			Sodium I bromide	7647-15-6	8120.81	77.4	3504.3	33.4	6998.2	66.7	104.92	33.4
106	14		Sodium I fluoride	7681-49-4	77.68	1.85	180.1	4.29	NA	NA	41.99	4.29
255			Sodium monochloroacetate	3926-62-3	168.90	1.45	75.7	0.65	NA	NA	116.48	0.65
227	46		Sodium oxalate	62-76-0	58.96	0.44	155.4	1.16	NA	NA	134	1.16
119			Sodium salicylate	54-21-7	693.28	4.33	1599.5	9.99	899.8	5.62	160.11	9.99
290			Sodium sulfite	7757-83-7	854.55	6.78	NA	NA	820.5	6.51	126.04	6.51
156			Stearyltrimethylammoniumchloride	112-03-8	2.09	0.006	NA	NA	536.1	1.54	348.13	1.54
265			Streptomycin sulfate	298-39-5	3979.25	2.73	NA	NA	495.6	0.34	1457.6	0.34
331			Strontium II chloride	10476-85-4	5770.13	36.4	2251.0	14.2	3107.0	19.6	158.52	14.2
5		K-	Strophantin		0.03	0.000044	NA	NA	NA	NA	710.9	
271			Styrene	100-42-5	343.73	3.3	4999.7	48	315.6	3.03	104.16	48
93			Sulfisoxazole	127-69-5	227.23	0.85	NA	NA	6790.2	25.4	267.33	25.4
330			Sulfuric acid	7664-93-9	3530.88	36	2138.1	21.8	NA	NA	98.08	21.8
135		2,3,7,8-	Tetrachloro-dibenzo-p-dioxin	1746-01-6	0.06	0.0002	NA	NA	0.1	0.00035	321.96	0.00035
289			Tetrachloroethene	127-18-4	1084.46	6.54	8854.8	53.4	8092.0	48.8	165.82	53.4

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
59			Tetracycline * HCl	64-75-5	67.33	0.14	6444.6	13.4	NA	NA	480.94	13.4
350			Tetrahydrofurfuryl alcohol	97-99-4	11338.65	111	2502.7	24.5	2298.4	22.5	102.15	24.5
247		(+)-	Thalidomide	731-40-8	209.18	0.81	NA	NA	400.3	1.55	258.25	1.55
203			Thallium I acetate	563-68-8	36.88	0.14	NA	NA	34.2	0.13	263.42	0.13
181	30		Thallium I sulfate	7446-18-6	27.26	0.054	NA	NA	28.8	0.057	504.8	0.057
105	21		Theophylline	58-55-9	329.75	1.83	NA	NA	600.0	3.33	180.19	3.33
303			Theophylline sodium	3485-82-3	2519.43	12.4	NA	NA	445.0	2.19	203.18	2.19
280			Theophylline sodium acetate	8002-89-9	1098.74	4.19	582.2	2.22	NA	NA	262.23	2.22
25			Thio-TEPA	52-24-4	2.08	0.011	NA	NA	37.8	0.2	189.24	0.2
279			Thioacetamide	62-55-5	313.33	4.17	301.3	4.01	NA	NA	75.14	4.01
140		6-	Thioguanine	154-42-7	0.10	0.00057	NA	NA	160.5	0.96	167.21	0.96
83			Thiopental	76-75-5	133.30	0.55	NA	NA	601.1	2.48	242.37	2.48
170	29		Thioridazine * HCl	130-61-0	11.81	0.029	NA	NA	358.2	0.88	407.07	0.88
80		2-	Thiouracil	141-90-2	41.01	0.32	999.6	7.8	NA	NA	128.16	7.8
347			Thiourea	62-56-6	6547.18	86	124.9	1.64	8526.6	112	76.13	1.64
214			Thymol	89-83-8	34.56	0.23	979.6	6.52	1802.9	12	150.24	6.52
256			Tin II chloride	7772-99-8	286.28	1.51	699.6	3.69	1200.1	6.33	189.59	3.69
104			Tolbutamide	64-77-7	489.39	1.81	NA	NA	2601.1	9.62	270.38	9.62
316			Toluene	108-88-3	1575.77	17.1	5003.7	54.3	NA	NA	92.15	54.3
194		p-	Toluylendiamine	95-70-5	11.49	0.094	101.4	0.83	NA	NA	122.19	0.83
1			Trenimon	68-76-8	0.00	0.0000033	NA	NA	NA	NA	231.28	
310			Tributylamine	102-82-9	2855.16	15.4	539.5	2.91	NA	NA	185.4	2.91
138			Tributyltin chloride	1461-22-9	0.18	0.00054	120.4	0.37	NA	NA	325.53	0.37
75			Trichlorfon	52-68-6	69.51	0.27	450.5	1.75	298.6	1.16	257.44	1.75
294			Trichloroacetic acid	76-03-9	1338.08	8.19	4999.4	30.6	5636.6	34.5	163.38	30.6
242		1,2,4-	Trichlorobenzene	120-82-1	128.82	0.71	756.6	4.17	765.7	4.22	181.44	4.17
297	11	1,1,1-	Trichloroethane	71-55-6	1374.02	10.3	10298.5	77.2	11245.6	84.3	133.4	77.2
228		2,4,5-	Trichlorophen- oxyacetic acid	93-76-5	112.41	0.44	298.9	1.17	388.3	1.52	255.48	1.17
126			Triethyl citrate	77-93-0	4061.90	14.7	6990.9	25.3	NA	NA	276.32	25.3
143			Triethylene melamine	51-18-3	0.16	0.00078	1.0	0.005	14.9	0.073	204.27	0.005
137			Triethyltin chloride	994-31-0	0.11	0.00046	5.1	0.021	NA	NA	241.35	0.021
318			Trifluoroacetic acid	76-05-1	2337.62	20.5	199.6	1.75	NA	NA	114.03	1.75
166			Triisooctylamine	2757-28-0	8.14	0.023	1620.2	4.58	NA	NA	353.76	4.58
354		1,3,5-	Trioxane	110-88-3	19189.17	213	800.0	8.88	NA	NA	90.09	8.88
132			Triphenyltin hydroxide	76-87-9	0.02	0.000049	44.0	0.12	245.9	0.67	367.03	0.12
182			Triton X-100	9002-93-1	35.59	0.055	1798.7	2.78	NA	NA	647	2.78
50			Trypan blue	72-57-1	91.66	0.095	6204.2	6.43	NA	NA	964.88	6.43
231			Tween 80	9005-65-6	641.90	0.49	NA	NA	25021.0	19.1	1310	19.1
208			Undecylenic acid	112-38-9	33.18	0.18	2506.6	13.6	8496.7	46.1	184.31	13.6

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
323			Urethan	51-79-6	2307.95	25.9	NA	NA	2504.0	28.1	89.11	28.1
250			Valproate sodium	1069-66-5	166.22	1	NA	NA	1695.4	10.2	166.22	10.2
196	40		VerapamilHCl	152-11-4	49.11	0.1	108.0	0.22	162.1	0.33	491.13	0.22
205			Versalide	88-29-9	38.77	0.15	315.3	1.22	NA	NA	258.44	1.22
4			Vincristine sulfate	2068-78-2	0.01	0.000015	NA	NA	NA	NA	923.14	
86	31		Warfarin	81-81-2	206.59	0.67	323.8	1.05	373.1	1.21	308.35	1.05
313			Xanthinol nicotinate	437-74-1	6865.26	15.8	14121.6	32.5	17336.9	39.9	434.51	32.5
301	17		Xylene	1330-20-7	1274.16	12	4300.3	40.5	NA	NA	106.18	40.5
55			Zinc II chloride	7646-85-7	17.72	0.13	350.2	2.57	350.2	2.57	136.27	2.57
186			Zineb	12122-67-7	16.27	0.059	5211.3	18.9	7610.1	27.6	275.73	18.9

Section 7.2
Table 7.4
Registry of Cytotoxicity Data - MEIC Chemicals (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
29	28		Mercury II chloride	7487-94-7	4.07	0.015	1.0	0.0037	10.0	0.037	271.49	0.0037
252	19		Potassium cyanide	151-50-8	72.93	1.12	9.8	0.15	8.5	0.13	65.12	0.15
153	26		Arsenic III trioxide	1327-53-3	0.83	0.0042	19.8	0.1	45.5	0.23	197.84	0.1
103	18		Nicotine	54-11-5	290.45	1.79	50.3	0.31	24.3	0.15	162.26	0.31
173	39		Pentachlorophenol	87-86-5	9.59	0.036	50.6	0.19	NA	NA	266.32	0.19
262	47		Amphetamine sulfate	60-13-9	726.02	1.97	55.3	0.15	24.0	0.065	368.54	0.15
235	25		Paraquat	4685-14-7	100.58	0.54	57.7	0.31	195.6	1.05	186.25	0.31
157	38		Hexachlorophene	70-30-4	3.21	0.0079	61.0	0.15	65.1	0.16	406.89	0.15
223	32		Lindane	58-89-9	119.24	0.41	75.6	0.26	87.2	0.3	290.82	0.26
229	22		Dextropropoxyphene * HCl	1639-60-7	184.23	0.49	82.7	0.22	82.7	0.22	375.98	0.22
196	40		VerapamilHCl	152-11-4	49.11	0.1	108.0	0.22	162.1	0.33	491.13	0.22
227	46		Sodium oxalate	62-76-0	58.96	0.44	155.4	1.16	NA	NA	134	1.16
118	24		Phenobarbital	50-06-6	884.91	3.81	162.6	0.7	167.2	0.72	232.26	0.7
106	14		Sodium I fluoride	7681-49-4	77.68	1.85	180.1	4.29	NA	NA	41.99	4.29
112	48		Caffeine	58-08-2	512.74	2.64	192.3	0.99	619.6	3.19	194.22	0.99
81	27		Cupric sulfate * 5 H2O	7758-99-8	82.40	0.33	299.6	1.2	NA	NA	249.7	1.2
261	3		Ferrous sulfate	7720-78-7	281.03	1.85	319.0	2.1	978.3	6.44	151.91	2.1
183	5		Amitriptyline	50-48-6	15.54	0.056	319.1	1.15	147.0	0.53	277.44	1.15
86	31		Warfarin	81-81-2	206.59	0.67	323.8	1.05	373.1	1.21	308.35	1.05
246	37		Barium II nitrate	10022-31-8	211.70	0.81	355.4	1.36	NA	NA	261.36	1.36
89	16	2,4-	Dichlorophenoxy- acetic acid	94-75-7	170.20	0.77	369.1	1.67	366.9	1.66	221.04	1.67
115	12		Phenol	108-95-2	283.30	3.01	414.1	4.4	300.2	3.19	94.12	4.4
230	42		Orphenadrine * HCl	341-69-5	149.88	0.49	425.2	1.39	125.4	0.41	305.88	1.39
53	43		Quinidine sulfate	50-54-4	50.70	0.12	456.3	1.08	595.8	1.41	422.54	1.08
70	49		Atropine sulfate	55-48-1	148.92	0.22	622.7	0.92	764.9	1.13	676.9	0.92
123	35		Isoniazid	54-85-3	1027.33	7.49	650.1	4.74	NA	NA	137.16	4.74
63	4		Diazepam	439-14-5	45.56	0.16	709.1	2.49	535.3	1.88	284.76	2.49
67	15		Malathion	121-75-5	66.08	0.2	885.4	2.68	776.4	2.35	330.38	2.68
308	33		Chloroform	67-66-3	1599.56	13.4	908.4	7.61	35.8	0.3	119.37	7.61
31	41		Chloroquine diphosphate	50-63-5	8.77	0.017	969.9	1.88	500.4	0.97	515.92	1.88
107	2		Acetylsalicylic acid	50-78-2	408.99	2.27	999.9	5.55	814.4	4.52	180.17	5.55
328	36		Dichloromethane	75-09-2	2964.06	34.9	1596.7	18.8	NA	NA	84.93	18.8
113	1		Acetaminophen	103-90-2	409.70	2.71	2403.8	15.9	338.6	2.24	151.18	15.9
346	50		Potassium I chloride	7447-40-7	6113.10	82	2601.8	34.9	1498.5	20.1	74.55	34.9
125	34		Carbon tetrachloride	56-23-5	1308.92	8.51	2799.3	18.2	12797.0	83.2	153.81	18.2
344	13		Sodium chloride	7647-14-5	4435.60	75.9	2998.0	51.3	3997.3	68.4	58.44	51.3
91	45		Chloramphenicol	56-75-7	255.29	0.79	3393.1	10.5	2640.1	8.17	323.15	10.5

Section 7.2
Table 7.4
Registry of Cytotoxicity Data - MEIC Chemicals (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
301	17		Xylene	1330-20-7	1274.16	12	4300.3	40.5	NA	NA	106.18	40.5
128	10	2-	Propanol	67-63-0	10038.37	167	5842.7	97.2	NA	NA	60.11	97.2
360	7		Ethylene glycol	107-21-1	34454.40	555	8567.0	138	7511.7	121	62.08	138
297	11	1,1,1-	Trichloroethane	71-55-6	1374.02	10.3	10298.5	77.2	11245.6	84.3	133.4	77.2
361	8		Methanol	67-56-1	29806.50	930	13012.3	406	NA	NA	32.05	406
130	9		Ethanol	64-17-5	17464.32	379	14008.3	304	7787.5	169	46.08	304
22	6		Digoxin	20830-75-5	6.64	0.0085	NA	NA	18.0	0.023	781.05	0.023
327	20		Lithium I sulfate	10377-48-7	3704.98	33.7	NA	NA	1187.4	10.8	109.94	10.8
105	21		Theophylline	58-55-9	329.75	1.83	NA	NA	600.0	3.33	180.19	3.33
54	23		Propranolol * HCl	318-98-9	35.50	0.12	NA	NA	470.4	1.59	295.84	1.59
170	29		Thioridazine * HCl	130-61-0	11.81	0.029	NA	NA	358.2	0.88	407.07	0.88
181	30		Thallium I sulfate	7446-18-6	27.26	0.054	NA	NA	28.8	0.057	504.8	0.057
82	44		Diphenylhydantoin	57-41-0	98.39	0.39	NA	NA	199.3	0.79	252.29	0.79

Calculation of the Regression Between Cytotoxicity and Acute Oral Toxicity

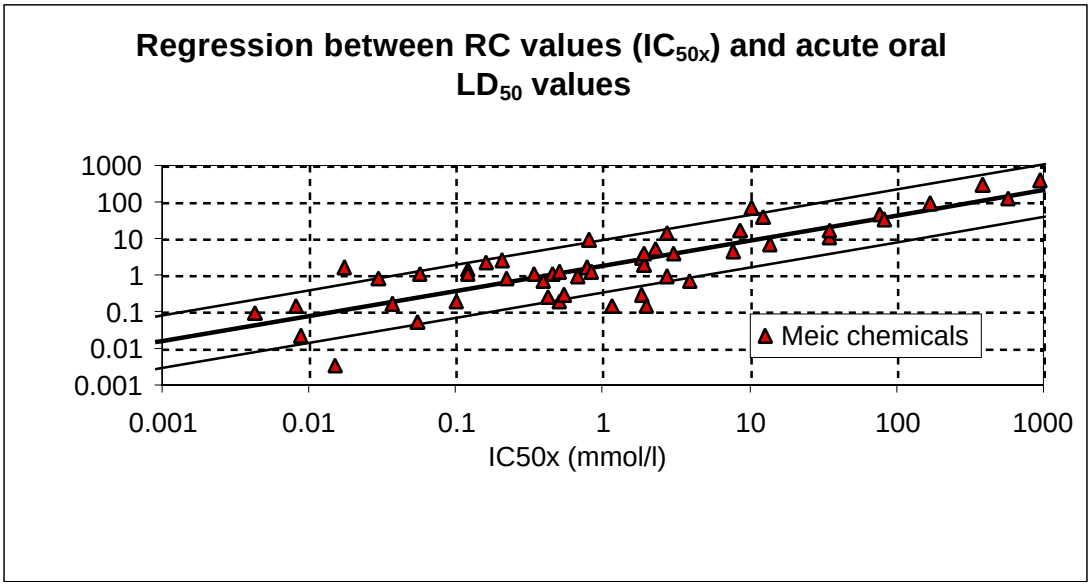


Figure 7.1 **Regression between RC values (IC50x) and acute oral LD50 values (MEIC chemicals)**

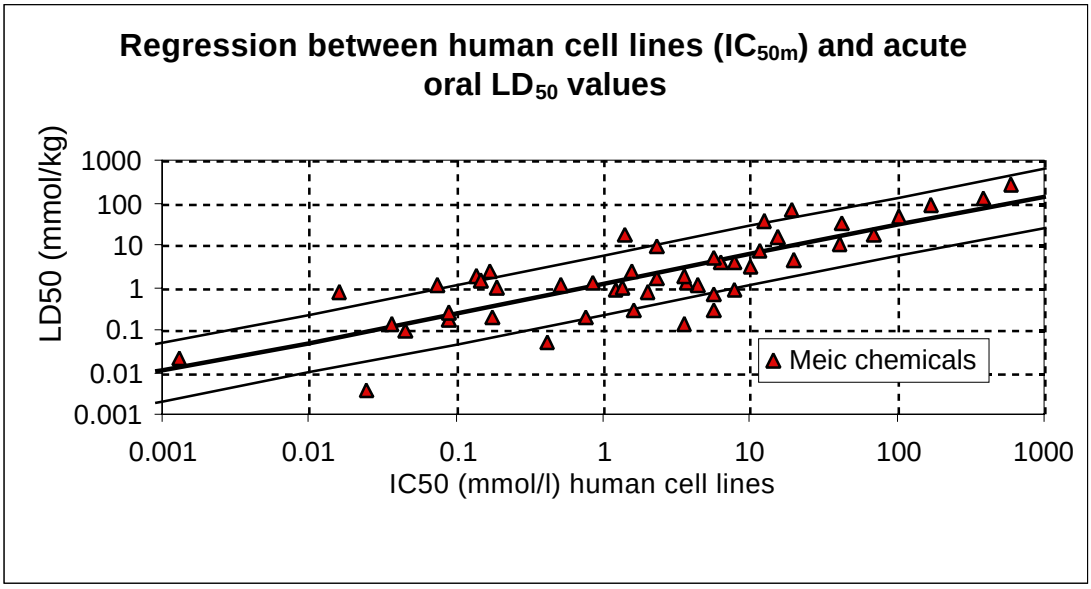


Figure 7.2 **Regression between human cell lines (IC50m) and acute oral LD50 values (MEIC chemicals)**

8.0 References

This reference list includes all cited references in the Breakout Group reports and key references provided to or recommended for *In Vitro* Workshop participants.

16 CFR 1500. 2000. Title 16: Commercial Practices. Chapter II. Consumer Product Safety Commission. Part 1500: Hazardous Substances And Articles; Administration And Enforcement Regulations. Government Printing Office, Washington, DC.
http://frwebgate.access.gpo.gov/cgi-bin/get-cfr.cgi

29 CFR 1910.1200. 1998. Title 29: Department of Labor. Chapter XVII. Part 1910: Occupational Safety And Health Administration. Subpart Z: Toxic And Hazardous Substances. Section 1200: Hazard Communication. Government Printing Office, Washington, DC.
http://frwebgate.access.gpo.gov/cgi-bin/get-cfr.cgi

40 CFR 156.10. 2000. Title 40: Protection Of Environment Agency. Code of Federal Regulations. Part 156: Labeling Requirements For Pesticides And Devices. Section 10: Labeling Requirements. Government Printing Office, Washington, DC.
http://frwebgate.access.gpo.gov/cgi-bin/get-cfr.cgi

40 CFR 159.165. 2000. Title 40: Protection Of Environment Agency. Part 159: Statements Of Policies And Interpretations. Section 165: Toxicological and Ecological Studies. Government Printing Office, Washington, DC.
http://frwebgate.access.gpo.gov/cgi-bin/get-cfr.cgi

49 CFR 173.132. 1999. Title 49: Department of Transportation. Code of Federal Regulations. Part 173: Shippers--General Requirements for Shipments and Packagings. Section 132: Class 6, Division 6.1 – Definitions. Government Printing Office, Washington, DC.
http://frwebgate.access.gpo.gov/cgi-bin/get-cfr.cgi

49 CFR 173.133. 1999. Title 49: Department of Transportation. Code of Federal Regulations. Part 173: Shippers--General Requirements for Shipments and Packagings. Section 133: Assignment of Packing Group and Hazard Zones for Division 6.1 Materials. Government Printing Office, Washington, DC.
http://frwebgate.access.gpo.gov/cgi-bin/get-cfr.cgi
Adegunloye, B.I., and O.A. Sofola. 1997. Differential Responses of Rat Aorta and Mesenteric Artery to Norepinephrine and Serotonin *In Vitro*. Pharmacology 55: 25-31.

Albert, A. 1985. Selective Toxicity, 7th edition. Chapman and Hall, London.

Amess, J. 1993. Haematotoxicology. In: General and Applied Toxicology. (Ballantyne, B., T. Marrs, and P. Turner, eds). Volume 1. Basingstoke, UK: Macmillan Press. 839-867.

Andres, M.I., A. Forsby, and E. Walum . 1997. Polygodial-Induced Noradrenaline Release in Human Neuroblastoma SH-SY5Y Cells. Toxicol. *In Vitro* 11: 509-511.

Archer, G., M. Balls, L.H. Bruner, R.D. Curren, J.H. Fentem, H-G. Holzhütter, M. Liebsch, D.P Lovell, and J.A. Southee. 1997. The Validation of Toxicological Prediction Models. ATLA 25: 505-516.

Arms, A.D., and C.C. Travis. 1988. Reference physiological parameters in pharmacokinetics modeling. U.S. Environmental Protection Agency (EPA).

Aszalos, A., J.A. Bradlaw, E.F. Reynaldo, G.C. Yang, and A.N. El-Hage. 1984. Studies on the Action of Nystatin on Cultured Rat Myocardial Cells and Cell Membranes. Biochem. Pharmacol. 33: 3779-3786.

Atterwill, C.K., A. Bruinink, J. Drejer, E. Duarte, P. McFarlene, E. Abdulla, C. Meredith, P. Nicotera, C. Regan, E. Rodriquez-Farre, M.G. Simpson, R. Smith, B. Veronesi, H. Vijverberg, E. Walum, and C. Williams. 1994. *In vitro* Neurotoxicity Testing. ATLA 22: 350-362.

Bach, P.H., A.E.M. Vickers, R. Fisher, A. Baumann, E. Brittebo, D.J. Carlile, H.J. Koster, B.G. Lake, F. Salmon, T.W. Sawyer, G. Skibinski. 1996. The use of tissue slices for pharmacotoxicology studies - The report and recommendations of ECVAM workshop 20.
ATLA- 24: (6) 893-923.

Balls, M. 1998. Commentary: Mechanistic Approaches and the Development of Alternative Toxicity Test Methods. Environ. Hlth. Perspect. 106 (Suppl. 2): 453-458.

Balls, M., C. Atterwill, J.Fentem, M. Garle, and F. Wiebel. 1992. Evaluation of Alternatives to Animal Tests for Assessing the Acute Lethal Potency and Neurotoxic Potential of Chemicals. A report prepared for DGXI, CEC; Contract Number B4-3081/91/8678. FRAME, Nottingham.

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. 1995a. Practical Aspects of the Validation of Toxicity Test Procedures. The report and recommendations of ECVAM Workshop 5. ATLA 23: 129-147.

Balls, M., P.A.Botham, L.H. Bruner, and H. Spielmann. 1995b. The EC/HO International Validation Study on Alternatives to the Draize Eye Irritation Test. Toxicol. *In Vitro* 9: 871-929.

Balls, M., and R.H. Clothier. 1992. Cytotoxicity Assays for Intrinsic Toxicity and Irritancy. In: *In Vitro* Methods of Toxicology. (R.R. Watson, ed). CRC Press, Boca Raton, FL. pp. 37-52.

Balls, M., and J.H. Fentem. 1992. The Use of Basal Cytotoxicity and Target Organ Toxicity Tests in Hazard Identification and Risk Assessment. ATLA 20: 368-388.

Balls, M., A.-M. van Zeller, and M.E. Halder, Eds. *Progress in the Reduction, Refinement, and Replacement of Animal Experimentation: Proceedings of the 3rd World Congress on Alternatives and Animal Use in the Life Sciences.* 2000. Elsevier Science, Amsterdam. pp. 667-717.

Balls, M., and E. Walum. 1999. Toward the Acceptance of *In Vitro* Neurotoxicity Tests. In: Neurotoxicology *In Vitro*. (V.W. Pentreath, ed). Taylor & Francis, Philadelphia. pp. 269-283.

Barile, F.A., D. Alexander, and A. Sookhoo. 1995. Potential of Human Lung Cells for Predicting Acute Cytotoxicity. ATLA 23: 461-468.

Barile, F.A., S. Arjun, and D. Hopkinson. 1993. *In Vitro* Cytotoxicity Testing: Biological and Statistical Significance. Toxicol. *In Vitro* 7(2): 111-116.

Barile, F.A., S. Arjun, and L. Borges. 1991. *In Vitro* Cytotoxicity Testing of 30 Reference Chemicals to Predict Acute Human and Animal Toxicity. FASEBJ 5(4): A553-A553, Part 1.

Barratt, M. 2000. Prediction of Toxicity from Chemical Structure. Cell Biol. Toxicol. 16: 1-13.

Barratt, M.D. 1998. Integration of QSAR and *In Vitro* Toxicology. Environ. Hlth Perspect. 106 (Suppl. 2): 459-466.

Barnes, M.D. 1988. Joint Soviet-US. Attack on Heart Muscle Dogma. Science 242: 193-195.

Barratt, M.D., P.G. Brantom, J.H. Fentem, I. Gerner, A.P. Walker, and A.P. Worth. 1998. The ECVAM International Validation Study on *In Vitro* Tests for Skin Corrosivity. 1. Selection and distribution of the test chemicals. Toxicol. *In Vitro* 12: 471-482.

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.

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

Blaauboer, B.J., Balls, M., Barratt, M., Casati, S., Coecke, S, Mohamed, M.K., Moore, J., Rall, D., Smith, K.R., Tennant, R., Schwetz, B.A., Stokes, W.S., Younes, M. 1998. 13th meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC): Alternative testing methodologies and conceptual issues. Environ. Health Persp. 106 (Suppl. 2): 413-418.

Blaauboer, B.J., M. Balls, V. Bianchi, G. Golcsfoldi, A. Guillouzo, G.A. Moore, L. Odland, C.A. Reinhardt, H. Spielmann, and E. Walum. 1994. The ECITTS Integrated Toxicity Testing Scheme: The Application of *In Vitro* Test Systems to the Hazard Assessment of Chemicals. Toxicol. *In Vitro* 8: 845-846.

Blaauboer, B.J., M.D. Barratt, J.B. Houston. 1999. The Integrated Use of Alternative Methods in Toxicological Risk Evaluation. ECVAM Integrated Testing Strategies Task Force Report 1. ATLA 27: 229-237.

Blaauboer, B.J., M.K. Bayliss, J.V. Castell, C.T.A. Evelo, J.M. Frazier, K. Groen, M. Guelden, A. Guillouzo, A.M. Hissink, J.B. Houston, G. Johanson, J. deJongh, G.L. Kedderis, C.A. Reinhardt, J.J.M. Van de Sandt, and G. Semino. 1996. The Use of Biokinetics and *In Vitro* Methods in Toxicological Risk Evaluation. ATLA 24: 473-497.

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.

Blaauboer, B.J., A. Forsby, J.B. Houston, M. Beckman, R.D. Combes, and J. DeJongh. 2000. An Integrated Approach to the Prediction of Systemic Toxicity using Biokinetic Models and Biological *In Vitro* Test Methods. In: Progress in the Reduction Refinement and Replacement of Animal Experimentation. (Balls, M., A-M van Zeller, and M.E. Halder, eds). Elsevier, Amsterdam, pp. 525-536.

Blaauboer, B.J., M. Balls, M. Barratt, S. Casati, S. Coecke, M.K. Mohamed, J. Moore, D. Rall, K.R. Smith, R. Tennant, B.A. Schwetz, W.S. Stokes, and M. Younes. 1998. Alternative Testing Methodologies and Conceptual Issues. Environ. Hlth Perspect. 106 (Suppl. 2): 413-418.

Blaauboer, B.J., M.D. Barratt, and J.B. Houston. 1999. The Integrated Use of Alternative Methods in Toxicological Risk Evaluation. ECVAM Integrated Testing Strategies Task Force Report 1. ATLA 27: 229-237.

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.

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.

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.

Borenfreund, E., and J.A. Puerner. 1986. Cytotoxicity of Metals, Metal-Metal and Metal-Chelator Combinations Assayed *In Vitro.* Toxicology 39: 121-124.

Brantom, P.G., L.H. Bruner, M. Chamberlain, O. DeSilva, J. Dupuis, L.K. Earl, D.P. Lovell, W.J.W. Pape, M. Uttley, D.M. Bagley, F.W. Baker, M. Bracher, P.Courtellemont, I. Declercq, S. Freeman, W. Steiling, A.P. Walker, G.J. Carr, N. Dami, G. Thomas, J. Harbell, P.A. Jones, U. Pfannenbecker, J.A. Southee, M. Tcheng, H. Argembeaux, D. Castelli, R. Clothier, D.J. Esdaile, H. Itigaki, K. Jung, Y. Kasai, H.Kojima, U. Kristen, M. Larnicol, R.W. Lewis, K. Marenus, O. Moreno, A. Peterson, E.S. Rasmussen, C. Robles, and M. Stern. 1997. A Summary Report of the COLIPA International Validation Study on Alternatives to the Draize Rabbit Eye Irritation Test. Toxicol. *In Vitro* 11: 141-179.

Brown, R. P., M. D. Delp, S.L. Lindstedt, L.R. Rhomberg, and R.P. Beliles. 1997. Physiological Parameter Values of Physiologically Based Pharmacokinetic Models. Toxicol. Ind. Health 13: 407-484.

Brown, R.A., and A.O. Savage. 1996. Effects of Acute Acetaldehyde, Chronic Ethanol and Pargyline Treatment on Agonist Responses of the Rat Aorta. Toxicol. Appl. Pharmacol. 136: 170-178.

Bruce R.D. 1985. An Up-and-Down Procedure for Acute Toxicity Testing. Fundam. Appl. Tox. 5: 151-157.

Bruner, L.H., G.J. Carr, M. Chamberlain, and R.D. Curren. 1996. Validation of Alternative Methods for Toxicity Testing. Toxicol. *In Vitro* 10: 479-501.

Bruner, L.H., G.J. Carr, R.D. Curren, and M. Chamberlain. 1998. Validation of Alternative Methods for Toxicity Testing. Environ. Hlth Perspect. 106 (Suppl. 2): 477-484.

CAAT - The Johns Hopkins Center for Alternatives to Animal Testing TestSmart Workshops.
TestSmart: A Humane and Efficient Approach to SIDS Data--April 26-27, 1999
TestSmart-Endocrine Disrupters--February 21-22, 2001
TestSmart-Pharmaceuticals--May 7-9, 2001
http://caat.jhsph.edu/programs/workshops/workshops.htm

California. Environmental Protection Agency. Quality Assurance Project Plan for Evaluating the Simplified *In Vitro* Test Method. Prepared for: The Solubility/Bioavailability Research Consortium. Revision 4. March 1999. Provided by Cal Willhite.

Champeroux, P., E. Martel, C. Vannier, V. Blanc, J.Y. Liguennec, J. Fowler, and S. Richard. 2000. The Preclinical Assessment of the Risk for QT Interval Prolongation. Therapie Jan-Feb. 55 (1): 101-109.

Chan, E.M, M.J. Thomas, B. Bandy, and G.F. Tibbits. 1996. Effects of Doxorubicin, 4-Epirubicin, and Antioxidant Enzymes on the Contractility of Isolated Cardiomuyocytes. Can. J. Physiol. Pharmacol. Aug: 74 (8): 904-910.

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.

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. 1998a. 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. 1998b. 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., 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. 1996a. 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. 1996b.

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.

Clewell, H.J. 1993. Coupling of computer modeling with *In vitro* Methodologies to Reduce Animal Usage in Toxicity Testing. Toxicol. Lett. 68: 101-117.

Clothier, R.H., L.M.Hulme, A.B. Ahmed, H.L. Reeves, M. Smith, and M. Balls. 1988. *In Vitro* Cytotoxicity of 150 Chemicals to 3T3-L1 Cells Assessed by the FRAME Kenacid Blue Method. ATLA 16: 84-95.

Clothier, R.H., L.M.Hulme, M. Smith, and M. Balls. 1987. Comparison of the *In Vitro* Cytotoxicities and Acute *In Vivo* Toxicities of 59 Chemicals. Molecular Toxicol. 1: 571-577.

Combes, R.D., and L.K. Earl. 1999. *In Vitro* Toxicology-Priorities for the Year 2000. Hum. and Exper. Toxicol.

Costa, L.G. 1998. Neurotoxicity Testing: A Discussion of *In Vitro* Alternatives. Environ. Hlth Perspect. 106 (Suppl. 2): 505-510.

Crass, M.F., S.M. Hulsey, and T.J. Bulkey. 1988. Use of a New Pulsatile Perfused Rat Aorta Preparation to Study the Characteristics of the Vasodilator Effect of Parathyroid Hormone. J. Pharmacol. Exp. Ther. 245: 723-734.

Cronin, M.T.D., and J.C. Dearden. 1995. QSAR in Toxicology. 2. Prediction of Acute Mammalian Toxicity and Interspecies Correlations. Mammalian QSAR Review. Quant. Struct-Act. Relat. 14: 17-120.

Cross, D.M., and M. K. Bayliss. 2000. A commentary on the Use of Hepatocytes in Drug Metabolism Studies during Drug Discovery and Development. Drug Metab. Rev. 32(2): 219-240.

Curren, R., L. Bruner, A. Goldberg, and E. Walum. 1998. 13th meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC): Validation and acute toxicity testing. Environ. Hlth Perspect. 106 (Suppl. 2): 419-425.

Curren, R.D., and J.W. Harbell. 1998. *In Vitro* Alternatives for Ocular Irritation. Environ. Hlth Perspect. 106 (Suppl. 2): 485-492.

Curren, R.D., J.A. Southee, H. Spielmann, M. Liebsch, J.H. Fentem, and M. Balls. 1995. The Role of Prevalidation in the Development, Validation and Acceptance of Alternative Methods. ATLA 23: 211-217.

Dearden, J.C., M.D. Barratt, R. Benigni, D.W. Bristol, R.D. Combes, M.T.D.Cronin, P.N. Judson, M.P. Payne, A.M. Richard, M. Tichy, A.P. Worth, and J.J. Yourick. 1997. The Development and Validation of Expert Systems for Predicting Toxicity. The report and recommendations of an ECVAM/ECB workshop (ECVAM workshop 24). ATLA 25: 223-252.

DeJongh, J., A. Forsby, J.B. Houston, M. Beckman, R. Combes, and B.J. Blaauboer. 1999. An Integrated Approach to the Prediction of Systemic Toxicity Using Computer-Based Biokinetic Models and Biological *In Vitro* Test Methods: Overview of a prevalidation study based on the ECITTS Project. Toxicol. *In Vitro* 13: 549-554.

DeJongh, J., M. Nordin-Andersson, B.A. Ploeger, and A. Forsby. 1999. Estimation of Systemic Toxicity of Acrylamide by Integration of *In Vitro* Toxicity Data with Kinetic Simulations. Toxicol. Appl. Pharmacol. 158: 261-268.

DeJongh, J., H.J.M. Verhaar, and J.L.M. Hermens. 1997. A Quantitative Property-Property Relationship (QPPR) Approach to Estimate *In Vitro* Tissue-Blood Partition Coefficients of Organic Chemicals in Rats and Humans. Archives of Toxicol. 72: 17-25.

Diener, W., U. Mischke, D. Kayser, and E. Schlede . 1995. The Biometric Evaluation of the OECD Modified Version of the Acute-Toxic-Class Method (Oral). Arch. Toxicol. 69: 729-734.

Diener, W., and E. Schlede. 2000. Acute Toxic Class Methods: Biometric Evaluations and Test Procedures for the New International Classification Systems. Manuscript in preparation.

Diener W. and E. Schlede. 1999. Acute toxic class methods: alternatives to LD/LC_{50} tests. ALTEX 16: 129-134.

Dierickx, P.J. 1989. Cytotoxicity Testing of 114 Compounds by the Determination of Protein Content in HepG2 Cell Cultures. Toxicol. *In Vitro* 3: 189-193.

Dixon, W.J. 1991. Staircase Bioassay: The Up-and-Down Method. Neurosci. Biobehav. Rev. 15: 47-50.

Dixon, W.J. 1991. Design and Analysis of Quantal Dose-Response Experiments (with Emphasis on Staircase Designs). Dixon Statistical Associates, Los Angeles CA, USA.

Dixon, W.J. 1965. The Up-and-Down Method for Small Samples. J. Amer. Statist. Assoc. 60: 967-978.

Dixon, W.J., and A.M. Mood. 1948. A Method for Obtaining and Analyzing Sensitivity Data. J. Amer. Statist. Assoc. 43: 109-126.

Eagle, H., and G.E. Foley. 1956. The Cytotoxic Action of Carcinolytic Agents in Tissue Culture. Amer. Jour. of Med. 21: 739-745.

Ehrich, M., L. Correll, and B. Veronesi. 1997. Acetylcholinesterase and Neuropathy Target Esterase Inhibitors in Neuroblastoma Cells to Distinguish Organophosphorus Compounds Causing Acute and Delayed Neurotoxicity. Fund. Appl. Toxicol. 38: 55-63.

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. 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. 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. 1983. Screening of Toxic Compounds in Mammalian Cell Cultures. Ann. New York Acad. Sci. 407: 64-77.

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., 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., C. Clemedson, B. Ekwall, P. Ring, and L. Romert. 1999. EDIT: A new international multicentre programme to develop and evaluate batteries on *in vitro* tests for acute and chronic systemic toxicity. ATLA 27: 339-349.

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. 1998a. 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. 1998b. 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.

Ekwall, B., B. Ekwall, and M. Sjostrom. 2000. MEIC Evaluation of Acute Systemic Toxicity. Part VIII. Multivariate partial least squares evaluation, including the selection of a battery cell line tests with a good prediction of human acute lethal peak blood concentrations for 50 chemicals. ATLA 28 (Suppl. 1): 201-234.

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, B. Ekwall, M. Sjostrom. 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

Elmore, E., T-T.Luc, V.E. Steele, G.J. Kelloff, and J.L. Redpath. 2000. The Human Epithelial Cell Cytotoxicity Assay for Determining Tissue Specific Toxicity. Meth. Cell Sci. 22: 17-24.

Elmore, E., T-T.Luc, V.E. Steele, G.J. Kelloff, and J.L. Redpath. Comparative Tissue-Specific Toxicities of Twenty Cancer Preventive Agents Using Cultured Cells From Eight Different Normal Human Epithelia. (in press: *In Vitro* & Molecular Toxicology).

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.

Fentem, J.H., G.E.B. Archer, M. Balls, P.A. Botham, R.D. Curren, L.K.Earl, D.J.Esdaile, H-G. Holzhütter, and M. Liebsch. 1998. The ECVAM International Validation Study on *In Vitro* Tests for Skin Corrosivity. 2. Results and evaluation by the Management Team. Toxicology *In Vitro* 12: 483-524.

Fentem, J.H., D. Briggs, C.Chesné, G.R. Elliott, J.W. Harbell, J.R. Heylings, P. Portes, R. Roguet, J.J.M. van de Sandt, and P.A.Botham. 2001. A Prevalidation Study on *In Vitro* Tests for Acute Skin Irritation: results and evaluation by the Management Team. Toxicology *In vitro* 15:57-93.

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.

Foex, P. 1988. Experimental Models of Myocardial Ischemia. Br. J. Anaesth. 61: 44-55.

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.

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

Fry, J.R., M.J. Garle, and A.H. Hammond. 1988. Choice of Acute Toxicity Measures for Comparison of *In Vivo-In Vitro* Toxicity. ATLA 16: 175-179.

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.

Fuller, J., and D.D. Woodman. 1993. Hematology in Toxicology Studies. In: General and Applied Toxicology. (Ballantyne, B., T. Marrs, P. Turner, eds). Volume 1. Basingstoke, UK: Macmillan Press Ltd. 267-301.

Galson, S. 2000. Historical and current regulatory perspectives. Opening Plenary Session, ICCVAM International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, October 17-20, 2000.

Gandolfi, A.L., K. Brendel, R.L. Fisher, and J.P. Michaud. 1995. Use of Tissue Slices in Chemical Mixture Toxicology and Interspecies Investigations. Toxicology 105: 285-290.

Garberg, P. 1998. *In Vitro* Models of the Blood-Brain Barrier. ATLA 26: 821-847.

Garle, M., 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.

Garle, M.J., A.H. Hammond, and J. R. Fry. 1987. The Cytotoxicity of 27 Chemicals to V79 Chinese Hamster Cells. ATLA 15: 30-32.

The German Institute for Medical Documentation and Information (DIMDI). (http://www.dimdi.de)

Goldberg, A.M., and H.I. Maibach. 1998. Dermal Toxicity: Alternative Methods for Risk Assessment. Environ. Hlth Perspect. 106 (Suppl. 2): 493-496.

Gonzalez-Gay, M. A., C. Garcia-Porrua, J. Llorca, A. H. Hajeer, F. Branas, A. Dababneh, E. Gonzalez-Rodriguez-Gil, P. Rodriguiz-Ledo, and W. E. Ollier. 2000. Visula Manifestations of Giant Cell Arteritis. Trends and Clinical Spectrum in 1. Medicine (Baltimore) 79(5): 283-292.

Gribaldo, L., J. Bueren, A. Deldar, P. Hokland, C. Meredith, D. Moneta, P. Mosesso, R. Parchment, D. Parent-Massin, A. Pessina, J. San Roman, and G. Schoeters. 1996. The Use of *In Vitro* Systems for Evaluating Haematotoxicity. ATLA 24: 211-231.

Gribaldo, L., S. Casati, F.A. Castoldi, and A. Pessina. 1999. Comparison of *In Vitro* Drug-Sensitivity of Human Granulocyte-Macrophage Progenitors from Two Different Origins: Umbilical cord blood and bone marrow. Exp Hematology 27: 1593-1598.

Gribaldo, L., P. Catalani, and E. Marafante. 1999. Metabolism of Doxorubicin in Long-Term Bone Marrow Cultures and SR-4987 Stromal Established Cell Line. Drug Metabolism and Drug Interactions 15: 279-291.

Gribaldo, L., M. Piccirillo, S. Casati, A. Collotta, E. Mineo, and A. Pessina. 1998a. Drug Sensitivity of Granulocyte-Macrophage Precursors (GM-CFU) from Fresh Murine Bone Marrow and from Long-Term Bone Marrow Cultures. Toxicol. *In Vitro* 12: 39-45.

Gribaldo, L., S. Casati, L.E. Figliuzzi, and E. Marafante. 1998b. *In Vitro* Myelotoxicity of Environmental Contaminants. Env Toxicol. Pharmacol. 6: 135-141.

Grisham, J.W., and G.J. Smith. 1984. Predictive and Mechanistic Evaluation of Toxic Responses in Mammalian Cell Culture Systems. Pharmacolog. Rev. 36 (Suppl.): 151S-171S.

Guillouzo, A. 1998. Liver cell models in *in vitro* toxicology. Environ. Health Prespect. 106 Suppl. 2: 511-532.

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.

Guillouzo, A. 1997. Biotransformation of Drugs by Hepatocytes. In: *In Vitro* Methods in Pharmaceutical Research, Academic Press Ltd.

Gupta, M. N., R. E. Sturrock and G. Gupta. 2000. Cutaneous Leucocytoclastic Vasculitis Caused by Cyclosporin A (Sandimmun). Ann. Rheum. Dis. 59(4): 319.

Guzzie, P.J. 1994. Lethality Testing. In: *In Vitro Toxicology*. (S.C. Gad, ed). Raven Press, New York. pp. 57-86.

Halle, W. 1998. Toxizitätsprüfungen in Zellkulturen für eine Vorhersage der akuten Toxizität (LD50) zur Einsparung von Tierversuchen. Life Sciences/ Lebens-wissenschaften, Volume 1, 94 pp. Jülich: Forschungszentrum Jülich.

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

Halle, W., M. Liebsch, D. Traue, and H. Spielmann. 1997. Reduktion der Tierzahlen bei der Einstufung von Stoffen in die EU-Toxizitätsklassen für akute orale Toxizität mit Hilfe von Daten aus dem Register der Zytotoxizität (RC). ALTEX 14: 8-15.

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

Harrison, D.G. 1997. Endothelial Function and Oxidant Stress (review). Clin. Cardiol. 20: 11-17.

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.

Hartely, R.S., M. Margulis, P.S. Fishman, V.M. Lee, and C.M. Tang. 1999. Functional Synapses are Formed Between Human NTera2 (NT2N, hNT) Neurons Grown on Astrocytes. J. Comp. Neurol. 407: 1-10.

Hawksworth G.M., P.H. Bach, J.F. Nagelkerke, W. Dekant, J.E. Diezi, E. Harpur, E.A. Lock, C. Macdonald, J.P. Morin, W.Pfaller, Fajjle Rutten, M.P. Ryan, H.J. Toutain, A. Trevisan. 1995. Nephrotoxicity Testing In-Vitro - The Report And Recommendations Of Ecvam Workshop-10. ATLA 23: (5) 713-727.

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.

Hester, R.K., and K. Ramos. 1991. Vessel Cylinders. In: Methods in Toxicology. (Tyson, C., and J. Frazier, eds). San Diego, CA, Academic Press.

Hopkinson, D., R. Bourne, and F.A. Barile. 1993. *In vitro* Cytotoxicity Testing: 24-hour and 72-hour studies with cultured lung cells. ATLA 21: 167-172.

Horwitz, L.D., J.S. Wallner, D.E. Decker, and S.E. Buxser. 1996. Efficacy of Lipid Soluble, Membrane-Protective Agents Against Hydrogen Peroxide Cytotoxicity in Cardiac Myocytes. Free Radic. Biol. Med. 21(6): 743-753.

Houston, J.B., and D.J. Carlile. 1997. Prediction of Hepatic Clearance from Microsomes, Hepatocytes, and Liver Slices. Drug Metabol. Rev. 29: 891-922.

Hukkanen, J., A. Lassila, K. Paivarinta, S. Valanne, S. Sarpo, J. Hakkola, O. Pelkonen, and H. Raunio. 2000. Induction and Regulation of Xenobiotic-Metabolizing Cytochrome P450s in the Human A549 Lung Adenocarcinoma Cell Line. Am. J. Respir. Cell Mol. Biol. 22: 360-366.

Hulme, L.M., H.L. Reeves, R.H. Clothier, M. Smith, and M. Balls. 1987. Assessment of Two Alternative Methods for Predicting the *In Vivo* Toxicities of Metallic Compounds. Molecular Toxicol. 1: 589-596.

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). 1997. Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods. NIH Publication 97-3981. National Institute of Environmental Health Sciences, Research Triangle Park, NC. Available on the Internet at http://iccvam.niehs.nih.gov/docs/guidelines/validate.pdf.

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). 1999. Evaluation of the validation status of toxicological methods: General Guidelines for Submissions to ICCVAM Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods. NIH Publication 99-4496. National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina. Available on the Internet at http://iccvam.niehs.nih.gov/docs/guidelines/subguide.htm.

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). 2000. The revised Up-and-Down Procedure: a test for determining the acute oral toxicity of chemicals and products. Proposed test method and background review document, April 14, 2000. ICCVAM / NIEHS, Research Triangle Park, NC 27709. Available on the Internet at http://iccvam.niehs.nih.gov/methods/udpdocs/AllBRDlk.pdf.

Ingwall, J.S., M. Deluca, H.D. Sybers, and K. Wildenthal. 1975. Fetal Mouse Hearts: A model for studying ischemia. Proc. Natl. Acad. Sci. USA 72: 2809-2813.

Itagaki, H., T.Ohno, M. Hatao et al. 1998a. Validation Study on Five Cytotoxicity Assays by JSAAE. V. Details of the crystal violet staining assay. AATEX 5: 87-98.

Itagaki, H., T.Ohno, M. Hatao et al. 1998b. Validation Study on Five Cytotoxicity Assays by JSAAE. VII. Details of the MTT assay. AATEX 5: 119-130.

Izumi, I., K. Mineura, K. Watanabe, and M. Kowada. 1994. Establishment of the Two Glioma Cell Lines YH and AM. Hum. Cell. 7: 101-105.

Jahangiri, A., W. R. Leifert, G. S. Patten, and E. J. McMurchie. 2000. Termination of Asynchronous Contractile Activity in rat Atrial Myocytes by n-acids. Mol. Cell. Biochem. 206(1):33-41.

Janigro, D., S. Leaman , and K.A. Stanness. 1999. *In Vitro* Modeling of the BBB. Pharm. Sci. Technol. Today 2: 7-12.

Jover, R., X. Ponsoda, J.V. Castell, and M.J. Gomez-Lechón. 1992. Evaluation of the Cytotoxicity of Ten Chemicals on Human Cultured Hepatocytes: Predictability of Human Toxicity and Comparison with Rodent Cell Culture Systems. Toxicology *InVitro* 6: 47-52.

Kaiser, K.L.E. 1998. Correlations of *Vibrio fischeri* Bacteria Test Data with Bioassay Data for Other Organisms. Environ. Hlth Perspect. 106 (Suppl. 2): 583-591.

Karol, M.H. 1998. Target Organs and Systems: Methodologies to assess immune system function. Environ. Hlth Perspect. 106 (Suppl. 2): 533-540.

Kedderis, G.L. 1997. Extrapolation of *In Vitro* Enzyme Induction Data to Humans *In Vivo*. Chem.-Biol. Interact. 107: 109-121.

Kedderis, G.L., M.A. Carfagna, S.D. Held, R. Batra, J.E. Murphy, and M.L. Gargas. 1993. Kinetic Analysis of Furan Biotransformation *In Vivo* and *In Vitro*. Toxicol. Appl. Pharmacol. 123: 274-282.

Kedderis, G.L., and S.D. Held. 1996. Prediction of Furan Pharmacokinetics from Hepatocyte Studies: Comparison of bioactivation and hepatic dosimetry in rats, mice, and humans. Toxicol. Appl. Pharmacol. 140: 124-130.

Klaassen, C.D., and D.L. Eaton. 1991a. Principles of Toxicology. In: Caserett and Doull's Toxicology: The Science of Poisons. 4th Ed (Amdur, M.O., J. Doull, and C.D. Klaassen, eds). Pergamon Press Inc., New York, pp. 16-17.

Klaassen, C.D., and D.L. Eaton. 1991b. Principles of Toxicology. In: Caserett and Doull's Toxicology: The Science of Poisons. 4th Ed (Amdur, M.O., J. Doull, and C.D. Klaassen, eds). Pergamon Press Inc., New York, pp. 22.

Knox, P., P.F. Uphill, J.R. Fry, J. Benford, and M. Balls. 1986. The FRAME Multicentre Project on *In Vitro* Cytotoxicity. Food Chem. Toxicol. 24: 457-463.

Koo, E.W.Y., and A.I. Gottlieb. 1992. The Use of Organ Cultures to Study Vessel Wall Pathobiology. Scan. Micro. 6: 827-835.

Lagadic. L., and T. Caquet. 1998. Invertebrates in Testing of Environmental Chemicals: Are They Alternatives? Environ. Hlth Perspect. 106 (Suppl. 2): 593-611.

Lambre, C. R., M. Aufderheide, R. E. Bolton, B. Fubini, H. P. Haagsman, P. M. Hext, M. Jorissen, Y. Landry, J-P. Morin, B. Nemery, P. Nettesheim, J. Pauluhn, R. J. Richards, A. Vickers, and R. Wu. 1996. *In Vitro* Tests for Respiratory Toxicity. The Report and Recommendations of ECVAM Workshop 18. ATLA 24:671-681.

Larivee, P., A. Cantin, A. Dufresne, and R. Begin. 1990. Enzyme Activities of Lung Lavage in Silicosis. Lung 168: 151-158.

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.

Liebsch, M., D. Traue, C. Barrabas, H. Spielmann, P. Uphill, S. Wilkins, J.P. McPherson, C. Wiemann, T. Kaufmann, M. Remmele, and H.G. Holzhutter. 2000. The ECVAM Prevalidation Study on the Use of EpiDerm for Skin Corrosivity Testing. ATLA 28: 371-402.

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.

Lipnick, R.L., M. Zeeman, and J.A. Cotruvo. 1995b. Structure-activity relationships in the validation of in vitro toxicology tests. In: *Animal Test Alternatives: Refinement Reduction Replacement* . H. Salem, ed., Marcel Dekker, New York. pp 47-55.

Loegering, Djm, C.A. Richard, C.B. Davison, and G.A. Wirth. 1995. Diethyldithiocarbamate Ameliorates the Effect of Lipo-Polysaccharide on both Increased Nitrite Production by Vascular Smooth Muscle Cells and Decreased Contractile Response of Aortic Rings. Life Sci. 57: 169-176.
MacGregor, J.T., J.M. Collins, Y. Sugiyama, C.A. Tyson, J. Dean, L. Smith, M. Andersen, R.D. Curren, J.B. Houston, F.F. Kadlubar, G.L. Kedderis, K. Krishnan, A.P. Li, R.E. Parchment, K. Thummel, J.E. Tomaszewski, R. Ulrich, A.E.M. Vickers, and S.A. Wrighton. *In Vitro* Human Tissue Models in Risk Assessment: Report of a consensus-building workshop. Submitted to Toxicological Sciences.

Maines, M.D., L.G. Costa, D.J. Reed, S. Sassa, I.G. Sipes, eds. Current Protocols in Toxicology. New York: John Wiley & Sons, Inc., 1999.

Malhotra, S.K., L.T. Loung, R. Bhatnagar, and T.K. Shnitka. 1997. Up-Regulation of Reactive Astrogliosis in the Rat Glioma 9L Cell Line by Combined Mechanical and Chemical Injuries. Cytobios. 89: 115-134.

Marom, Z., J.H. Shelhamer, and M. Kaliner. 1984. Human Pulmonary Macrophage-Derived Mucus Secretagogue. J. Exp. Med. 159: 844-860.
Matsumura, K., and K. Kawamoto. 1994. The Establishment and Charcteristics Lioma Cell Line (KMU100) with a Long Term Culture. Hum. Cell 7: 62-67.

Markovic, B., G. Malich, and C. Winder. 1994. Quantitative Methods of Detecting Cytokine Messenger RNA and Protein Changes in Lymphocytes as an Alternative to Acute Toxicity Testing in Animals. *In Vitro* Toxicology 7: 198.

Mazziotti, I., A.L. Stammati, and F. Zucco. 1990. *In Vitro* Cytotoxicity of 26 Coded Chemicals to HEp-2 Cells: A Validation Study. ATLA 17: 401-406.
OECD (Organisation for Economic Co-operation and Development). 1987. OECD Guideline for Testing of Chemicals 401: Acute oral toxicity. OECD, Paris.

MEIC, MEMO and EDIT information: http://wwwcctoxconsulting.a.se/nica.htm.

Meyer, K.H. 1937. Contributions to the Theory of Narcosis. *Faraday Soc. Trans.* 33: 1062-1064.

Miescher, P.A. 1980. Drug-Induced Thrombocytopenia. Clin Haematol. 9: 505-519.

Munir, M., L. Lu, J. Luo, B.B. Wolfe, and P. McGonigle. 1996. Pharmacological and Immunological Characterization of N-methyl-D-aspartate Receptors in Human NT2-N Neurons. J. Pharmacol. Exp. Ther. 6: 819-829.

Nakamura, H., Y. Kawasaki, N. Arakawa, M. Saeki, S. Maeda, Y. Koyama, A. Baba, and T. Matsuda. 2000. The Na+-Ca2+ Exchange Inhibitor KB-R7943 Inhibits High K+-induced Increases in Intracellular Ca2+ Concentration and [3H]Noradrenaline Release in the Human Neuroblastoma SH-SY5Y. Neurochem. Res. 25: 385-387.

Naltner, A., M. Ghaffari, J. Whitsett, and C. Yan. 2000. Retinoic Acid Stimulation of the Human Surfactant Protein B Promoter Is Thyroid Transcription Factor 1 Site-Dependent. J. Biol. Chem. 275: 56-62.

Napoli, C. 1997. Low Density Lipoprotein Oxidation and Atherogenesis: From experimental models to clinical studies. Giornale Italiano di Cardiologia 27: 1302-1314.

National Center for Toxicogenomics. (http://www.niehs.nih.gov/nct/)

NIEHS (National Institute of Environmental Health Sciences), 2001. Guidance document on using in vitro data to estimate in vivo starting doses for acute toxicity. NIH Publication 01-4500. NIEHS, Research Triangle Park, North Carolina.

Neelands, T.R., L.J. Greenfield Jr., J. Zhang, R.S. Turner, and R.L. Macdonald. 1998. GABAA Receptor Pharmacology and Subtype mRNA Expression in Human Neuronal NT2-N cells. J. Neurosci. 18: 4993-5007.

Norden-Andersson, M., A. Forsby, N. Heldring, J. DeJongh, P. Kjellstrand, and E. Walum. 1998. Neurite Degeneration in Differentiated Human Neuroblastoma Cells. Toxicol. *In Vitro* 12: 557-560.
Noah, T.L., A.M. Paradiso, M.C. Madden, K.P. McKinnon, and R.B. Devlin. 1991. The Response of a Human Bronchial Epithelial Cell Line to Histamine: Intracellular calcium changes and extracellular release of inflammatory mediators. Am. J. Respir. Cell Mol.Biol. 5: 484-492.
Ozdem, S.S., and C. Ogutman. 1997. Responsiveness of Aortic Rings of Cadmium-Hypertensive Rats to Endothelin-1. Pharmacology 54: 328-332.

OECD. October, 2000; Draft OECD Guideline for Testing of Chemicals, 423: Acute Oral toxicity - Acute Toxic Class Method; http://www.oecd.org/ehs/test/health.htm).

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.

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. 1996. OECD Guideline for Testing of Chemicals. 423. Acute Oral Toxicity - Acute Toxic Class Method. OECD, Paris.

OECD. 1992. OECD Guideline for Testing of Chemicals. 420. Acute Oral Toxicity - Fixed Dose Method. OECD, Paris.

OECD. 1987. OECD Guideline for Testing of Chemicals. 401. Acute Oral Toxicity. OECD, Paris.

OECD. 1981. OECD Guideline for Testing of Chemicals. 401. Acute Oral Toxicity. OECD, Paris. [updated in 1987]

Ohno, T., M. Asakura, T. Awogi et al. 1998a. Validation Study on Five Cytotoxicity Assays by JSAAE. I. Overview of the study and analyses of variations of ED50 values. AATEX 5: 1-38.

Ohno, T., Y. Futamura, A. Harihara et al. 1998b. Validation Study on Five Cytotoxicity Assays by JSAAE. VI. Details of the LDH release assay. AATEX 5: 99-118.

Ohno, T., Y. Futamura, A. Harihara et al. 1998c. Validation Study on Five Cytotoxicity Assays by JSAAE. VIII. Details of the Neutral Red Uptake assay. AATEX 5: 131-145.

Olson, H., G. Betton, D. Robinson, K. Thomas, A. Monro, G. Kolaja, P. Lilly, J. Sanders, G. Sipes. 2000. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul. Toxicol. Pharm. 32: 56-67.

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.

Parchment, R.E. 1998. Alternative Testing Systems for Evaluating Noncarcinogenic, Hematologic Toxicity. Environ. Hlth Perspect. 106 (Suppl. 2): 541-558.

Parchment, R.E., and M.J. Murphy. 1997. Human Hematopoietic Stem Cells: Laboartory assessment and response to toxic injury. In: Comprehensive Toxicology. (Sipes, G.I., C.A. McQueen, and A.J. Gandolfi, eds). Volume 4. New York: Pergamon. 335-362.

Parchment, R.E., D.A. Volpe, P.M. LoRusso, C.L. Erickson-Miller, M.J. Murphy, and C.K. Grieshaber. 1994. *In Vitro-In Vivo* Correlation of Myelotoxicity of 9-Methoxypyrazoloacridine to Myeloid and Erythroid Hematopoietic Progenitors from Human, Murine and Canine Marrow. J. Natl. Cancer Inst. 86: 273.

Parent-Massin, D. 2000. Relevance of Clonogenio Assays in Food Haematotoxicology. In: Progress in the Reduction, Refinement and Replacement of Animal Experimentation. (Balls, M., A-M van Zeller, M.E. Halder, eds). Amsterdam: Elsevier. 709-714.

Parent-Massin D, and D. Thouvenot. 1995. *In Vitro* Toxicity of Tricothecenes on Rat Haematopoietic Progenitors. Food Additives and Contaminants 12: 41-49.

Parent-Massin D, and D. Thouvenot. 1993. In Vitro Study of Pesticide Hematotoxicity in Human and Rat Progenitors. Jour. of Pharm. and Toxicol. Meth. 30: 203-207.

Parrish, A.R., A. J. Gandolfi and K. Brendel. 1995. Precision-cut Tissue Slices; Applications in Pharmacology and Toxicology. Life Sci. 57(21): 1887-901.

Paterson, S., and D. Mackay. 1989. Correlation of Tissue, Blood, and Air Partition Coefficients of Volatile Organic Chemicals. Brit. J. Indust. Med. 46: 321-328.

Peloux, A. F., C. Federici, N. Bichet, D. Gouy, and J.P. Cano. 1992. Hepatocytes in Primary Culture: An Alternative to LD50 Testing? Validation of a Predictive Model by Multivariate Analysis. ATLA 20: 8-26.

Pennie, W.D., J.D. Tugwood, G.J.A. Oliver, and I. Kimber. 2000. The Principles and Practice of Toxicogenomics: Applications and Opportunities. Toxicol. Sci. 54: 277-283.

Pessina, A., B. Albella, J. Bueren, P. Bramtom, S. Casati, G. Corrao, L. Gribaldo, R. Parchment, D. Parent-Massin, M. Piccirillo, B. Rio, S. Sacchi, G. Schoeters, and R. van den Heuvel. 2000. Method Development for a Prevalidation Study of the *In Vitro* GM-CFU Assay for Predicting Myelotoxicity. In: Progress in the Reduction, Refinement and Replacement of Animal Experimentation. (Balls, M., A-M van Zeller, M.E. Halder, eds). Amsterdam: Elsevier. 679-692.

Pessina, A., M.G. Neri, E. Mineo, M. Piccirillo, L. Gribaldo, P. Brambilla, G. Zaleskis, and P. Ujhazy. 1997. Expression of B Cell Markers on SR-4987 Cells Derived from Murine Bone Marrow Stroma. Exp. Haematol. 25: 436-541.

Pessina, A., M. Piccirillo, E. Mineo, P. Catalani, L. Gribaldo, E. Marafante, M.G. Neri, and A. Raimondi. 1999. Role of SR-4987 Stromal Cells in the Modulation of Doxorubicin Toxicity to *In Vitro* Granulocyte-Macrophage Progenitors (GM-CFU). Life Sci. 65: 513-523.

Pfaller, W., and G. Gtraunthaler. 1998. Nephrotoxicity Testing *In Vitro*—What We Know and What We Need to Know. Environ. Hlth Perspect. 106 (Suppl. 2): 559-570.

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.

Piacibello, W., D. Ferrero, F. Sanavio, R. Badoni, A. Stacchini, A. Sverino, and M. Aglietta. 1991. Responsiveness of Hghly Enriched CFU-GM Subpopulations from Bone Marrow, Peripheral Blood, and Cord Blood to Hemopoietic Growth Inhibitors. Exp. Hematol. 19: 1084.

Piper, H.M., I. Probst, P. Schwartz, F.J. Hutter, and P.G. Spieckermann. 1982. Culturing of Calcium Stable Adult Cardiac Myocytes. J. Mol. Cell. Cardiol. 14: 397-412.

Poliquin, J.F., and J. Crepeau. 1985. Immune Defence Mechanisms of the Nasal Mucosa. J. Otolaryngol. 14: 80-84.

Pomerat, C., and C.D. Lake. 1954. Short Term Cultures for Drug Assays: general considerations. Ann. New York Acad. Sci. 58: 1110-1128.

Reddel, R.R., Y. Ke, M.E. Kaighn, L. Malan-Shibley, J.F. Lechner, J.S. Rhim, and C.C. Harris. 1988. Human Bronchial Epithelial Cells Neoplastically Transformed by v-Ki- ras: Altered response to inducers of terminal squamous differentiation. Oncogene Res. 3: 401-408.

Rhee, H.M., B.J. Song, S. Cushman, and S.E. Shoaf. 1995. Vascular Reactivity in Alcoholic Rat Aortas: *In vitro* reactions between catecholamines and alcohol. Neurotoxicology 16: 179-185.

Rhodes, C. 1988/89. Current Status of *In Vitro* Toxicity Tests in the Industrial Setting: A European Viewpoint. *In Vitro* Toxicology 2: 151-160.

Riddell, R.J., D.S.Panacer, S.M. Wilde, R.H. Clothier, and M. Balls. 1986. The Importance of Exposure Period and Cell Type in *In Vitro* Cytotoxicity Tests. ATLA 14: 86-92.

Rodriguez-Barbero, A., B. L'Azou, J. Cambar, and J.M. Lopez-Novoa. 2000. Potential Use of Isolated Glomeruli and Cultured Mesangial Cells as *In Vitro* Models to Assess Nephrotoxicity. Cell Biol. and Toxicol. 16(3): 145-153.

Rose, M.C., F.M. Piazza, Y.A. Chen, M.Z. Alimam, M.V. Bautista, N. Letwin, and B. Rajput. 2000. Model Systems for Investigating Mucin Gene Expression in Airway Diseases. J. Aerosol Med. 13: 245-262.

Rowan, A.N., and A.M. Goldberg. 1985. Perspectives on Alternatives to Current Animal Testing Techniques in Preclinical Toxicology. Ann. Rev. Pharmacol. Toxicol. 25: 225-247.

Satoh, N., T.M Suter, R. Liao, and W.S. Colucci. 2000. Chronic Alpha-Adregenergic Receptor Stimulation Modulates the Contractile Phenotype of Cardiac Myocytes *In Vitro*. Circulation Oct. 31, (18): 2249-2254.

Saunders, K.B., and P.A. D'Amore. 1992. An *In Vitro* Model for Cell-Cell Interactions. *In Vitro* Cell. Dev. Biol. 28: 521-528.

Sawyer, D.B., R. Fukazawa, M.A. Arstall, and R.A. Kelly. 1999. Daunorubicin-Induced Apoptosis in Rat Cardiac Myocytes in Inhibited by Dexrazoxane. Circ. Res. Feb. 19, 84(3): 257-265.

Scandinavian Society of Cell Toxicology. 1999. Programs and Abstracts from the 17th SSCT Annual Workshop on *In Vitro* Toxicology in Röstånga, Sweden. September 23-26, 1999.

Schlede, E., U. Mishke, W. Diener, and D. Kayser. 1994. The International Validation Study of the Acute-Toxic-Class Method (oral). Arch. Toxicol. 69: 659-670.

Schlede, E., U. Mishke, R. Roll, and D. Kayser. 1992. A National Validation Study of the Acute-Toxic-Class Method—An Alternative to the LD50 Test. Arch. Toxicol. 66: 455-470.

Schwetz, B., and D. Gaylor. 1998. Alternative Tests: Carcinogenesis as an Example. Environ. Hlth Perspect. 106 (Suppl. 2): 467-471.

Seibert, H., M. Balls, J.H. Fentem, V. Bianchi, R.H. Clothier, P.J. Dierickx, B. Ekwall, M.J. Garle, M.J. Gómez-Lechón, L. Gribaldo, M. Gülden, M. Liebsch, E. Rasmussen, R. Roguet, R. Shrivastava, and E. Walum. 1996. Acute Toxicity Testing *In Vitro* and the Classification and Labeling of Chemicals: The report and recommendations of ECVAM Workshop 16. ATLA 24: 499-510.

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. 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.

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

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.

Smith, C.G., J.E. Grady, and J.I. Northam. 1963. Relationship between Cytotoxicity *In Vitro* and Whole Animal Toxicity. Cancer Chemother. Rep. 30: 9-12.

Smith, E.L., and A.H. Hainsworth. 1998. Acute Effects of Interleukin-1 Beta on Noradrenaline Release from the Human Neuroblastoma Cell Line SH-SY5Y. Neurosci. Lett. 251: 89-92.

Soni, M.G., S.K. Ramaiah, M.M. Mumtaz, H.J. Clewell, and H.M. Mehendale. 1999. Toxicant-Inflicted Injury and Stimulated Tissue Repair Are Opposing Toxicodynamic Forces in Predictive Toxicology. Reg. Toxicol. Pharmacol. 29:165-174.

Speralakis, N., and R. Shigenoubu. 1974. Organ Cultured Chick Embryonic Heart Cells of Various Ages. Part I. Electrophysiology. J. Mol. Cell. Cardiol. 6: 449-471.

Spielmann, H. 1998. Reproduction and Development. Environ. Hlth Perspect. 106 (Suppl. 2): 571-575.

Spielmann, H., M. Balls, J. Dupuis, W.J.W. Pape, G. Pechovitch, O. de Silva, H-G. Holzhütter, R. Clothier, P. Desolle, F. Gerberick, M. Liebsch, W.W. Lovell, T. Maurer, U. Pfannenbecker, J.M. Potthast, M. Csato, D. Sladowski, W. Steiling, and P. Brantom. 1998. EU/COLIPA "*In Vitro P*hototoxicity" Validation Study, Results of Phase II (blind trial), Part 1: the 3T3 NRU phototoxicity test. Toxicol. *In Vitro* 12: 305-327.

Spielmann, H., N.P. Bochkov, L. Costa, L. Gribaldo, A. Guillouzo, J.H. Heindel, M. Karol, R. Parchment, W. Pfaller, P. Prieto Peraita, and T. Zacharewski. 1998. Alternative Testing Methodologies for Organ Toxicity. Env Hlth Persp. 106 (suppl 2): 427-439.

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.

Spielmann, H., M. Liebsch, and S. Kalweits. 1996. Results of a Validation Study in Germany on Two *In Vitro* Alternatives to the Draize Eye Irritation Test, the HET-CAM Test and the 3T3-NRU Cytotoxicity Test. ATLA 24: 741-858.

Stanness, K., L. Westrum, P. Mascagni, E. Fornaciari, J. Nelson, and D. Janigro. 1997. Morphological and Functional Characterization of an *In Vitro* Blood-Brain Barrier Model. Brain Res. 771: 329-342.

Stokes, W.S. and E. Marafante. 1998. Introduction and Summary of the 13th Meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC): Alternative testing methodologies. Environ. Hlth Perspect. 106 (Suppl. 2): 405-412.

Svendsen, C.N., M.A.Caldwell, J. Shen, M.G. ter Borg, A.E. Rosser, P. Tyers, S. Karmiol, and S.B. Dunnett. 1997. Long-Term Survival of Human Central Nervous System Progenitor Cells Transplanted into a Rat Model of Parkinson's Disease. Exp. Neurol. 148: 135-146.

Tanaka, N. M. Asakura, C. Hattori et al. 1998. Validation Study on Five Cytotoxicity Assays by JSAAE. IV. Details of the colony formation assay. AATEX 5: 74-86.

Taylor, T.J., N.C. Smoth, M.J. Langford, and G.W. Parker Jr. 1995. Effect of Palytoxin on Endothelium-Dependent and -Independent Relaxation in Rat Aortic Rings. J. Appl. Toxicol. 15: 5-12.

Tennant, R.W. 1998. Evaluation and Validation Issues in the Development of Transgenic Mouse Carcinogenicity Bioassays. Environ. Hlth Perspect. 106 (Suppl. 2): 473-476.

Trevan, J.W. 1927. The Error of Determination of Toxicity. Proceedings of the Royal Society (London). Series B 101: 483-514.

Trollinger, D.R., W.E. Cascio, and J.J. Lemasters. 2000. Mitochondrial Calcium Transients in Adult Rabbit Cardiac Myocytes: Inhibition by ruthenium red and artifacts caused by lysosomal loading of Ca(2+)-indicating fluorophores. Biophys J Jul, 79:39-50

Ulrich, R.G., J. A. Bacon, C. T. Cramer, G. W. Peng, D. K. Petrella, R. P. Stryd and E. L. Sun. 1995. Toxicol. Lett. 82-83: 107-115.

Vainchenker, W. 1995. Megakaryocytopoiesis: Cellular aspects and regulation. Crit. Rev. Oncol. Hematol. 20: 165-192.

van der Heuvel, M.J. 1990. Editorial: An Alternative to the LD50? Human & Experimental Toxicol. 9: 369-370.

Vermes, I., C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger. 1995. A Novel Assay for Apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. Jour. Immunol. Meth. 184: 39-51.

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.

Wade, J.A., P.F. Vaughan, and C. Peers. 1998. Hypoxia Enhances [3H]Noradrenaline Release Evoked by Nicotinic Receptor Activation from the Human Neuroblastoma SH-SY5Y. J. Neurochem. 71: 1482-1489.

Walker, C.H. 1998. Biomarker Strategies to Evaluate the Environmental Effects of Chemicals. Environ. Hlth Perspect. 106 (Suppl. 2): 613-620.

Walker, C., K. Kaiser, W. Klein, L. Lagadic, D. Peakall, S. Sheffield, T. Soldan, and M. Yasuno. 1998. 13th Meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC): Alternative Testing Methodologies for Ecotoxicity. Environ. Hlth Perspect. 106 (Suppl. 2): 441-451.

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

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.

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.

Walum, E., and A. Peterson. 1984. On the Application of Cultured Neuroblastoma Cells in Chemical Toxicity Screening. Jour. of Toxicol.and Environ. Hlth 13: 511-520.

Wakuri, S., J. Izumi, K. Sasaki, N. Tanaka, and H. Ono. 1993. Cytotoxicity Study of 32 MEIC Chemicals by Colony Formation and ATP Assays. Toxicol. *In Vitro* 7: 517-521.

Wang, S., G. Wright, W. Geng, and G.L. Wright. 1997. Retinol Influences Contractile Function and Exerts an Anti-Proliferative Effect on Vascular Smooth Muscle Cells through an Endothelium-Dependent Mechanism. Pflugers Archiv - Eur J. Physiol. 434: 669-677.

Wang, Y-C, N. Neckelmann, A. Mayne, A. Herskoqit, A. Srinivasan, K.W. Sell, and A. Ahmed Ansair. 1991. Establishment of a Human Fetal Cardiac Myocyte Cell Line. *In Vitro* Cell Dev. Biol. 27: 63-74.

Waring, J.F.and R.G. Ulrich. 2000. The impact of genomics-based technologies on drug safety evaluation. Annual Review of Pharmacology and Toxicology. 40: 335-352.

Werle, B., W. Ebert, W. Klein, and E. Spiess. 1994. Cathepsin B in Tumors, Normal Tissue and Isolated Cells from the Human Lung. Anticancer Res. 14: 1169-1176.
Wesche, D.L., B.G. Schuster, W.X. Wang, and R.L. Woosley. 2000. Mechanism of Cardiotoxicity of Halofantrine. Clin. Pharmacol. Ther. May, 67(5): 521-529.

Wormser, U., and Ben-Zakine S. 1990. The Liver Slice System: An *In Vitro* Acute Toxicity Test for the Assessment of Hepatotoxins and the Antidotes. Toxicol. *In Vitro* 4: 449-451.

Worth, A.P., M.D. Barratt, and J.B. Houston. 1998. The Validation of Computational Prediction Techniques. ATLA 26: 241-247.

Younkin, D.P., C-M.Tang, M. Hardy, U.R. Reddy, Q-Y. Shi, S.J. Pleasure, V. Lee, and D. Pleasure. 1993. Inducable Expression of Neuronal Glutamate Receptor Channels in the NT2 Human Cell Line. Proc. Natl. Acad. Sci. USA 90: 2174-2178

Yu, P.H., C.T. Lai, and D.M. Zuo. 1997. Formation of Formaldehyde from Adrenalin *In Vivo*: A potential risk factor for stress-related angiopathy. Neurochem. Res. 22: 615-20.

Zacharewski, T. 1998. Identification and Assessment of Endocrine Disruptors: Limitations of *In Vivo* and *In Vitro* Assays. Environ. Hlth Perspect. 106 (Suppl. 2): 577-579.

Zanetti, C., I. De Angelis, A-L. Stammati, and F. Zucco. 1992. Evaluation of Toxicity Testing of 20 MEIC Chemicals on Hep-2 Cells Using Two Viability Endpoints. ATLA 20: 120-125.

Zbinden, G., and M. Flury-Roversi. 1981. Significance of the LD50 Ttest for the Toxicological Evaluation of Chemical Substances. Arch. of Toxicol. 47: 77-99.

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

Hyatt Regency Crystal City Hotel, Arlington, VA

Detailed Workshop Agenda

Tuesday, October 17, 2000

7:30 a.m.	**Registration**
8:30 a.m.	**Opening Plenary Session – Regency Ballroom F**
8:30 a.m.	Welcome from the National Toxicology Program (NTP) Dr. John Bucher, Deputy Director, ETP, NIEHS
8:35 a.m.	Workshop Introduction Dr. Philip Sayre, U.S. EPA, OPPTS, Co-Chair Workshop Organizing Committee
8:45 a.m.	Special Presentation on Dr. Bjorn Ekwall: Contributions to *In Vitro* Toxicology Dr. Erik Walum, Pharmacia & Upjohn AB, Stockholm, Sweden
8:55 a.m.	Role of ICCVAM and the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) in the Validation and Acceptance of New Methods Dr. William Stokes, NIEHS, Co-Chair ICCVAM
9:10 a.m.	Acute Toxicity: Historical and Current Regulatory Perspectives Dr. Steve Galson, Director, Office of Science Policy and Coordination, U.S. EPA
9:40 a.m.	Acute Toxicity Data: A Clinical Perspective Dr. Jim Cone, Chief, Occupational Health Branch, California Dept. of Health Services
10:10 a.m.	**Coffee Break**
10:30 a.m.	***In Vitro* Approaches to Estimate the Acute Toxicity Potential of Chemicals** Estimating Starting Doses for *In Vivo* Studies using *In Vitro* Data Dr. Manfried Liebsch, ZEBET – Center for Documentation and Evaluation of Alternative Methods to Animal Experiments
11:00 a.m.	An Integrated Approach for Predicting Acute Systemic Toxicity Dr. Bas Blaauboer, Research Institute of Toxicology (RITOX), Utrecht University
11:30 a.m.	Opportunities for Future Progress Dr. Oliver Flint, Bristol-Meyers Squibb
12:00 p.m.	**Public Comment**
12:15 p.m.	**Breakout Group Charges** Dr. John Frazier, DOD Tri-Service Toxicology Lab, USAF, Co-Chair Workshop Organizing Committee
12:30 p.m.	**Lunch Break**
1:45 p.m.	**Breakout Groups: Identify Needs** 1. Screening Methods (Regency Ballroom F) 2. Toxicokinetic Determinations (Arlington Room) 3. Predicting Organ Specific Toxicity and Mechanisms (Fairfax Room) 4. Chemical Data Sets for Validation (Prince William Room)
3:30 p.m.	**Coffee Break**
4:00 p.m.	**Breakout Groups (Cont'd)**
5:30 p.m.	**Adjourn for Day**
6:00 p.m.	**Shuttle Begins between Hyatt Regency and Potowmack Landing Restaurant**
7:00 p.m.	**Dinner (Chart Room) – Pre-registration was required by October 9th.**
8:00 p.m.	**Dinner Speaker – Professor Michael Balls, ECVAM *"In Vitro Toxicology: Perspectives on Past and Future Progress"***
8:45 p.m.	**Shuttle Begins between Potowmack Landing Restaurant and Hyatt Regency**

Wednesday, October 18, 2000

8:00 a.m. **Plenary Session – Status Reports by Breakout Group Co-Chairs – Regency Ballroom F**
(Moderator: Dr. Philip Sayre, U.S. EPA, OPPTS, Co-Chair Workshop Organizing Committee)
8:40 a.m. **General Discussion**
9:00 a.m. **Breakout Group: Current Status**
1. Screening Methods (Regency Ballroom F)
2. Toxicokinetic Determinations (Arlington Room)
3. Predicting Organ Specific Toxicity and Mechanisms (Fairfax Room)
4. Chemical Data Sets for Validation (Prince William Room)

10:30 a.m. **Coffee Break**
10:45 a.m. **Breakout Groups (Cont'd)**
12:00 p.m. **Lunch Break**
1:30 p.m. **Breakout Groups (Cont'd)**
3:30 p.m. **Coffee Break**
4:00 p.m. **Breakout Groups (Cont'd)**
5:30 p.m. **Adjourn for the Day**

Thursday, October 19, 2000

8:00 a.m. **Current Status Plenary Session – Status Reports by Breakout Group Co-Chairs – Regency Ballroom F**
(Moderator: Dr. John Frazier, DOD Tri-Service Toxicology Lab, USAF, Co-Chair Workshop Organizing Committee)
8:40 a.m. **General Discussion**
9:00 a.m. **Breakout Groups: Future Directions**
1. Screening Methods (Regency Ballroom F)
2. Toxicokinetic Determinations (Arlington Room)
3. Predicting Organ Specific Toxicity and Mechanisms (Fairfax Room)
4. Chemical Data Sets for Validation (Prince William Room)

10:30 am **Coffee Break**
10:45 a.m. **Breakout Groups (Cont'd)**
12:00 p.m. **Lunch Break**
1:30 p.m. **Breakout Groups (Cont'd)**
3:30 p.m. **Coffee Break**
4:00 p.m. **Breakout Groups (Cont'd)**
5:30 p.m. **Adjourn for the Day**

Friday, October 20, 2000

8:00 a.m. **Closing Plenary Session – Reports by Breakout Group Co-Chairs – Regency Ballroom A/B**
(Moderator: Dr. William Stokes, NIEHS, Co-Chair ICCVAM)
8:00 a.m. Screening Methods *(30 min/15 min discussion)*
8:45 a.m. Toxicokinetic Determinations *(30 min/15 min discussion)*
9:30 a.m. Predicting Organ Specific Toxicity and Mechanisms *(30 min/15 min discussion)*
10:15 a.m. **Coffee Break**
10:45 a.m. **Closing Plenary Session – Reports by Breakout Group Co-Chairs (Cont'd)**
10:45 a.m. Chemical Data Sets for Validation of *In Vitro* Testing Methods for Assessing Acute Toxicity *(30 min/15 min discussion)*
11:30 a.m. **Public Comment**
12:00 p.m. **Closing Comments**
12:15 p.m. **Adjourn**

Summary of Opening Plenary Session and Public Comments

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

October 17-20, 2000

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

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

National Institute of Environmental Health Sciences (NIEHS)

Opening Plenary Session

Speakers:

- Dr. John Frazier, USAF/ICCVAM, Workshop Co-Chair
- Dr. Philip Sayre, EPA/OPPT/ICCVAM, Workshop Co-Chair
- Dr. William Stokes, NIEHS/ICCVAM/NICEATM
- Dr. John Bucher, NIEHS
- Dr. Steve Galson, EPA/OPPT
- Dr. James Cone, California Department of Health Services
- Dr. Manfred Liebsch, ZEBET
- Dr. Bas Blaauboer, Research Institute of Toxicology, Utrecht University
- Dr. Oliver Flint, Bristol-Meyers Squibb

Call to Order and Introductions

Dr. William Stokes called the workshop to order at 8:38 a.m. Dr. Stokes explained that the Workshop was organized by ICCVAM and NICEATM and was co-sponsored by the U.S. Environmental Protection Agency (EPA), the National Institute of Environmental Health Sciences (NIEHS) and the National Toxicology Program (NTP). He thanked everyone for their participation and attendance. He discussed the goals of ICCVAM and NICEATM stating that the overall goal is to validate and achieve regulatory acceptance of test methods that will provide improved protection of human health and the environment, while incorporating the three Rs for the use of animals (refinement, reduction and replacement) whenever scientifically feasible. He stated that the purpose of the workshop was to evaluate the validation status of *in vitro* test methods for assessing acute systemic toxicity. He reviewed the functions of ICCVAM, which include the technical evaluation of new methods including independent scientific peer reviews, and organizing expert panel meetings to review test methods at various stages of development and validation. Dr. Stokes concluded by stating that ICCVAM also organizes workshops to identify additional research and validation efforts necessary to develop and further enhance the usefulness of new methods.

Welcome from the National Toxicology Program (NTP)

Dr. Stokes introduced Dr. John Bucher of NIEHS as the next speaker. Dr. Bucher thanked Dr. Stokes and welcomed the participants of the workshop. He conveyed the regrets of Dr. Christopher Portier of NIEHS/NTP who was unable to attend the workshop and then thanked the ICCVAM agencies and the

U.S. EPA for the effort provided for the workshop. Dr. Bucher remarked that the purpose of the workshop was to seek scientific advice and opinion concerning alternative test methods. He expressed hope that the scientists would work to advance alternatives for acute toxicity testing and provide information to move *in vitro* alternative tests forward. He concluded by thanking the workshop participants for their knowledge, experience and time.

Workshop Objectives

Dr. Sayer reintroduced the objectives of the workshop, provided background remarks and listed points for the participants to consider: 1) determine the hazards of chemicals by alternative methods; 2) find non-lethal acute toxicity testing endpoints; and 3) ascertain which *in vitro* methods might be helpful and could be validated. He challenged the scientists to review *in vitro* screening methods for toxicokinetics and specific organ toxicity and to recommend applicable methods for pre-validation and validation studies. Dr. Sayre asked the scientists to recommend validation study designs, to determine lists of reference chemicals and to prioritize *in vitro* methods.

Dr. Sayre discussed the general structure of the workshop. Four breakout groups would investigate their respective topics and the invited expert scientists would lead the discussions. Time would be made available for public comment at the meetings. The workshop would begin each morning with a short plenary session to discuss the previous day's activities and would end each evening with a meeting of the co-chairs and rapporteurs. A final report from each breakout group would be compiled as a workshop report ready for publishing by January 2001. He also said that a workshop monograph could be published by NIEHS' Environmental Health Perspectives Supplements in April 2001. Dr. Sayre concluded his remarks by naming the organizing committee for the workshop and then thanked everyone for their work.

Memoriam for Björn Ekwall

Dr. Stokes thanked Dr. Sayre and continued the session by mentioning the recent untimely death of Dr. Björn Ekwall. He spoke of Dr. Ekwall's extensive contributions and dedication to alternative test method development. Dr. Stokes then introduced Dr. Erik Walum, a close friend and colleague of Dr. Ekwall.

Dr. Walum described Dr. Ekwall as a medical doctor and toxicologist who pushed seriously for implementation of *in vitro* test methods. He discussed Dr. Ekwall's life and work in Uppsala, Sweden and related Dr. Ekwall's belief that the United States must accept *in vitro* alternative testing methods in order for the world to embrace the methodology. Dr. Ekwall established the Scandinavian Cell Toxicology Society whose mission is to gather scientists for meetings and show that chemical effects on cells should translate to *in vivo* effects. He initiated the Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC) to test 50 chemicals and collect the results. Sixty-five different test methods were employed for testing the chemicals. He introduced the concept to test compounds in simple systems such as cell cultures and to extrapolate the results to human toxicity. He felt that one could break down systems to elementary parts then analyze them by *in vitro* methods. Dr. Walum concluded his remarks by relating that Dr. Ekwall knew that if he were not able to continue his work, then someone else would take over. Dr. Stokes thanked Dr. Walum for his remarks.

The Role of ICCVAM

Dr. Stokes described the evolution, structure, and function of ICCVAM, and its role in facilitating the development and validation of alternative test methods. The driving forces for the establishment and need for ICCVAM were listed: 1) the opportunity to incorporate new science and technologies into toxicological testing practices; 2) the potential benefits of improved prediction of toxicity, improved efficiency and improved animal welfare; 3) legislation including the NIH Revitalization Act of 1993

(Public Law 103-43); and 4) the need for development and validation of test methods for new endpoints of concern, such as the Endocrine Disruptor Screening and Testing Program at EPA. ICCVAM also fulfills other mandates provided to NIEHS by Public Law 103-43, such as alternative test method development and validation.

Dr. Stokes related that ICCVAM began as an ad hoc committee comprised of representatives from 15 Federal regulatory and research agencies in September 1994. The committee developed a report on criteria and processes for the validation and regulatory acceptance of toxicological test methods that was published in 1997. A standing ICCVAM committee was established in May 1997 to implement the Public Law 103-43 mandate that NIEHS establish a process to achieve the regulatory acceptance of scientifically valid alternative methods. The committee evaluates proposed test methods and provides recommendations to Federal agencies, which in turn decide the regulatory acceptability of the methods. He explained that NICEATM is located at NIEHS and provides operational and technical support for ICCVAM by co-organizing workshops and peer reviews of test methods, disseminating information, and developing partnerships with stakeholders.

Dr. Stokes reviewed the prerequisites for using new methods which include: 1) adequate validation, which involves determining the reliability and relevance of test methods for specific purposes, and 2) acceptance, which involves determination of the acceptability for regulatory risk assessment purposes. The evolution process for new testing includes: the review of existing risk assessment methods, research, development, pre-validation, validation, peer review, regulatory acceptance, and implementation. The current ICCVAM/NICEATM role in test method development and validation is to provide information, to evaluate test methods, and to provide recommendations to agencies. The objectives of ICCVAM Workshops include: to evaluate the adequacy of current test methods; to identify toxicological endpoints; to identify promising methods which need further development and validation; to recommend appropriate validation studies; and to recommend research and model development efforts needed to support improved test methods for specific toxicity endpoints. ICCVAM/NICEATM has completed independent peer review evaluations for the following tests: 1) the murine local lymph node assay (LLNA); 2) Corrositex□; 3) FETAX; and 4) the revised UDP. Dr. Stokes concluded his presentation by acknowledging the contributions of the ICCVAM Agency Representatives, the ICCVAM Workshop Organizing Committee, and the NICEATM staff.

Acute Toxicity Testing: Historical and Current Regulatory Perspectives

Dr. Galson began by saying that the workshop represents the working relationship of EPA and NIEHS. He thanked Dr. Richard Hill of the EPA and Dr. Stokes for their work and participation in the workshop. He acknowledged the animal welfare groups for their role in pushing forward the objectives of alternative testing. He also thanked Dr. Amy Rispin of the EPA for her contributions to forwarding alternative testing. Dr. Galson said the EPA committee assures that the 3Rs will be the primary objective of the workshop and the committee will work toward regulatory acceptance with the protection of public health foremost in mind.

Dr. Galson spoke of alternative methods for determining acute toxicity being used by the regulatory agencies to revise acute toxicity studies. The long-term goal is to develop *in vitro* methods to replace animals and recommendations from the workshop participants will move *in vitro* methods forward. He outlined the current methods used for determining acute toxicity as the "classical" LD50 test and OECD Acute Oral Toxicity Tests 401, 420, 423 and 425. He related that OECD 401 test was to be dropped and that U.S. agencies will accept this decision.

Regulatory uses of acute toxicity data include hazard labeling (only EPA requires), hazard classification (LD50 dose points – required by some EPA offices, e.g., Office of Pesticide Programs), and risk

assessment. Dr. Galson listed the regulatory agencies and illustrated how they use hazard labels, and how they receive data and perform risk assessment. It is important to harmonize test methods between the various federal agencies (CPSC, DOT, OSHA, EPA, FDA, NIOSH, and ATSDR). Dr. Galson concluded by urging the workshop participants to revise methods for determining acute toxicity and to meet the scientific challenges. Recommendations of the workshop would be relevant to the federal regulatory agencies, in particular, the EPA for the HPV chemical program. Dr. Stokes thanked Dr. Galson and then introduced Dr. James Cone who would speak about clinical perspectives in occupational health.

Acute Toxicity Data -- A Clinical Perspective

Dr. Cone defined acute toxicity as health effects resulting from exposure over a short period of time. Though no single definition for acute exposure had been agreed upon, he felt that unintended releases of chemicals into the environment and poisonings would constitute a working definition. Many chemicals have acute toxicity human data and he related the clinician's experience with acute toxicity data by listing the available tools: Physicians Desk Reference (PDR), Material Safety Data Sheets (MSDS), poison control centers (PCC), Medline searches, the internet and the telephone. Knowledge is often based on human exposure. The clinician views acute toxicity as an immediate exposure to a substance while chronic toxicity occurs from exposure over a long period of time.

Dr. Cone discussed two incidents of toxic exposure that occurred in California. One incident involved a four-hour release/spill of oleum into the environment and required the evaluation of 20,000 residents at local emergency facilities. A second case study resulted from the release of 19,000 gallons of metam sodium into a river. Problems faced by agencies responding to these incidents included determining: the toxic agent, the acute health effects of the release, medical treatment and whether evacuation of the area was necessary. Exposure assessment was difficult in these cases because of differences in the odor threshold and the irritant threshold. It was important to know whether the substance traveled as a plume or flowed in the waterways. Dr. Cone discussed the examination of personnel close to the spills and the difficulty in detecting acute exposure in the individuals.

Dr. Cone suggested that the clinician's tools for measuring acute toxicity are mostly crude. Data from HSDB may be too old, as are data for threshold limit values (TLV) and legal permissible exposure limits (PELs). The limitations of the existing toxicity data include the lack of acute toxicity data for some chemicals and the lack of toxicity information for exposure to multiple chemicals, which is a common exposure scenario for humans. Dr. Cone also provided sources/websites of acute toxicity data. Dr. Cone stated that the clinician is challenged on how to interpret acute toxicity data on chemicals and on how to keep updated on human data. Dr. Cone ended his presentation by reminding the participants of the Nuremberg Code for Medical Experimentation on Humans. Dr. Stokes thanked Dr. Cone and dismissed the participants for a break.

In Vitro Approaches to Estimate the Acute Toxicity Potential of Chemicals

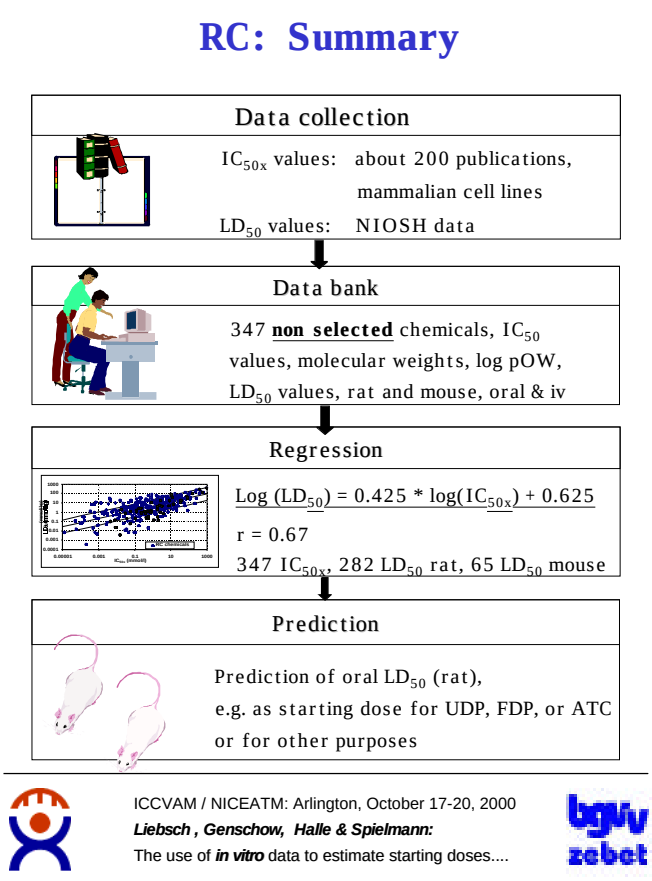
Dr. John Frazier opened the second phase of the plenary session by introducing Dr. Manfred Liebsch from the Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET).

Estimating Starting Doses for *In Vivo* Studies using *In Vitro* Data

Dr. Liebsch began his presentation with an overview of ZEBET, which is part of the Federal Institute for Health Protection of Consumers and Veterinary Medicine of Germany. The three divisions of ZEBET are for documentation, evaluation and research. ZEBET uses *in vitro* data for prediction of *in vivo* toxicity. One hundred ten chemicals were evaluated in 1954 and another 15 chemicals were evaluated in 1956

using data from Dr. Willi Halle (Registry of Cytotoxicity) and Dr. Björn Ekwall (MEIC). Dr. Halle produced a monograph, which include a registry of 347 chemicals, in 1998. Dr. Liebsch provided the scheme used for predicting starting doses for acute toxicity tests for these chemicals: NIOSH data → concentration response curve → databank → regression → prediction of starting dose.

The Registry of Cytotoxicity (RC) acceptance criteria includes: 1) *in vitro* IC50 data gathered from the literature; 2) data from mammalian primary cells or cell lines (no hepatocytes); 3) chemical incubation time ≥ 16 hours; and 4) data from two different laboratories or two different cell types or two cytotoxicity endpoints. *In vitro* cytotoxicity endpoints include cell profiles, viability (MTT, Neutral Red, Trypan Blue data) and markers for differentiation. *In vivo* LD50 data includes only values found in NIOSH databases. If more than one LD50 value is available, then the largest value is used. LD50 data from rats and mice (oral and iv route) were collected; rat data are preferred. The ZEBET chemical list was shown and IC50x (i.e., geometric mean of IC50s for each chemical) values were discussed.

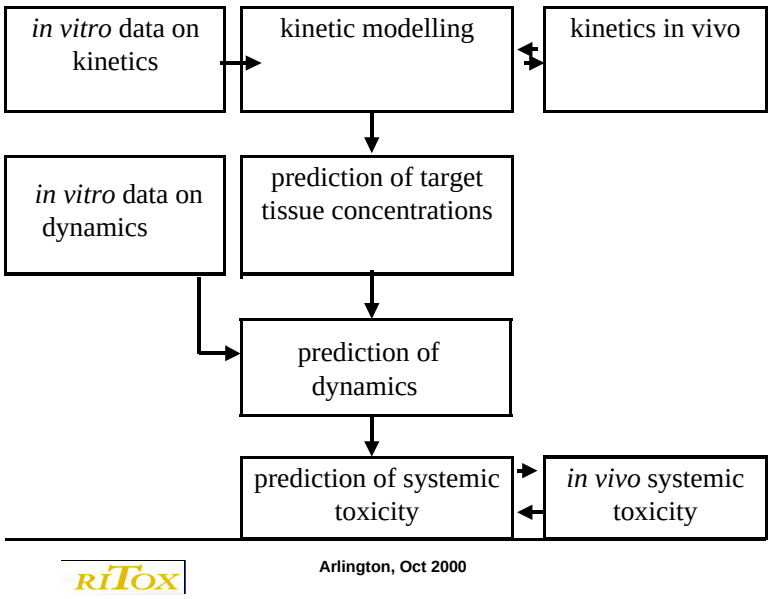


Dr. Liebsch presented the RC method of validation: LD50 = a + b x log IC50x (a = intercept, b = regression coefficient, r = correlation coefficient). Changes in the estimates of a, b, and r were small for the four regression analyses of the RC using 102, 117, 230, and 347 chemicals. The regression analysis provides a better prediction of LD50 for less toxic chemicals. Dr. Liebsch continued by discussing ECVAM Workshop 16 (1994) that produced 10 recommendations for determining starting doses. He discussed the UDP test, which uses sequential dosing starting close to the LD50 value, and said that the RC data could predict acute oral LD50s. One would determine the IC50 in a cytotoxicity test, predict the LD50 using the RC, and then determine the LD50 in the animal. A tiered approach to the LD50, as shown in Dr. Liebsch's slide on the left, would use a cytotoxicity test to determine the starting dose for non-toxic chemicals where only the highest dose is applied (Limit Test). In a classification of 1115 industrial chemicals for acute toxicity in Europe, the majority were found to be non-toxic. Dr. Liebsch concluded his presentation with the following points: 1) the use of basal cytotoxicity to predict the oral LD50 for use as a starting dose will save 30-40% of animals used; 2) basal cytoxicity tests can be used to determine whether a Limit Test should be performed; 3) the increased number of toxicity classes in OECD-HCL guidelines will increase the animal saving effect of the tiered in vitro/in vivo approach; and 4) lower animal use is predicted and validation of animal reduction is needed. His final point was that all of the effort is worth it to reduce animal testing. Dr. Frazier thanked Dr. Liebsch and then introduced Dr. Bas Blaauboer as the next speaker.

An Integrated Approach for Predicting Systemic Toxicity

Dr. Blaauboer introduced his presentation on how to integrate *in vitro* data in predictive toxicology. He challenged the workshop participants to eliminate animal use and discussed the Institute of Risk Assessment Sciences, the development of computer based biokinetic models, and *in vitro* tests. He provided a brief discussion of the ECITTS (ERGATT/CFN Integrated Toxicity Testing Scheme) project.

Dr. Blaauboer explained that the aim of "classical" toxicological risk assessment is to establish safety factors for human exposure. Classical *in vitro* toxicology methods are limited because they find concentration for effect instead of determining dose and it is difficult to extrapolate the data to an intact organism. There is also a lack of biotransformation/kinetics data and the tests concentrate on cytotoxicity rather than on mechanisms of importance *in vivo*. He presented the necessary building blocks to produce integrated models: 1) biokinetic modeling; 2) prediction of tissue concentration; 3) knowledge of effective concentration for relative targets; 4) prediction of these effective concentrations; and 5) calculation of doses relevant for risk assessment. He briefly discussed the European Research Group for Alternatives in Toxicity Testing (ERGATT) and the Swedish National Board for Laboratory Animals (CFN).



The ECITTS project building blocks are: 1) experimental – QSAR and *in vitro* data for biokinetics model; 2) modeling – *in vitro* data for PBBK models, determination of target tissue concentration; and 3) validation – validate against *in vivo* kinetics. The stepwise approach is: 1) determine the relevant parameters for biokinetic model, building model using non-animal data – physiochemical properties (e.g. tissue partition, air/blood partition) and data from cell culture systems (e.g., biotransformation, passage of cellular layers with barrier functions); 2) validate with *in vitro*/*in vivo* comparisons; 3) use *in vivo* data to construct or improve biokinetic model; extrapolate data from non-toxic doses; 4) estimate tissue concentration especially in target tissues; 5) use *in vitro* assays to get response surrogates; 6) integrate kinetic and dynamic data, as shown in Dr. Blaauboer's slide above; and 7) predict surrogate dose.

Dr. Blaauboer produced a list of compounds tested with a neural aspect (e.g., pesticides) and explained that the test strategy included: determination of basal cytotoxicity and morphological changes; determination of changes in cell physiology and neurochemistry; and determination of neurotoxic concentration (EC20). He illustrated this strategy using acrylamide as an example.

The following schematic would be used for the integrated use of alternative methods in toxicological risk assessment: structure of compound → chemical functionalities → QSAR → *in vitro* testing → classification of compound. This approach would lead to an *in vitro* test battery that could produce EC50 ratios, ultimately leading to limited *in vivo* testing. Dr. Blaauboer concluded that integrating *in vitro* data

in risk evaluation is valid provided biokinetics are taken into account and that the integration of all available data in a stepwise manner will improve risk assessment. Dr. Frazier thanked Dr. Blaauboer and introduced Dr. Oliver Flint.

Opportunities for Future Progress - *In Vitro* Approaches to Predicting Acute Toxicity

Dr. Flint opened his presentation by stating that *in vitro* tests used in a focused way could predict acute toxicity. He provided a test example: Taxol® Neuropathy – Successful *In Vitro* Prediction of Acute Toxicity. The objective was to characterize the neurotoxic effect of Taxol®. The *in vitro* model uses dorsal root ganglia cells and examines cytotoxicity, mitochondrial transport, morphology, and LDH leakage as endpoints. Dr. Flint discussed prediction of lethality as described by the MEIC project. He listed MEIC websites and suggested that mirror sites for the data be established. The basal cytotoxicity hypothesis for lethality using the 50 MEIC compounds correlates with human lethal plasma concentration. Problems with the basal cytotoxicity hypothesis are confounding factors such as interspecies differences in liver toxicity and specific toxicity for cell types; not all cell lines are alike.

He presented lessons in lethality predictions: 1) *in vitro* systems can make general predictions of *in vivo* toxicity; 2) human toxicity is best predicted by human cells; 3) variability is an unavoidable confounding factor; and 4) choosing the right cell is of critical importance. Future directions for predicting acute and other toxicities include computational predictions, molecular biology and *in vitro* systems targeting specific toxicological areas. *In silico* predictive toxicity is good for mutagenicity and carcinogenicity predictions, but weak for acute and reproductive toxicology. Dr. Flint presented the table, on the left, for the changing paradigm illustrating the great reduction of testing time using *in silico* predictions. He also discussed emerging technologies such as transcriptome, proteome, and metabonome and stated the usefulness and limitations of the techniques. Dr. Flint concluded by stating the need to develop new technologies to characterize predictive biomarkers and to investigate transcriptome and proteome for *in vitro* and metabonomics for *in vivo*.

The Changing Paradigm

	MUTA-GENICITY	CARCINO-GENICITY	TERATO-GENICITY
TRADITIONAL	1-Month Ames	2-Year Rodent Bioassay	4-Month Segment II Rodent Assay
PARADIGM SHIFT - ***In silico*** followed by:	1-Day DNA Damage Assay	2-6 Week Cell Transformation Assay	5-Day Cell Differentiation Assay

Public Comments:

Ms. Mary Beth Sweetland (PETA)

Ms. Sweetland spoke of the January 1997 Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC) conference on alternatives and the focus on the need to increase the rate of development of alternatives for toxicology. She expressed concern for the EPA

endocrine disruptor screening program's use of numerous animals. She appreciated Dr. Galson's assurance that the EPA supported dropping OECD's TG 401 but feels that the ICCVAM validation principles are being applied arbitrarily resulting in a double standard. Ms. Sweetland stated that the non-standardized developmental neurotoxicity test uses up to two-thousand animals and is required by the EPA in the pesticide testing program even though testers can't agree on many points of the test. She believes that the EPA should support and practice full validation of all tests, animal and non-animal. Additionally, she feels that transgenics are not a true reduction method. She expressed frustration at the EPA, FDA and DOT for the agencies' continued use of animals in testing and dismay that *in vitro* cytotoxicity testing was being viewed as a novel concept instead of a time tested one. She again expressed appreciation for Dr. Galson's recommendation that *in vitro* cytotoxicity be used for dose setting as an interim step to total replacement. She urged regulatory agencies and companies to not wait for others to solve the problem and move forward on enhancing the cell tests.

Dr. Andrew Rowan (U.S. Humane Society)
Dr. Rowan explained that the Helsinki Declaration has been significantly revised in terms of animal welfare and appropriate animal testing and thus has been significantly modified from the old Nuremburg Code.

Dr. Giles Klopman (Case Western Reserve University; Multicase, Inc.)
Dr. Klopman stated that computer models wouldn't come into play if the validation is as lax as validation of short-term assays. He predicted that computer models will replace short-term assays and said that the FDA has a database for short-term assays. He was confident that the scientific community would solve the testing problems in the long run.

Adjournment

Dr. Frazier concluded the morning plenary session by restating the charge for the breakout groups and workshop participants. He stated the workshop objectives and described the nature of the four breakout groups. He explained that the workshop was to have the breakout groups answer the prepared questions provided by the Organizing Committee and to produce reports that will eventually be published. The morning session ended at 12:18 p.m.

Closing Plenary Session

Dr. Stokes opened the closing plenary session at 8:04 a.m. and introduced the Co-Chairs of the breakout groups. Co-Chairs presented their workshop reports (See **Sections 2-5)** and an opportunity for public comments was permitted.

Public Comments:

Ms. Jessica Sandler (PETA)
Ms. Sandler spoke of money available for development of non-animal tests: NIEHS committed $1.5 million for fiscal year 2000 and $3.0 million for fiscal year 2001; the EPA committed $0.5 million over two years, and stated that the MEIC study would receive high priority. She expressed concern that the EPA had no single project in development for developing non-animal tests, yet continued requiring massive animal testing programs, in particular the HPV program. Ms. Sandler urged the ICCVAM to take a more aggressive role in developing alternative testing methods. She praised the

workshop for bringing together international and American scientists to persuade government regulators to seriously consider alternative testing methods.

Dr. Martin Stevens (Humane Society of the U.S.)
Dr. Stevens complimented ICCVAM for its role in organizing the workshop and hoped to be involved with ICCVAM in moving forward with the recommendations put forth by the workshop. He spoke of three hurdles in the evolution of replacing the LD50 test: 1) use of cytotoxicity data to accurately predict starting doses to reduce animal use; 2) use of limit tests to confirm non-toxicity; and 3) total replacement of the LD50 test.

Ms. Mary Beth Sweetland (PETA)
Ms. Sweetland made comments directly to ICCVAM concerning European Union acceptance of four validated test methods (three for corrosion and one for phototoxicity): Episkin™, EpiDerm™, rat skin TER, and 3T3 Neutral Red Uptake. She stated that the United States should accept the ECVAM validations and present these methods to the OECD as accepted methods. She concluded by thanking those who put the effort forth for the workshop.

In response to Ms. Sweetland's comments, Dr. Stokes stated that ICCVAM has an interagency Corrosivity Working Group that has provided extensive comments on the OECD proposals for the corrosivity methods mentioned, and U.S. government scientists also provided comments on the phototoxicity method. ICCVAM is currently developing an expedited process by which methods reviewed, validated, and accepted in Europe could be reviewed and considered by U.S. agencies.

Conclusion and Adjournment

Dr. Stokes presented the closing comments for the workshop, stating that the Breakout Groups had made remarkable progress. He thanked the co-chairs of the breakout groups, the agency representatives and the scientists attending the workshop. He stated that the objectives of the workshop had been met or exceeded in all areas, and that the Workshop's advice will lead to refinement in the near term and contribute to progress toward replacement. He stated that a report of the workshop would be published in 2001 and made available to the public. Dr. Stokes also recognized and thanked the ICCVAM Organizing Committee, Dr. Philip Sayre, Dr. John Frazier, and the NICEATM staff. The meeting was adjourned at 12:00 noon.

International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity
October 17-20, 2000
Arlington, VA. U.S.A.

Guidance for Breakout Groups

Breakout Groups will address the applicable Workshop objectives and develop responses to the questions provided for each Breakout Group.

A. Workshop Objectives:

1. 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;
2. Recommend candidate methods for future evaluation in prevalidation and validation studies;
3. Recommend validation study designs that can be used to adequately characterize the usefulness and limitations of proposed *in vitro* methods;
4. Identify reference chemicals that can be used for development and validation of *in vitro* methods for assessing *in vivo* acute toxicity;
5. 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.

B. Breakout Group Questions

Breakout Group 1: *In Vitro* Screening Methods for Assessing Acute Toxicity

This Breakout Group is asked to evaluate the validation status of available in vitro methods for estimating in vivo acute toxicity. The Group will identify methods and appropriate validation studies that might be completed within the next 1-2 years. The potential uses of QSAR as part of an in vitro strategy will also be evaluated.

Session 1-1: Identifying Needs

1. What are the near-term (< 2 years) goals and potentially attainable objectives for validation and use of *in vitro* methods that might reduce animal use for assessing acute toxicity?
2. What types of in vitro endpoints would be most effective for assessing in vivo acute toxicity; those that relate to general toxicity (e.g., cell death, growth inhibition) or those that are more cell or function specific (e.g., DNA damage/repair/synthesis; mitochondrial functionality; inhibition of other metabolic pathways)?
3. What other issues need to be considered for selecting protocols, e.g., robustness of protocol, reproducibility, stability of cell line?
4. What is the role of QSAR (and other prediction models) in predicting acute toxicity?

Session 1-2: Current Status

1. What are the available in vitro methods that might be useful in estimating acute in vivo toxicity.? Are standardized and/or optimized protocols available?
2. What are the strengths and limitations of available in vitro cytotoxicity assays (e.g., MEIC; ZEBET's validation efforts to extend cytotoxicity data to obtain better starting dose estimations; other mechanism-based cytotoxicity assays)?
3. What is the validation status of available in vitro screening methods (see Validation Criteria)?
4. Have any of these available in vitro methods been adequately evaluated for their usefulness for a specific purpose? If so, is their performance sufficient to recommend their use at this time?
5. What are the relative advantages and disadvantages for the use of human cells/tissues versus human cell lines versus animal cells/tissues versus animal cell lines?

6. To what extent do available methods take into consideration metabolic activation/inactivation of chemicals?
7. How have QSAR and other prediction models been used to estimate acute toxicity? What commercially available software exists? What are their advantages and disadvantages?
8. Are the available toxicity databases adequate to develop useful QSARs for industrial chemicals, consumer products, drugs? If not, what are the data needs?

Session 1-3: Future Directions

1. What are the most promising *in vitro* methods that should be further evaluated for their usefulness in reducing and/or refining animal use for acute toxicity?
   a.. What validation studies would be necessary to adequately evaluate the usefulness and limitations of these proposed methods for their proposed use?
   b. What research and/or developmental needs are required for candidate *in vitro* tests?
   c. What other mechanism-based *in vitro* methods or endpoints should be evaluated in future validation studies (e.g., microarray evaluation of altered gene expression patterns)? If so, which *in vitro* methods or endpoints should be given priority?
2. Which are the most promising *in vitro* methods for further evaluation or validation as replacements for *in vivo* acute toxicity test methods?
   a. What additional validation studies would be necessary to adequately evaluate the usefulness and limitations of these methods as replacements?
   b. What research and/or developmental needs are required for candidate *in vitro* tests?
   c. What other mechanism-based *in vitro* methods or endpoints should be evaluated in future validation studies (e.g., microarray evaluation of altered gene expression patterns)? If so, which *in vitro* methods or endpoints should be given priority?
3. How should individual tests be evaluated to determine their usefulness for integration into an overall acute toxicity testing strategy?
4. What criteria should be used to evaluate QSAR methods? To what extent could QSAR's be improved by an improved understanding of the molecular and cellular mechanisms of action of toxicity? What knowledge gaps exist that should be addressed by future research?

Breakout Group 2: *In Vitro* Methods for Assessing Acute Toxicity –Toxicokinetic Determinations

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 organ 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.

Session 2-1: Identify Needs

1. How can *in vitro* methods for evaluating chemical kinetics in biological systems contribute to the hazard and risk assessment process?
2. What is the role of toxicokinetics in the overall mechanisms by which chemicals illicit acute toxicity?
3. What toxicokinetic techniques should be considered as *in vitro* assays to improve predictivity and increase understanding of toxicity mechanisms? What is the role of QSAR in predicting chemical kinetics?

Session 2-2: Current Status

1. What *in vitro* methods are available for *in vitro* estimations of chemical-specific toxicokinetic parameters in animals and humans?
2. What are the strengths, limitations, and validation status of these available methods?
3. What mathematical approaches are available to predict or model toxicokinetics of

chemicals in mammalian systems based on data from *in vitro* systems?
4. What are the potential strengths and limitations of these approaches?
5. How would the approaches have to be modified/improved to meet acute toxicity testing needs?
6. How effective are the available QSAR systems for predicting *in vivo* toxicokinetic parameters?

Session 2-3: Future Directions

1. Which *in vitro*, QSAR or PBBK methods are the most promising for future use or development?
2. How should candidate methods be further developed/validated?
3. What are the more important issues to focus on in the long run (e.g., GI absorption, blood-brain barrier penetration)?
4. What research and development efforts are needed to achieve the ability to predict chemical kinetics in animals and humans?

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

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 develop recommendations for priority research efforts necessary to support the development of methods that can accurately assess target organ toxicity.

Session 3-1: Identify Needs

1. How can *in vitro* methods for assessing target organ toxicity contribute to hazard identification and dose-response assessment processes?
2. What is the relationship between *in vitro* mechanisms of toxicity and mechanisms by which chemicals are acutely toxic to animals and humans?
3. How can *in vitro* toxicity assays be used to predict acute organ-specific toxicity?
4. Can mechanism-based *in vitro* methods be developed to evaluate the range of *in vivo* toxicity processes and estimate those which may lead to injury or lethality?
5. What *in vitro* procedures and endpoints should be considered to improve predictability of *in vivo* effects and increase understanding of toxicity mechanisms?

Session 3-2: Current Status

1. What *in vitro* methods are available for target tissue-based estimations of animal and human responses to chemicals?
2. What is the validation status of these available methods?
3. What are their potential strengths and limitations?
4. How would they have to be modified/improved to enhance their usefulness?
5. Are techniques available to extrapolate *in vitro* cell toxicity data to predict acute systemic responses and ultimately system failure?

Session 3-3: Future Directions

1. Which are the most promising assays or methodologies to evaluate further?
2. How should each one be further developed/validated?
3. What are the research needs to attain the ability to predict acute toxicity in animals and humans?
4. What new methods or approaches are available that might improve mechanism-based in vitro estimations of animal and human responses to chemicals? How should they be developed for acute toxicity testing purposes?
5. How might the potential usefulness of microarray technology/differential gene expression for predicting systemic toxicity be further evaluated?
6. What research needs must be supported to improve QSAR methods for predicting target organ toxicity?

Breakout Group 4: Chemical Data Sets for Validation of *In Vitro* Toxicity Tests

This Breakout Group will have 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.

Session 4-1: Identify Needs

1. What are the characteristics of chemical [sets] that should be used in the validation of *in vitro* test methods for acute toxicity? For predicting organ-specific toxicity or toxicity based on specific mechanisms?
2. What criteria should be used for selecting chemical classes and chemicals to validate *in vitro* methods for assessing acute toxicity? Considering the different purposes of various *in vitro* methods, which sets of chemicals should be used to evaluate these different purposes?
3. To what extent and how should product classes/chemical classes (as used by regulatory agencies) be used to guide chemical selection?
4. To what extent and how should mode of action and biological target data be used to identify chemicals for use in validation studies?
5. How can QSAR methods help in the selection of validation chemicals?

Session 4-2: Current Status

1. What chemical data sets are available (e.g., EPA-HPV industrial chemicals, pesticides, drugs, food additives, NTP chemicals) that could be used for the validation of acute toxicity testing methods?
2. Are sufficient toxicity data available on existing chemicals or will additional data need to be obtained.
3. Do the available chemical data sets adequately represent the range of regulatory classifications for toxicity?
4. What QSAR models are currently available for such an effort?

Session 4-3: Future Directions

1. What are the characteristics of chemical data sets that could be used for validation of in vitro tests for in vivo toxicity (e.g., estimation of acute toxicity; identification of organ-specific toxic effects; determination of ADME parameters)?
2. To the extent possible, identify reference chemicals for which sufficient information is available that they should be considered for validation of assays/methodologies for predicting starting doses for in vivo studies, assays, or other assays that can be implemented in the near term? Are existing chemical sets adequate? Are additional chemicals needed, and if yes, are additional in vivo acute toxicity data needed?
3. To the extent possible, which reference chemicals should be used in the development/validation of assays/methods developed to predict in vivo acute toxicity in the longer term? Are different sets of chemicals needed to evaluate methods to predict target organ toxicity?
4. Should there be established chemical data sets for use in validation studies, or should they be selected or developed according to the specific test to be evaluated?
5. What additional chemical data sets need to be compiled or developed?
6. How should these chemical data sets be developed, and by whom?

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]

APPENDIX E

NICEATM Summary of The Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC)

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, TAB 6].

The following ATLA (Alternatives To Laboratory Animals) excerpts are reprinted with permission from Professor Michael Balls, editor of ATLA.

- Clemedson et al., 1998. MEIC Evaluation of Acute Systemic Toxicity, Part IV. ATLA 26: 131-183. **[Table 1]**

- Ekwall et al., 1998. MEIC Evaluation of Acute Systemic Toxicity, Part V. ATLA 26: 571-616. **[Tables II, III, IV, V, VI, IX]**

- Ekwall et al., 2000. MEIC Evaluation of Acute Systemic Toxicity, Part VIII, ATLA 28 Suppl 1, 201-234**. [Figures 1 and 10]**

- Ekwall et al., 1999. EDIT: A new international multicentre programme to develop and evaluate batteries of in vitro tests for acute chronic systemic toxicity. ATLA 27: 339-349. **[Table 1 and Figure 1]**

The following table was reproduced with permission from Dr. Gary Hook (NIEHS).

- Wallum, E. 1998. Acute Oral Toxicity. EHP 106: 497-503**. [reproduction of Table 1]**

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

Summary

September 2000

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

TABLE OF CONTENTS

LIST OF APPENDICES

1.0 Introduction

The Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC) program was organized by the Scandinavian Society for Cell Toxicology in 1989. MEIC was started with two goals. The first was to investigate the relevance of results from *in vitro* tests for predicting the acute toxic action of chemicals in humans. The second was to establish batteries of existing *in vitro* toxicity tests as replacements for acute toxicity tests on animals (LD50). Achievement of the second goal, the practical and ethical one, was considered to be entirely dependent on a successful outcome of the first, scientific goal. At the same time, it was recognized that a demonstrated high relevance of *in vitro* toxicity tests for human acute toxicity did not mean that all problems of replacement of animal tests would be solved. MEIC was a voluntary effort involving 96 international laboratories that evaluated the relevance and reliability of *in vitro* cytotoxicity tests originally developed as alternatives to or supplements for animal tests for acute systemic toxicity, chronic systemic toxicity, organ toxicity, skin irritancy, or other forms of general toxicity. In establishing the framework for this program, a minimum of methodological directives was provided in order to maximize protocol diversity among the participating laboratories. The collection of test method data was completed in 1996. The multiple publications originating from these studies are provided in chronological order in Section 12. All *in vitro* toxicity test results collected during MEIC are available on the Cytotoxicology Laboratory, Uppsala (CTLU) website (www.ctlu.se) as a searchable database.

2.0 Test Chemicals

Fifty reference chemicals were selected for testing (**Appendix 1**). Selection was based on the availability of reasonably accurate human data on acute toxicity. Due to the anticipated five-year duration of MEIC, it was recognized that multiple samples (lots) of each chemical would be needed. However, it was decided that the chemicals would not be provided by a central supplier, but rather that each laboratory would purchase each chemical at the highest purity obtainable with the proviso that storage duration would be kept to a minimum. The decision to not have a central supplier was based on the rationale that most reference chemicals are drugs, which presents fewer impurity problems. It is also based on the recognition that the results would be evaluated against human poisonings, which involve chemicals of different origin and purity.

3.0 *In Vitro* Test Assays

By the end of the project in 1996, 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 (**Appendix 2**). Slight variants of four of the assays were also used to test some chemicals. The primary 82 assays included:

- Twenty human cell line assays utilizing Chang liver, HeLa, Hep 2, Hep G2, HFL1, HL-60, McCoy, NB-1, SQ-5, and WI-1003 cells;
- Seven human primary culture assays utilizing hepatocytes, keratinocytes, and polymorphonuclear leukocytes;
- Nineteen animal cell line assays utilizing 3T3, 3T3-L1, Balb 3T3, BP8, ELD, Hepa-1c1c7, HTC, L2, LLC-PK1, LS-292, MDBK, PC12h, and V79 cells;
- Eighteen animal primary culture assays utilizing bovine spermatozoa, chicken neurons, mouse erythrocytes, rat hepatocytes, and rat muscle cells; and
- Eighteen ecotoxicological tests utilizing bacteria (*Bacillus subtilis*, *Escherichia coli* B, *Photobacterium phosphoreum*, *Vibrio fisheri*), rotifer (*Brachionus calyciflorus*), crustacea (*Artemia salina*, *Daphnia magna*, *Streptocephalus proscideus*), plant (*Alium cepa* root, tobacco plant pollen tubes), and fish (trout hepatocytes, trout R1 fibroblast-like cells).

4.0 Assay Endpoints

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 reduce the endpoint in question by 50%) (**Appendix 2**).

These values were generated by the participating laboratories and were not independently verified; original data were not presented in the MEIC publications. 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, and included:

- Cell viability as measured by the metabolism of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT), neutral red uptake (NRU), lactate dehydrogenase (LDH) release, cell morphology, adenosine triphosphate (ATP) content or leakage, trypan blue exclusion, viable cell count, tritiated-proline uptake, 86Rb leakage, creatine kinase activity, and glucose consumption;
- Cell growth as measured by protein content, macromolecule content, cell number, pH change, and optical density;
- Colony formation as measured by plating efficiency;
- An organotypic cellular endpoint (i.e., contractility of rat skeletel muscle cells);
- Motility and velocity for bovine sperm;
- Bioluminescence; and
- Mortality in lower eukaryotic organisms.

5.0 Comparative Data

The types of comparative data used to evaluate the predictive accuracy of the *in vitro* IC50 toxicity data for human acute toxicity included:

- Oral rat and mouse LD50 values obtained from Registry of Toxic Effects of Chemical Substances (RTECS) (**Appendix 3,** which contains rat and mouse LD50 data and average human lethal dose data for the 50 MEIC chemicals, ranked in three consecutive tables according to potency for rat, then mouse, and finally human. It also contains an U.S. Environmental Protection Agency (EPA) classification scheme for the acute toxicity of chemicals in humans.);
- Acute oral lethal doses in humans obtained from nine reference handbooks (**Appendix 4**);
- Clinically measured acute lethal serum concentrations in humans obtained from ten reference handbooks (**Appendix 5**);
- Acute lethal blood concentrations in humans measured post-mortem obtained from one forensic handbook and six forensic tabulations (**Appendix 6**);
- Human pharmacokinetics following single doses, including absorption, peak time, distribution/elimination curves, plasma half-life, distribution volume, distribution to organs (notably brain), and blood protein binding (**Appendix 7**);
- Peaks from curves of an ~50% lethal blood/serum concentration over time after ingestion (LC50 curves derived from human acute poisoning case reports) (**Appendix 8**);
- Qualitative human acute toxicity data, including lethal symptoms, main causes of death, average time to death, target organs, presence of histopathological injury in target organs, presence of toxic metabolites, and known or hypothetical mechanisms for the lethal injury (**Appendix 9**).

Early in the MEIC project, the *in vitro* cytotoxicity results were compared with average lethal blood concentrations (LCs) from acute human poisoning. However, these LCs were of limited value because they were averages of data with a wide variation due to different time between exposure and sampling (clinical) or death (forensic medicine). Therefore, a project was started to collect published and unpublished (from poison information centers and medico-legal institutes) case reports from human poisonings for the 50 MEIC reference chemicals that had lethal or sublethal blood concentrations with known time between ingestion and sampling/death. The aim was to compile enough case reports to be able to construct time-related lethal concentration

curves to be compared with the IC50 values for different incubation times *in vitro*. The results from the project were presented and analyzed in a series of 50 MEIC monographs. All monographs with sufficient case reports contain five tables presenting blood concentrations and two figures presenting LC curves. Three tables present (i) clinically measured, time-related sublethal blood concentrations, (ii) clinically measured, time-related lethal blood concentrations, and (iii) post-mortem, time-related blood concentrations. In these tables, blood concentration and the time interval between exposure and sampling for these concentrations are listed, as well as other important information on the cases. One table contains case reports with blood concentrations without a known time after ingestion and one table presents average blood concentrations calculated from the values presented in the other tables. The two figures presented in each of the monographs are scatter plots of sublethal and lethal blood concentrations. Based on these plots, concentration curves over time were drawn for the highest no lethal concentrations (NLC100); the lowest lethal concentrations (LC0); and the median curve between NLC100 and LC0, which is called the approximate LC50 even though it is not equivalent to a 50% mortality.

6.0 Statistical Analyses

The statistical analyses conducted by the MEIC management team involved:

- Principal components analysis (PCA);
- Analysis of Variance (ANOVA) and pairwise comparison of means using Tukey's method;
- Linear regression and ANOVA linear contrast analysis; and
- Multivariable partial least square (PLS) modeling with latent variables.

7.0 Results (based on IC50 response)

The MEIC management team, based on their analyses of the *in vitro* IC50 data, obtained the following results:

- The 1st PCA component described 80% of the variance of all the cytotoxicity data.
- Tukey's ANOVA indicated a similar sensitivity (~80%) for the assays.
- The toxicity of many chemicals increased with exposure time, making it necessary to perform a test at several exposure times to fully characterize the cytotoxicity.
- In general, human cytotoxicity was predicted well by animal cytotoxicity.
- Prediction of human cytotoxicity by ecotoxicological tests was only fairly good.
- One organotypic endpoint (muscle cell contractility) gave different results to those obtained with viability/growth assays.
- Sixteen comparisons of similar test systems involving different cell types and exposure times revealed similar toxicities, regardless of cell type.
- Nine of ten comparisons of test systems with identical cell types and exposure times revealed similar toxicities, regardless of the viability or growth endpoint measurement used.
- Nine comparisons of similar test systems employing different primary cultures and cell lines indicated that they shared similar toxicities.
- A high correlation between an intracellular protein denaturation test and average human cell line toxicity test suggested that denaturation may be a frequently occurring mechanism in basal cytotoxicity.

The following results were based on comparisons between *in vitro* data and *in vivo* data:

- Simple human cell tests were shown to be relevant for human acute lethal action for as many as 43 of the 50 MEIC reference chemicals (86%). The exceptions were atropine, digoxin, malathion, nicotine, cyanide, paracetamol, and paraquat -- all specific receptor-mediated toxicants.
- A battery of three of these human cell line tests (nos. 1, 9, 5/16) was found to be highly predictive (R^2 = 0.77) of the peak human lethal blood concentrations (LC50) of chemicals. The prediction increased markedly (R^2 = 0.83) when a simple

algorithm based on the knowledge of passage across the blood-brain barrier was used to adapt *in vitro* to *in vivo* concentrations (**Appendix 7**). The battery involved four endpoints and two exposure times (protein content/24 hours; ATP content/24 hours; inhibition of elongation of cells/24 hours; pH change/7 days). Prediction was better than the prediction of human lethal doses by rat and mouse LD50-values ($R^2 = 0.65$). The correlation between calculated oral LD50 doses in rats and mice and acute lethal dose in humans is presented graphically in **Appendix 10**, while the correlation between IC50 values and peak lethal blood concentrations in humans is presented graphically in **Appendix 11**.

- In the *in vitro* -- *in vivo* MEIC evaluation of chemicals that do easily not cross the blood-brain barrier, the 24 hour cytotoxic concentrations for rapidly acting chemicals correlated well with the human lethal peak blood concentrations, while the corresponding cytotoxicity for the slow-acting chemicals did not correlate as well with the peak concentrations. The prediction of human toxicity by the tests of slow-acting chemicals was much improved when 48-hour cytotoxic concentrations were compared with 48-hour human lethal blood concentrations. Thus, an *in vitro* test providing a discrimination between a rapid and a slow cytotoxic action would increase the predictive power of a cell test battery on acute toxicity.
- The findings from both the *in vitro-in vitro* comparisons and the *in vitro-in vivo* comparisons strongly supported the basal cytotoxicity concept.

8.0 MEIC Conclusions and Recommendations

Based on the analyses conducted, the MEIC management team made the following conclusions:

- The MEIC 1, 9, 5/16 test battery can be used directly as a surrogate for a LD50 test. However, since the battery predicts lethal blood concentrations, not lethal dosages, it is not a direct counterpart of the animal LD50 test. Thus, the 1, 9, 5/16 battery must be supplemented with data on gut absorption as well as the distribution volumes (Vd) of chemicals. Vd essentially depends on whether chemicals penetrate cells or not, and the degree of accumulation in the cell for chemicals that enter cells. Binding to proteins, lipids, bone and intracellular matrix will also influence Vd. Probably, a simple test of accumulation in cells over time would provide adequate Vd data. There is sufficient *knowledge of kinetics and Vd to enable an evaluation of results from such an assay for most of the 50 MEIC chemicals.
- An ongoing evaluation is being conducted to address the issue of predicting human oral lethal doses rather than human lethal blood concentrations. One MEIC manuscript in preparation will focus on the importance of the kinetic determinants of target organs for basal cytotoxicity. A second MEIC manuscript will describe how human lethal doses may be predicted by cellular tests on basal cytotoxicity (the 1, 9, 5/16 battery) and kinetic data.
- If human lethal doses are shown to be well predicted by the 1, 9, 5/16 battery, when combined with absorption and distribution data, a new but simple *in vitro* test to predict distribution volumes must be developed. An effective *in vitro* test on absorption is stated to already exist. Development of new *in vitro* methods is not addressed by MEIC, which only evaluated existing methods.
- In MEIC, only two of the 50 reference chemicals (ethylene glycol and methanol) were biotransformed to more toxic metabolites, contributing to the acute lethal action. The occurrence of toxic metabolites for the two chemicals did not affect the prediction of human lethal peak concentrations by human cell line inhibitory concentrations, but seemed to interfere with the correlation between *in vitro* delayed effects and the prediction of

later lethal effects of the chemicals. These results confirm the proposed usefulness of an *in vitro* test that could measure the formation and release of a toxic metabolite by metabolically competent cells within the time frame of acute toxicity. One design of such a test would be to use human hepatocytes in co-cultures with a target cell line. Since so few metabolically active chemicals were tested in MEIC, future studies will need to include additional metabolically activated chemicals.

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

In recognition that additional *in vitro* tests were needed to enhance the accuracy of the proposed *in vitro* battery for predicting human acute toxicity, a second voluntary multicenter program was initiated by the CTLU. The CTLU has designed a blueprint for an extended battery and has invited all interested laboratories to develop the "missing" tests of this battery within the

framework of the EDIT program (**Appendix 12** and **13**). The EDIT research program is published on the Internet (www.ctlu.se). The aim of EDIT is to provide a full replacement of the animal acute toxicity tests. The most urgently needed developments are assays on the accumulation of chemicals in cells (test of Vd), passage across the intestinal and blood-brain barriers, and biotransformation to more toxic metabolites. CTLU will provide interested laboratories with human reference data and will evaluate results as single components of complex models. The Internet version of the general EDIT research program contains additional, regularly updated information on the project. Purported advantages of the project are as follows. First, the evaluation-guided test development in EDIT is rational since tests are designed according to obvious needs and as elementary tests of single events integrated into whole models, which is the potential strength of the *in vitro* toxicity testing strategy. Second, the direct testing of MEIC chemicals in newly developed *in vitro* assays will lead to a rapid evaluation of the potential value of each assay.

10.0 Recommended Integration of MEIC/EDIT into the EPA High Production Volume (HPV) Program

Dr. Ekwall, the principle scientist for the MEIC program, has provided several suggestions for using MEIC results and the forthcoming EDIT results to reduce animal testing in the HPV program. These suggestions include the following:

1. Formal validation by ECVAM/ICCVAM of the existing 3 test MEIC battery. If considered validated, use of the battery to test every chemical in the HPV program would provide inexpensive and useful supplementary data.
2. Evaluate some of the HPV chemicals in a battery of *in vitro* toxicity and toxicokinetic tests on acute toxicity (EDIT and similar models) as follows:
   - Engage poison information experts to select a set of HPV chemicals with sound human acute toxicity data, including time-related lethal blood concentrations.
   - Give priority to standard testing of the same chemicals in the HPV program.
   - Testing of the same chemicals in the newly developed *in vitro* systems (EDIT, etc.), including modeling of acute toxicity by the new assays.
   - Comparison of HPV standard animal data and the *in vitro* data with the human data for the selected set of chemicals.

   If the new *in vitro* models can be shown to predict human acute toxicity better than the HPV animal tests, *in vitro* batteries may totally replace the animal acute toxicity tests in further HPV testing.

11.0 MEIC Evaluation Guidelines Checklist

A complete and formal assessment of the validation status of MEIC in regard to the ICCVAM evaluation guidelines would require the following to be reviewed and evaluated:

ICCVAM Evaluation Guidelines

1.0 Introduction and Rationale of each Test Method
1.1 Scientific basis for each test method
1.1.1 Purpose of each proposed method, including the mechanistic basis
1.1.2 Similarities and differences of modes and mechanisms of action in each test system as compared to the species of interest (e.g., humans for human health-related toxicity testing).
1.2. Intended uses of each proposed test method.
1.2.1 Intended regulatory use(s) and rationale.
1.2.2 Substitute, replace, or complement existing test methods.
1.2.3 Fits into the overall strategy of hazard or safety assessment. If a component of a tiered assessment process, indicate the weight that will be applied relative to other measures.
1.2.4 Intended range of materials amenable to test and/or limits according to chemical class or physico-chemical factors.
2.0 Proposed Each Test Method Protocol(s)
2.1 Detailed protocol for each test method, duration of exposure, know limits of use, and nature of the response assessed, including:
2.1.1 Materials, equipment, and supplies needed
2.1.2 Suggested positive or negative controls.
2.1.3 Detailed procedures for conducting the test
2.1.4 Dose-selection procedures, including the need for any dose range-finding studies or acute toxicity data prior to conducting the test, if applicable;
2.1.5 Endpoint(s) measured
2.1.6 Duration of exposure
2.1.7 Known limits of use
2.1.8 Nature of the response assessed
2.1.9 Appropriate vehicle, positive and negative controls and the basis for their selection
2.1.10 Acceptable range of vehicle, positive and negative control responses
2.1.11 Nature of the data to be collected and the methods used for data collection
2.1.12 Type of media in which data are stored
2.1.13 Measures of variability
2.1.14 Statistical or non-statistical method(s) used to analyze the resulting data (including methods to analyze for a dose response relationship). The method(s) employed should

be justified and described
2.1.15 Decision criteria or the prediction model used to classify a test chemical (e.g., positive, negative, or equivocal), as appropriate
2.1.16 Information that will be included in the test report
2.2 Basis for each test system
2.3 Confidential information
2.4 Basis for the decision criteria established for each test
2.5 Basis for the number of replicate and repeat experiments; provide the rationale if studies are not replicated or repeated
2.6 Basis for any modifications to each proposed protocol that were made based on results from validation studies
3.0 Characterization of Materials Tested
3.1 Rationale for the chemicals/products selected for evaluation. Include information on suitability of chemicals selected for testing, indicating any chemicals that were found to be unsuitable
3.2 Rationale for the number of chemicals that were tested
3.3 The chemicals/products evaluated, including:
3.3.1. Chemical or product name; if a mixture, describe all components.
3.3.2 CAS number(s)
3.3.3 Chemical or product class
3.3.4 Physical/chemical characteristics
3.3.5 Stability of the test material in the test medium
3.3.6 Concentration tested.
3.3.7 Purity; presence and identity of contaminants.
3.3.8 Supplier/source of compound.
3.4 If mixtures were tested, constituents and relative concentrations should be provided whenever possible
3.5 Describe coding used (if any) during validation studies.
4.0 Reference Data Used for Performance Assessment
4.1 Clear description of the protocol for the reference test method. If a specific guideline has been followed, it should also be provided. Any deviation should be indicated, including the rationale for the deviation.
4.2. Provide reference data used to assess the performance of the proposed test method.
4.3 Availability of original datasheets for the reference data
4.4 Quality of the reference test data, including the extent of GLP compliance and any use of coded chemicals.
4.5 Availability and use of relevant toxicity information from the species of interest.
5.0 Test Method Data and Results
5.1 Complete, detailed protocol used to generate each set of data for each proposed test method.

Any deviations should be indicated, including the rationale for the deviation. Any protocol modifications made during the development process and their impact should be clearly stated for each data set.
5.2 Provide all data obtained using each proposed test method. This should include copies of original data from individual animals and/or individual samples, as well as derived data. The laboratory's summary judgement as to the outcome of each test should be indicated. The submission should also include data (and explanations) from unsuccessful, as well as successful, experiments.
5.3 Statistical approach used to evaluate the data from each proposed test method
5.4 Provide a summary, in graphic or tabular form, of the results.
5.5 For each set of data, indicate whether coded chemicals were tested, experiments were conducted blind, and the extent to which experiments followed GLP procedures.
5.6 Indicate the lot-to-lot consistency of the test materials, the time frame of the various studies, and the laboratory in which the study or studies were done. A coded designation for each laboratory is acceptable.
5.7 Any data not submitted should be available for external audit, if requested
6.0 Test Method Performance Assessment
6.1 Describe performance characteristics (e.g., accuracy, sensitivity, specificity, positive and negative predictivity, and false positive and negative rates) of each proposed test method separately and in combination compared with the reference test method currently accepted by regulatory agencies for the endpoint of interest. Explain how discordant results from each proposed test were considered when calculating performance values.
6.2 Results that are discordant with results from the reference method.
6.3 Performance characteristics of each proposed test method compared to data or recognized toxicity from the species of interest (e.g., humans for human health-related toxicity testing), where such data or toxicity classification is available. In instances where the proposed test method was discordant from the reference test method, describe the frequency of correct predictions of each test method compared to recognized toxicity information from the species of interest.
6.4 Strengths and limitations of the method, including those applicable to specific chemical classes or physical/chemical properties
6.5 Salient issues of data interpretation, including why specific parameters were selected for inclusion
7.0 Test Method Reliability (Repeatability/Reproducibility)
7.1 Rationale for the chemicals selected to evaluate intra- and inter-laboratory reproducibility for each test method, and the extent to which they represent the range of possible test outcomes.
7.2 Analyses and conclusions reached regarding inter- and intra-laboratory repeatability and reproducibility for each test method
7.3 Summarize historical positive and negative control data for each test method, including number of trials, measures of central tendency and variability.
8.0 Test Method Data Quality
8.1 Extent of adherence to GLPs

8.2. Results of any data quality audits
8.3 Impact of deviations from GLPs or any non-compliance detected in data quality audits
9.0 Other Scientific Reports and Reviews
9.1 All data from other published or unpublished studies conducted using the proposed test method should be included.
9.2 Comment on and compare the conclusions published in independent peer-reviewed reports or other independent scientific reviews of the test method. The conclusions of such scientific reports and/or reviews should be compared to the conclusions reached in this submission. Any other ongoing evaluations of the method should be mentioned.
10.0 Animal Welfare Considerations (Refinement, Reduction, and Replacement)
10.1 Describe how the proposed test methods will refine (reduce pain or distress), reduce, and/or replace animal use compared to the current methods used.
11.0 Other Considerations
11.1 Aspects of test method transferability. Include an explanation of how this compares to the transferability of the reference test method.
11.1.1 Facilities and major fixed equipment needed to conduct the test.
11.1.2 Required level of training and expertise needed for personnel to conduct the test.
11.1.3 General availability of other necessary equipment and supplies.
11.2 Cost involved in conducting each test. Discuss how this compares to the cost of the reference test method.
11.3 Indicate the amount of time needed to conduct each test and discuss how this compares with the reference test method.
12.0 Supporting Materials
12.1 Provide copies of all relevant publications, including those containing data from the proposed test method or the reference test method.
12.2 Include all available non-transformed original data for both each proposed test method and the reference test method.
12.3 Summarize and provide the results of any peer reviews conducted to date, and summarize any other ongoing or planned reviews.
12.4 Availability of laboratory notebooks or other records for an independent audit. Unpublished data should be supported by laboratory notebooks.

12.0 MEIC Related Publications (in chronological order)

Bernson, V., Bondesson, I., Ekwall, B., Stenberg, K., and Walum, E. (1987) A multicentre evaluation study of in vitro cytotoxicity. ATLA, 14, 144-145.

Bondesson, I., Ekwall, B., Stenberg, K., Romert, L. and Walum, E. (1988) Instruction for participants in the multicentre evaluation study of in vitro cytotoxicity (MEIC). ATLA, 15, 191-193.

Bondesson, I., Ekwall, B., Hellberg, S., Romert, L., Stenberg, K., and Walum, E. (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.

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

Ekwall, B., Gómez-Lechón, M.J., Hellberg, S., Bondsson, I., Castell, J.V., Jover, R., Högberg, J., Ponsoda, X., Stenberg, K., and Walum, E. (1990) Preliminary results from the Scandinavian multicentre evaluation of in vitro cytotoxicity (MEIC). Toxicol. In Vitro, 4, 688-691.

Hellberg, S., Bondesson, I., Ekwall, B., Gómez-Lechón, M.J., Jover, R., Högberg, J., Ponsoda; X., Romert, L., Stenberg, K., and Walum, E. (1990) Multivariate validation of cell toxicity data: The first ten MEIC chemicals. ATLA, 17, 237-238.

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

Shrivastava, R., Delomenie, C., Chevalier, A., John, G., Ekwall, B., Walum, E., and Massingham, R. (1992) Comparison of in vivo acute lethal potency and in vitro cytotoxicity of 48 chemicals. Cell Biol. Toxicol., 8(2), 157-170.

Ekwall, B., Abdulla, E., Barile, F., Bondesson, I., Clemedson, C., Clothier, R.,Curren, R., Dierickx, P., Fiskesjö, G., Garza-Ocanas, L., Gómez-Lechón, M.J., Gülden, M., Imai, K., Janus, J., Kristen, U., Kunimoto,, M., Kärenlampi, S., Lavrijsen, K., Lewan, L.,Malmsten, A., Miura, T., Nakamura, M., Ohno, T., Ono, H., Persoone, G., Rouget, R., Romert, L., Sandberg, M., Sawyer, T., Seibert, H., Shrivastava, R., Stammati, A., Tanaka, N., Walum, E., Wang, X & Zucco, F. (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 Proc. of JSAAE (Japanese Society for Alternatives to Animal Experiments) 6th annual meeting in Tokyo, Dec 17-18, 1992, (Ed. S. Sato), pp 114-115, Tokyo.

Ekwall, B., Abdulla, E., Barile, F.,Chesne, C., Clothier, Cottin, M., Curren, R., Daniel- Szolgay, E., Dierickx, P., Ferro, M., Fiskesjö, G., Garza-Ocanas, L., Gómez-Lechón, M.J., Gülden, M. Isomaa, B., Kahru, A., Kemp, R.B., Kerszman, G., Kristen, U., Kunimoto,, M., Kärenlampi, S., Lavrijsen, K., Lewan, L., Ohno, T., Persoone, G., Pettersson, R., Rouget, R., Romert, L., Sawyer, T., Seibert, H., Shrivastava, R., Sjöström, M., Tanaka, N., Zucco, F., Walum, E., & Clemedson, C. (1994) A comparative cytotoxicity analysis of the results from tests of the first 30 MEIC reference chemicals in 68 different in vitro toxicity systems, pp 117-118 in Alternatives Research - Proceedings of the 8th Annual Meeting of the Japanese Society for Alternatives to Animal Experiments, Nov. 28-29, 1994, Tokyo.

Ekwall, B. (1995) The basal cytotoxicity concept, pp 721-725. 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, Vol 11. Mary Ann Liebert, New York, 1995.

Balls, M, Blaauboer, BJ, Fentem, JH, Bruner, L, Combes, RD, Ekwall, B, Fielder, RJ, Guillouzo, A, Lewis, RW, Lovell, DP, Reinhardt, CA, Repetto, G, Sladowski, D, Spielmann, H & Zucco, F (1995) Practical aspects of the

validation of toxicity test procedures - The report and recommendations of ECVAM Workshop 5. ATLA 23, 129-147.

Walum, E, Nilsson, M, Clemedson, C & Ekwall, B. (1995) The MEIC program and its implications for the prediction of acute human systemic toxicity, pp 275-282 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, Vol 11. Mary Ann Liebert, New York, 1995.

Clemedson, C, McFarlane-Abdulla, E., Andersson, M., Barile, F.A., Calleja, M.C., Chesné, C., Clothier, R., Cottin, M., Curren, R., Daniel-Szolgay, E., Dierickx, P., Ferro, M., Fiskesjö, G., Garza-Ocanas, L., Gómez-Lechón, M.J., Gülden, M., Isomaa, B., Janus, J., Judge, P., Kahru, A., Kemp, R.B., Kerszman, G., Kristen, U., Kunimoto, M., Kärenlampi, S., Lavrijsen, K., Lewan L., Lilius, H., Ohno, T., Persoone, G.,Roguet, R., Romert, L., Sawyer, T., Seibert, H., Shrivastava, R., Stammati, A., Tanaka, N., Torres Alanis, O., Voss, J-U., Wakuri, S., Walum, E., Wang, X., Zucco, F. and Ekwall, B. (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, 1996, 249-272.

Clemedson, C, McFarlane-Abdulla, E., Andersson, M., Barile, F.A., Calleja, M.C., Chesné, C., Clothier, R., Cottin, M., Curren, R., Dierickx, P., Ferro, M., Fiskesjö, G., Garza-Ocanas, L., Gómez-Lechón, M.J., Gülden, M., Isomaa, B., Janus, J., Judge, P., Kahru, A., Kemp, R.B., Kerszman, G., Kristen, U., Kunimoto, M., Kärenlampi, S., Lavrijsen, K., Lewan L., Lilius, H., Malmsten, A., Ohno, T., Persoone, G., Pettersson, R., Roguet, R., Romert, L., Sandberg, M., Sawyer, T., Seibert, H., Shrivastava, R., Sjöström, M., Stammati, A., Tanaka, N., Torres Alanis, O., Voss, J-U., Wakuri, S., Walum, E., Wang, X., Zucco, F. and Ekwall, B. (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, 1996, 273-311.

Ekwall, B, Clemedson, C, Crafoord, B, Ekwall, Ba, Hallander, S, Sjöström, M & Walum, E (1997) Correlation between in vivo and in vitro acute toxicity tests; Results of the MEIC project, pp. 82-83 in Development of Ecotoxicity and Toxicity Testing of Chemicals - Proceeding of the 2nd Network Meeting, TemaNord 1997:524, Nordic Council of Ministers, Copenhagen, 1997.

Clemedson, C., Barile, F.A., Ekwall, B., Gómez-Lechón, M.J., Hall, T., Imai, K., Kahru, A., Logemann, P., Monaco, F., Ohno, T., Segner, H., Sjöström, M., Valentino, M., Walum, E., Wang, X. and Ekwall, B. (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., Aoki, Y., Andersson, M., Barile, F.A., Bassi, A.M., Calleja, M.C., Castano, A., Clothier, R.H., Dierickx, P., Ekwall, Ba., Ferro, M., Fiskesjö, G., Garza-Ocanas, L.Gómez-Lechón, M.J., Gülden, M., Hall, T., Imai, K., Isomaa, B., Kahru, A., Kerszman, G., Kjellstrand, P., Kristen, U., Kunimoto, M., Kärenlampi, S., Lewan, L., Lilius, H., Loukianov, A., Monaco, F., Ohno, T., Persoone, G., Romert, L., Sawyer, T.W., Shrivastava, R., Segner, H., Seibert, H., Sjöström, M., Stammati, A., Tanaka, N., Thuvander, A., Torres-Alanis, O., Valentino, M., Wakuri, S., Walum, E., Wieslander, A., Wang, X., Zucco, F. and Ekwall, B. (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.

Ekwall, B., Clemedson, C., Crafoord, B., Ekwall, Ba., Hallander, S., Walum E.and Bondesson, I. (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., Barile., F.A., Castano, A., Clemedson, C., Clothier, R.H., Dierickx, P., Ekwall, Ba., Ferro, M., Fiskesjö, G., Garza-Ocanas, L., Gómez-Lechón, M-J., Gülden, M., Hall, T., Isomaa, B., Kahru, A, Kerszman, G., Kristen, U., Kunimoto, M., Kärenlampi, S., Lewan, L, Loukianov, A., Ohno, T., Persoone, G., Romert, L.,. Sawyer, T.W., Segner, H., Shrivastava, R., Stammati, A., Tanaka, N., Valentino, M., Walum, E. and Zucco, F. (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.

Walum, E. (1998) Acute oral toxicity. Environ. Health Persp. 106, Suppl. 2, 497-503.

Ekwall, B., Clemedson, C., Ekwall, Ba., Ring, P. and Romert, L. (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.

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

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. Toxicology In Vitro, 13, 665-673.

Clemedson, C., Barile, F.A., Chesné, C., Cottin, M., Curren, R., Ekwall, B., Ferro, M., Gomez-Lechon, M.J., Imai, K., Janus, J., Kemp, R.B., Kerszman, G., Kjellstrand, P., Lavrijsen, K., Logemann, P., McFarlane-Abdulla, E., Roguet, R., Segner, H., Seibert, H., Thuvander, A., Walum, E. and Ekwall, Bj. (1999) 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.

Appendix I
First Fifty Reference Chemicals

Acetaminophen	Arsenic trioxide
Aspirin	Cupric sulfate
Ferrous sulfate	Mercuric chloride
Diazepam	Thioridazine HCl
Amitriptyline	Thallium sulfate
Digoxin	Warfarin
Ethylene glycol	Lindane
Methyl alcohol	Chloroform
Ethyl alcohol	Carbon tetrachloride
Isopropyl alcohol	Isoniazid
1,1,1-Trichloroethane	Dichloromethane
Phenol	Barium nitrate
Sodium chloride	Hexachlorophene
Sodium fluoride	Pentachlorophenol
Malathion	Varapamil HCl
2,4-Dichlorophenoxyacetic acid	Chloroquine phosphate
Xylene	Orphenadrine HCl
Nicotine	Quinidine sulfate
Potassium cyanide	Diphenylhydantoin
Lithium sulfate	Chloramphenicol
Theophylline	Sodium oxalate
Dextropropoxyphene HCl	Amphetamine sulfate
Propranolol HCl	Caffeine
Phenobarbital	Atropine sulfate
Paraquat	Potassium chloride

Appendix II: Descriptions of the Essential Traits of 67 *in vitro* Methods

Table I: Descriptions of the essential traits of 67 *in vitro* methods

Method							
No.	Old No.[a]	Cell type/ test system	Tissue of origin	Species	Endpoint	Incubation time	Testing laboratory[b]	Reference
Human cell lines								
1.	II:1	Hep G2	Hepatoma	Human	Protein content/Lowry	24 hours	Dierickx	3
2.	III:2	Hep G2	Hepatoma	Human	Protein content/ Sulphorhodamine B	24 hours	Hall, Cambridge & James	5
3.	II:2	Hep G2	Hepatoma	Human	MTT	24 hours	Gómez-Lechón, Jover, Ponsoda & Castell[c]	3, 12
4.	II:4	WI-1003/Hep G2[d]	Lung/Hepatoma	Human	Morphology	24 hours	Garza-Ocañas & Torres-Alanis	3
5.	II:3	Chang liver cells	Liver	Human	Morphology	24 hours	Garza-Ocañas & Torres-Alanis	3
6.	II:5	HeLa	Cervical carcinoma	Human	Morphology	24 hours	Ekwall & Malmsten	3
7.	II:6	Hep 2	Epithelial carcinoma of larynx	Human	Protein content/ Coomassie blue staining	24 hours	Stammati, Zucco, Zanetti & De Angelis	3
8.	II:7	Hep 2	Epithelial carcinoma of larynx	Human	LDH release	24 hours	Stammati, Zucco, Zanetti & De Angelis	3
9.	II:8	HL-60	Promyelocytic leukaemia	Human	ATP content	24 hours	Tanaka, Wakuri, Izumi, Sasaki & Ono	3
10.	III:10	HFL1	Fetal lung cells	Human	MTT	24 hours	Barile & Sookhoo[e]	5, 13
11.	III:11A	SQ-5	Lung squamous carcinoma	Human	LDH content[f]	48 hours	Ohno, Wang, Sasaki & Hirano	3, 14
12.	III:12	SQ-5	Lung squamous carcinoma	Human	Killing index[g]	48 hours	Ohno, Wang, Sasaki & Hirano	3, 14
13.	II:10	NB-1	Neuroblastoma	Human	Protein content/ Crystal violet staining	48 hours	Kunimoto, Miura, Aoki & Kunimoto	3
14.	II:11	McCoy	Epithelial cells from synovial fluid	Human	Morphology/Trypan blue exclusion[h]	72 hours	Shrivastava & Chevalier	3
15.	II:13	WI-1003/Hep G2[d]	Lung/Hepatoma	Human	Morphology/pH changes	168 hours	Garza-Ocañas & Torres-Alanis	3

Source: Clemedson et al. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part IV. ATLA 26:131-183. (reprinted with permission from the editor)

16.	II:12	Chang liver	Liver	Human	Morphology/pH changes	168 hours	Garza-Ocañas & Torres-Alanis	3
17.	II:14	HeLa	Cervical carcinoma	Human	pH changes (phenol red)	168 hours	Ekwall & Malmsten	3
18.	II:15	MRC-5 (finite cell line)	Epithelial cells from embryonic lung	Human	Protein content/Lowry	6 weeks	Dierickx	3, 15
Human primary cultures								
19.	III:21	Polymorphonuclear leukocytes[j]	Blood	Human	Viable cell count fluorescein diacetate/ Ethidium bromide	3 hours	Valentino, Monaco, Pieragostini, Amati & Governa	5
20.	III:22	Polymorphonuclear leukocytes[j]	Blood	Human	Locomotion stimulated by chemotactic peptide	3 hours	Valentino, Monaco, Pieragostini, Amati & Governa	5
Animal cell lines								
21.	II:19	ELD	Subline of Ehrlich ascites tumour cells	Mouse	ATP leakage	10 minutes	Lewan & Andersson	3
22.[i]	II:20	ELD	Subline of Ehrlich ascites tumour cells	Mouse	ATP leakage	10 minutes	Lewan & Andersson	3
23.	II:23	HTC	Hepatoma	Rat	Macromolecular content	24 hours	Ferro, Bassi & Canepa[k]	3
24.	II:25	L2	Lung epithelial cells	Rat	[^{3}H]-proline uptake	24 hours	Barile, Borges, Arjun & Hopkinson[l]	3, 16
25.	II:30	3T3	Fibroblasts	Mouse	MTT	24 hours	Gómez-Lechón, Jover, Ponsoda & Castell[c]	3, 42
26.	III:40	LLC-PK1	Kidney	Pig	Protein content/ Sulphorhodamine B	24 hours	Hall, Cambridge & James	5
27.	II:31	BP8	Ascites sarcoma	Mouse	Cell number/ Coulter counter	48 hours	Romert, Jansson & Jensseen	3
28.	II:32	PC12h	Pheochromocytoma	Rat	Protein content	48 hours	Kunimoto, Miura, Aoki & Kunimoto	3
29.	II:33	MDBK	Kidney	Bovine[m]	Morphology/Trypan blue exclusion[h]	72 hours	Shrivastava & Chevalier	3
30.	II:34	Hepa-1c1c7 (Subclone of Hepa-1)	Hepatoma	Mouse	Protein content/ Coomassie blue staining	72 hours	Kärenlampi & Malmivuori	3 *INVITTOX* protocol 112[m]

Table I: continued

Method No.	Old No.[a]	Cell type/ test system	Tissue of origin	Species	Endpoint	Incubation time	Testing laboratory[b]	Reference
31.	II:35	3T3-L1 (Subclone of 3T3)	Embryonal fibroblasts	Swiss mouse	Protein content/Kenacid blue staining	72 hours	Clothier	3
32.	II:36	Balb 3T3 A31-1-1	Whole embryo	Balb/c mouse	Colony formation	168 hours	Tanaka, Wakuri, Izumi, Sasaki & Ono	3
Animal primary cultures								
33.		Muscle cells	Skeletal muscle	Rat	Spontaneous contractility	1 hour	Gülden, Seibert & Voss	3, *INVITTOX* protocol 93[m]
34.	II:45A	Neurons	Embryonal forebrain	Chicken	Neutral red uptake	20 hours	Sawyer & Weiss	3
35.	II:46A	Neurons	Embryonal forebrain	Chicken	MTT	21 hours	Sawyer & Weiss	3
36.	II:50	Hepatocytes[n]	Liver	Male rat	MTT	24 hours	Gómez-Lechón, Jover, Ponsoda & Castell[f]	3, 12
37.	II:51	Hepatocytes[n]	Liver	Male rat	Morphology/Trypan blue exclusion/LDH release[h]	24 hours	Shrivastava & Chevalier	3
38.	II:52	Erythrocytes	Peripheral blood of 9-week males	Balb/c mouse	ATP content	24 hours	Tanaka, Wakuri, Izumi, Sasaki & Ono	3
39.		Muscle cells	Skeletal muscle	Rat	Intracellular creatine kinase activity	24 hours	Gülden, Seibert & Voss	3, *INVITTOX* protocol 93[m]
40.		Muscle cells	Skeletal muscle	Rat	Glucose consumption	24 hours	Gülden, Seibert & Voss	3, *INVITTOX* protocol 93[m]
41.		Muscle cells	Skeletal muscle	Rat	Spontaneous contractility	24 hours	Gülden, Seibert & Voss	3, *INVITTOX* protocol 93[m]

Appendix III: Oral LD50 Doses for Rat and Mouse and Mean Oral Lethal Doses for Humans and Toxicity Categories

Oral LD50 Doses for Rat and Mouse and Mean Oral Lethal Doses for Humans

Chemical Number	Chemical	Rat LD50		Mouse LD50		Ave. Human Dose	
		mg/kg	umol/kg	mg/kg	umol/kg	mg/kg	umol/kg
28	Mercuric chloride	**1**	**4**	6	22	25.7	94.7
31	Warfarin	**2**	**5**	3	10	107.1	347.4
18	Potassium cyanide	**5**	**77**	9	131	2.9	43.9
26	Arsenic trioxide	**15**	**74**	31	159	4.1	20.9
30	Thallium sulfate	**16**	**32**	24	47	14.0	27.7
39	Pentachlorophenol	**27**	**101**	28	105	28.6	107.3
6	Digoxin	**28**	**36**	18	23	0.1	0.17
17	Nicotine	**50**	**308**	3	21	0.7	4.4
13	Sodium fluoride	**52**	**1238**	57	1357	92.8	2210.9
47	Amphetamine sulfate	**55**	**149**	24	65	20.0	54.3
38	Hexachlorophene	**56**	**138**	67	165	214.3	526.6
32	Lindane	**76**	**261**	44	151	242.9	835.1
21	Propoxyphene HCL	**84**	**223**	255	678	24.6	65.4
25	Paraquat	**100**	**537**	120	644	40.0	214.7
40	Varapamil HCL	**108**	**220**	163	331	122.3	249.1
23	Penobarbital	**162**	**697**	137	590	111.4	479.7
48	Caffeine	**192**	**989**	127	654	135.7	698.8
2	Acetylsalicylic acid	**200**	**1110**	232	1287	385.7	2140.5
20	Theophylline	**244**	**1354**	235	1304	157.1	872.1
42	Orphenadrine HCL	**255**	**834**	100	327	50.0	163.4
43	Quinidine sulfate	**258**	**610**	286	676	79.2	187.4
14	Malathion	**290**	**878**	190	575	742.8	2248.4
11	Phenol	**317**	**3369**	270	2869	157.2	1670.0
3	Ferrous sulfate	**319**	**2100**	680	4477	392.1	2581.0
5	Amitriptyline	**320**	**1154**	140	505	37.1	133.8
4	Diazepam	**352**	**1236**	45	159	71.4	250.8
37	Barium nitrate	**355**	**1358**	266	1016	37.1	142.1
15	2,4-Dichlorophenoxy-acetic acid	**375**	**1697**	347	1570	385.8	1745.3
22	Propamolol HCL	**466**	**1575**	320	1082	71.5	241.7
27	Cupric sulfate	**469**	**1880**	502	2012	290.6	1163.6
19	Lithium sulfate	**492**	**4478**	1190	10,828	1065.5	9691.8
49	Altropine sulfate	**585**	**864**	456	674	1.7	2.5
41	Chloroquine phosphate	**623**	**1208**	500	969	84.3	163.4
33	Chloroform	**908**	**7605**	36	302	999.8	8375.2
29	Thioridazine HCL	**995**	**2445**	385	946	68.6	1684
35	Isoniazid	**1250**	**9117**	133	970	171.5	1250.4
36	Dichloromethane	**1601**	**18,846**	873	10,280	1386.2	16,321.7
44	Diphenylhydantoin	**1635**	**6480**	150	595	300.0	1189.1
34	Carbon tetrachloride	**2350**	**15,280**	8264	53,726	1314.4	8545.4
1	Paracetamol	**2404**	**15,899**	338	2235	271.4	1795.2
45	Chloramphenicol	**2500**	**7735**	1500	4641	285.7	884.0
50	Potassium chloride	**2598**	**34,853**	1499	20,107	285.5	3830.0
12	Sodium chloride	**3002**	**51,370**	4003	68,493	2287.3	39,138.9

Oral LD50 Doses for Rat and Mouse and Mean Oral Lethal Doses for Humans

16	Xylene	**4299**	**40,490**	2119	19,953	899.8	8474.6
7	Ethylene glycol	**4698**	**75,684**	5498	88,567	1570.9	25,304.8
8	Methanol	**5619**	**175,327**	7289	227,414	1569.0	48,954.2
9	Ethanol	**7057**	**153,145**	3448	74,837	4712.2	102,262.2
46	Sodium oxalate	**11160**	**83,284**	5095	38,019	357.1	2665.3
10	1,1,1-Trichloroethane	**11196**	**83,927**	7989	59,884	5707.6	42,785.8

Source: E. Walum. 1998. Acute oral toxicity. EHP 106:497-503. (reprinted with permission from the editor)

Oral LD50 Doses for Rat and Mouse and Mean Oral Lethal Doses for Humans

Chemical Number	Chemical	Rat LD50		Mouse LD50		Ave. Human Dose	
		mg/kg	umol/kg	mg/kg	umol/kg	mg/kg	umol/kg
31	Warfarin	2	5	**3**	**10**	107.1	347.4
17	Nicotine	50	308	**3**	**21**	0.7	4.4
28	Mercuric chloride	1	4	**6**	**22**	25.7	94.7
18	Potassium cyanide	5	77	**9**	**131**	2.9	43.9
6	Digoxin	28	36	**18**	**23**	0.1	0.2
30	Thallium sulfate	16	32	**24**	**47**	14.0	27.7
47	Amphetamine sulfate	55	149	**24**	**65**	20.0	54.3
39	Pentachlorophenol	27	101	**28**	**105**	28.6	107.3
26	Arsenic trioxide	15	74	**31**	**159**	4.1	20.9
33	Chloroform	908	7605	**36**	**302**	999.8	8375.2
32	Lindane	76	261	**44**	**151**	242.9	835.1
4	Diazepam	352	1236	**45**	**159**	71.4	250.8
13	Sodium fluoride	52	1238	**57**	**1357**	92.8	2210.9
38	Hexachlorophene	56	138	**67**	**165**	214.3	526.6
42	Orphenadrine HCL	255	834	**100**	**327**	50.00	163.4
25	Paraquat	100	537	**120**	**644**	40.00	214.7
48	Caffeine	192	989	**127**	**654**	135.7	698.8
35	Isoniazid	1250	9117	**133**	**970**	171.5	1250.4
23	Penobarbital	162	697	**137**	**590**	111.4	479.7
5	Amitriptyline	320	1154	**140**	**505**	37.1	133.8
44	Diphenylhydantoin	1635	6480	**150**	**595**	300.0	1189.1
40	Varapamil HCL	108	220	**163**	**331**	122.3	249.1
14	Malathion	290	878	**190**	**575**	742.8	2248.4
2	Acetylsalicylic acid	200	1110	**232**	**1287**	385.7	2140.5
20	Theophylline	244	1354	**235**	**1304**	157.1	872.1
21	Propoxyphene HCL	84	223	**255**	**678**	24.6	65.4
37	Barium nitrate	355	1358	**266**	**1016**	37.1	142.1
11	Phenol	317	3369	**270**	**2869**	157.2	1670.0
43	Quinidine sulfate	258	610	**286**	**676**	79.2	187.4
22	Propamolol HCL	466	1575	**320**	**1082**	71.5	241.7
1	Paracetamol	2404	15,899	**338**	**2235**	271.4	1795.2
15	2,4-Dichlorophenoxy-acetic	375	1697	**347**	**1570**	385.8	1745.3
29	Thioridazine HCL	995	2445	**385**	**946**	68.6	168.5
49	Altropine sulfate	585	864	**456**	**674**	1.7	2.5
41	Chloroquine phosphate	623	1208	**500**	**969**	84.3	163.4
27	Cupric sulfate	469	1880	**502**	**2012**	290.6	1163.6
3	Ferrous sulfate	319	2100	**680**	**4477**	392.1	2581.0
36	Dichloromethane	1601	18,846	**873**	**10,280**	1386.2	16,321.7
19	Lithium sulfate	492	4478	**1190**	**10,828**	1065.5	9691.8
50	Potassium chloride	2598	34,853	**1499**	**20,107**	285.5	3830.0
45	Chloramphenicol	2500	7735	**1500**	**4641**	285.7	884.0
16	Xylene	4299	40,490	**2119**	**19,953**	899.8	8474.6
9	Ethanol	7057	153,145	**3448**	**74,837**	4712.2	102,262.2
12	Sodium chloride	3002	51,370	**4003**	**68,493**	2287.3	39,138.9
46	Sodium oxalate	11160	83,284	**5095**	**38,019**	357.1	2665.3
7	Ethylene glycol	4698	75,684	**5498**	**88,567**	1570.9	25,304.8
8	Methanol	5619	175,327	**7289**	**227,414**	1569.0	48,954.2
10	1,1,1-Trichloroethane	11196	83,927	**7989**	**59,884**	5707.6	42,785.8
34	Carbon tetrachloride	2350	15,280	**8264**	**53,726**	1314.4	8545.4

Source: E. Walum. 1998. Acute oral toxicity. EHP 106:497-503. (reprinted with permission from the editor)

Oral LD50 Doses for Rat and Mouse and Mean Oral Lethal Doses for Humans

Chemical Number	Chemical	Rat LD50		Mouse LD50		Ave. Human Dose	
		mg/kg	umol/kg	mg/kg	umol/kg	mg/kg	umol/kg
6	Digoxin	28	36	18	23	**0.1**	**0.2**
17	Nicotine	50	308	3	21	**0.7**	**4.4**
49	Altropine sulfate	585	864	456	674	**1.7**	**2.5**
18	Potassium cyanide	5	77	9	131	**2.9**	**43.9**
26	Arsenic trioxide	15	74	31	159	**4.1**	**20.9**
30	Thallium sulfate	16	32	24	47	**14.0**	**27.7**
47	Amphetamine sulfate	55	149	24	65	**20.0**	**54.3**
21	Propoxyphene HCL	84	223	255	678	**24.6**	**65.4**
28	Mercuric chloride	1	4	6	22	**25.7**	**94.7**
39	Pentachlorophenol	27	101	28	105	**28.6**	**107.3**
5	Amitriptyline	320	1154	140	505	**37.1**	**133.8**
37	Barium nitrate	355	1358	266	1016	**37.1**	**142.1**
25	Paraquat	100	537	120	644	**40.0**	**214.7**
42	Orphenadrine HCL	255	834	100	327	**50.0**	**163.4**
29	Thioridazine HCL	995	2445	385	946	**68.6**	**168.5**
4	Diazepam	352	1236	45	159	**71.4**	**250.8**
22	Propamolol HCL	466	1575	320	1082	**71.5**	**241.7**
43	Quinidine sulfate	258	610	286	676	**79.2**	**187.4**
41	Chloroquine phosphate	623	1208	500	969	**84.3**	**163.4**
13	Sodium fluoride	52	1238	57	1357	**92.8**	**2210.9**
31	Warfarin	2	5	3	10	**107.1**	**347.4**
23	Penobarbital	162	697	137	590	**111.4**	**479.7**
40	Varapamil HCL	108	220	163	331	**122.3**	**249.1**
48	Caffeine	192	989	127	654	**135.7**	**698.8**
20	Theophylline	244	1354	235	1304	**157.1**	**872.1**
11	Phenol	317	3369	270	2869	**157.2**	**1670.0**
35	Isoniazid	1250	9117	133	970	**171.5**	**1250.4**
38	Hexachlorophene	56	138	67	165	**214.3**	**526.6**
32	Lindane	76	261	44	151	**242.9**	**835.1**
1	Paracetamol	2404	15,899	338	2235	**271.4**	**1795.2**
50	Potassium chloride	2598	34,853	1499	20,107	**285.5**	**3830.0**
45	Chloramphenicol	2500	7735	1500	4641	**285.7**	**884.0**
27	Cupric sulfate	469	1880	502	2012	**290.6**	**1163.6**
44	Diphenylhydantoin	1635	6480	150	595	**300.0**	**1189.1**
46	Sodium oxalate	11160	83,284	5095	38,019	**357.1**	**2665.3**
2	Acetylsalicylic acid	200	1110	232	1287	**385.7**	**2140.5**
15	2,4-Dichlorophenoxy-acetic	375	1697	347	1570	**385.8**	**1745.3**
3	Ferrous sulfate	319	2100	680	4477	**392.1**	**2581.0**
14	Malathion	290	878	190	575	**742.8**	**2248.4**
16	Xylene	4299	40,490	2119	19,953	**899.8**	**8474.6**
33	Chloroform	908	7605	36	302	**999.8**	**8375.2**
19	Lithium sulfate	492	4478	1190	10,828	**1065.5**	**9691.8**
34	Carbon tetrachloride	2350	15,280	8264	53,726	**1314.4**	**8545.4**
36	Dichloromethane	1601	18,846	873	10,280	**1386.2**	**16,321.7**
8	Methanol	5619	175,327	7289	227,414	**1569.0**	**48,954.2**
7	Ethylene glycol	4698	75,684	5498	88,567	**1570.9**	**25,304.8**
12	Sodium chloride	3002	51,370	4003	68,493	**2287.3**	**39,138.9**
9	Ethanol	7057	153,145	3448	74,837	**4712.2**	**102,262.2**
10	1,1,1-Trichloroethane	11196	83,927	7989	59,884	**5707.6**	**42,785.8**

Source: E. Walum. 1998. Acute oral toxicity. EHP 106:497-503. (reprinted with permission from the editor)

Toxicity Categories

Category	Signal Word	Oral LD_{50} (mg/kg)	Dermal LD_{50} (mg/kg)	Inhalation LD_{50} (mg/L)[2]	Oral Lethal Dose	Eye Irritation	Skin Irritation
I - Highly Toxic	DANGER, POISON (skull & crossbones), WARNING	0 to 50	0 to 200	0 to 0.05	A few drops to a teaspoonful	Corrosive (irreversible destruction of ocular tissue) or corneal involvement or irritation persisting for more than 21 days	Corrosive (tissue destruction into the dermis and/or scarring)
II - Moderately Toxic	CAUTION	>50 to 500	>200 to 2,000	> 0.05 to 0.5	Over a teaspoonful to one ounce	Corneal involvement or irritation clearing in 8-21 days	Severe irritation at 72 hours (severe erythema or edema)
III - Slightly Toxic	CAUTION	>500 to 5,000	>2,000 to 20,000	>0.5 to 2	Over one ounce to one pint	Corneal involvement or irritation clearing in 7 days or less	Moderate irritation at 72 hours (moderate erythema)
IV - Relatively Non-toxic	none	>5,000	>20,000	> 2	Over one pint to one pound	Moderate irritation at 72 hours (moderate erythema)	Mild or slight irritation at 72 hours (no irritation or slight erythema)

1 EPA/OPP does not currently use the inhalation toxicity values in 40 CFR 150.10(h). Instead, OPP uses values that are from a 2/1/94 Health Effects Division paper entitiled "Interim Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity Studies".

[2] Four hour exposure.

Sources:
(1) U.S. EPA, Office of Pesticide Programs. Label Review Manual. Chapter 8: Precautionary Labeling. http://www.epa.gov/oppfead1/labeling/lrm/chap-0.8.htm.
(2) National Ag Safety Database. Toxicity of Pesticides. http://www.cdc.gov/niosh/nasd/docs2/as18700.html.
(3) 40 CFR 156.10(h) – Labeling Requirements for Pesticides and Devices. Warnings and precautionary statements.

Appendix IV: Oral Acute Single Lethal Doses in Humans

Table II: Oral acute single lethal doses in humans

No.	Chemical	LD/MLD	Dose values (g) Reference numbers 10	11	12	13	14	15	16	17	18	19	Other references	Mean dose
1.	Paracetamol	LD	19											19
		MLD	10	10	>10	17.5	22.5	>10	17.5			10		15
2.	Acetylsalicylic acid	LD	33.6	17.5	30			25						27
		MLD	35	17.5			20							22
3A.	Fe^{2+} in iron (II) sulphate	LD	16.8	17.5			6	15.7	11.5	7.7	23.2			14
		MLD			2.1	1.5	1.5				4.28			2.3
3B.	Iron (II) sulphate	LD												38
4.	Diazepam	LD												55*
		MLD						>2						2
5.	Amitriptyline hydrochloride	LD	5					1				2		2.6
		MLD			>2.1	2	2		1.75					2
6.	Digoxin	LD				0.005		0.015	0.0075					0.0092
		MLD			0.001									0.0011
7.	Ethylene glycol	LD			100			111		111	111			110
		MLD	111	111					111			111		110
8.	Methanol	LD		70	123		67	150	123	119				110
		MLD	23.6	23.8							17.5	59		31
9.	Ethanol	LD	455	280	276			455	455					330
		MLD										196		
10.	Isopropanol	LD	132	188	196		196	188	157	188			98 (9, 59)	98
		MLD												180
														196
11.	1,1,1-Trichloroethane	LD											193 (60), 802 (61)	500
		MLD	20	>42	>6.7									24
12.	Phenol	LD	4.8	4.5							8.8			11
		MLD	140	140	2	11.5						8		6.6
13.	Sodium chloride	LD											210[b]	160
		MLD												nr

Source: Ekwall et al. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part V. ATLA 26:571-616. (reprinted with permission from the editor)

14.	Sodium fluoride	LD	7.5			5	5	7.5	7.5		4.5		6.2
		MLD		4.6	1.2		2	1		7.5		1	2.9
15.	Malathion	LD	60					17.5		60			70 (62)	52
		MLD											25 (9, 59)[c]	25
16.	2,4-Dichloro-	LD	28[d]					24.1		28				27[e]
	phenoxyacetic acid	MLD	5.6		6.5								5.6 (9)[f]	5.9
17.	Xylene	LD			245			19.4	53					63[e]
		MLD	120							12.9		21.5		51
18.	Nicotine	LD	0.060			0.040		0.060	0.045	0.05	0.05	0.045		0.05
		MLD	0.050	0.045		0.045		0.005						0.036
19.	Potassium cyanide	LD	0.25	0.20	0.14			0.20			0.25			0.21
		MLD								0.20		0.2		0.20
20A.	Lithium	LD						9.4[f]						9.4
		MLD												nr
20B.	Lithium sulphate	LD								42[g]				58
21.	Theophylline	LD								11[g]			11 (63)	11
		MLD							4			7	5.2 (9, 59)[f]	5.4
22.	Dextropropoxyphene	LD							0.78[h]		0.64			0.71
	hydrochloride	MLD	1.1		0.5	0.75		1.28		0.65				0.86
23.	Propranolol	LD				E9.6		E5.1	4		1.2			5
	hydrochloride	MLD			>1									1
24.	Phenobarbital	LD	8	8			8	7.5	7.5					7.8
		MLD			1.5					6	5	5		4.8
25.	Paraquat	LD	4.5	2.1					3.1	1.5		1.5		2.5
		MLD			0.28							0.075		0.18
26.	Arsenic trioxide	LD						0.25	0.33				0.3[h]	0.29
		MLD	0.21	0.23	0.12				0.2	0.2		0.1		0.18
27.	Copper (II) sulphate	LD						15	15	15	10			14
		MLD										15	3.5 (59)[c]	9.3
28.	Mercury (II) chloride	LD		2.1	1			2.5		1	1			1.5
		MLD		0.5				0.5				0.5		0.5
29.	Thioridazine	LD	4.8						3.5					4.2
	hydrochloride	MLD							>3					3
30.	Thallium sulphate	LD	1	0.85	1			1	1	1	1			0.98
		MLD		0.56								0.8		0.68

Table II: continued

No.	Chemical	LD/ MLD	Dose values (g) Reference numbers 10	11	12	13	14	15	16	17	18	19	Other references	Mean doses
31.	Warfarin	LD							7.5[i]	7.5[i]				7.5
		MLD												nr
32.	Lindane	LD	15					8.75		28				17
		MLD			3.5									3.5
33.	Chloroform	LD						44			96			70
		MLD	44	14.8	14.8					14.8		22		22
34.	Carbon tetrachloride	LD	151					32.8[e,j]						92
		MLD	12		6.4			3.2	6.4			6.4		6.9
35.	Isoniazid	LD	12.5					14	8	12.5[f]	14			12
		MLD	8	8		8		8				10		8.4
36.	Dichloromethane	LD			33.2				146[e]			110[e]		97
		MLD									24			24
37.	Barium nitrate	LD		2	2					3.9[e]				2.6
		MLD										1		1
38.	Hexachlorophene	LD			5					17.5	21			15
		MLD										2.25		2.3
39.	Pentachlorophenol	LD								2			2 (62)	2
		MLD	2		1									1.5
40.	Verapamil hydrochloride	LD							8.6[f]					8.6
		MLD		3					>3	3.8[e]				3.4
41.	Chloroquine phosphate	LD	2.5			7.2	8		5.6		6.4			5.9
		MLD											2.2 (9)[c]	2.2
42.	Orphenadrine hydrochloride	LD	2.8	2.8	5.5[d]	2.8			2.8					3.3
		MLD								1				1
43.	Quinidine sulphate	LD				11.5			8		11.5			10
		MLD	4						>6	8[e]	2			5

44.	Diphenylhydantoin	LD			E21				8.5[j]				21
		MLD	E7.5	9.1						20		2	6.8
45.	Chloramphenicol	LD	20										20
		MLD										10 (62), 28 (9)[c]	19
46.	Sodium oxalate	LD		30	15					23			23
		MLD									15	5 (64)	8.3
47.	Amphetamine	LD							0.5		1.4[d]		0.95
	sulphate	MLD		0.1	0.1			0.15	0.12			0.25	0.14
48.	Caffeine	LD	7.5		15	7.5	7.5	10	12				9.9
		MLD	E6.5	10							10	10	9.1
49.	Atropine sulphate	LD	E0.10[k]		E0.1[k]	E0.2[k]					0.1[k]	0.075	0.12[k]
		MLD						0.10	0.2	0.050			0.12
50.	Potassium chloride	LD							E45		16.2		28
		MLD								18[k]			18

[a] *high variability as well as tolerance makes it difficult to establish human LD.*
[b] *"POISINDEX", Information Systems (ed. B.H. Rumack & D.G. Spoerke), Micromedex (Denver, CO, USA).*
[c] *Low LD.*
[d] *Extrapolated from animal dosage.*
[e] *Geometric mean value, when the quotient between original values (range) is larger than ten.*
[f] *Two lethal poisonings.*
[g] *One survivor and one dead.*
[k] *One death.*
[j] *12.5mg/kg lethal in 14 days (16), 1g lethal in 13 days (17).*
[l] *Several survivors.*
[k] *Very variable.*
LD = mean lethal dose; MLD = minimal lethal dose; E = extrapolated; nr = not reported.

Appendix V: Clinically Measured Acute Lethal Serum Concentrations in Humans

Table III: Clinically measured acute lethal serum concentrations in humans

No.	Chemical	LC/MLC	Concentrations (mg/l) References numbers 10	11	12	13	14	15	16	17	18	19	Other references	Mean concentration (mg/ml)
1.	Paracetamol	LC	300[a]	300[a]		300[a]						400		330
		MLC		160[a]	300[a]									250[a]
2.	Salicylic acid	LC	1300[b]				300[a]					600		950
		MLC		1000	900[b]	800[b]	1000							930
3.	Iron	LC										8		8
		MLC	10[c]	10[c]	5									7.6
4.	Diazepam	LC						5	8.1[c]			20		20
		MLC	15	20						20				18
5.	Amitriptyline	LC		5								10		7.5
		MLC								2.5[d]				2.5
6.	Digoxin	LC							0.003	0.027	0.003	0.01		0.018
		MLC	0.015											0.007
7.	Ethylene glycol	LC	4370							4370		2000		3600
		MLC												nr
8.	Methanol	LC	1750	1000						1600	1800	800		1400
		MLC								400				450
9.	Ethanol	LC		5000			4000	5000				4500		4600
		MLC	5000		5000				5000		3700			4600
10.	Isopropanol	LC	3400	2000								3000		2800
		MLC						1500	2000					1800
11.	1,1,1-Trichloro-ethane	LC											180 (26)[e]	E180
		MLC												nr
12.	Phenol	LC												nr
		MLC	42						50					46[f]

Source: Ekwall et al. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part V. ATLA 26:571-616. (reprinted with permission from the editor)

13.	Sodium lauryl sulfate	LC	10800	10800		10800							nr
		MLC											11000[f]
14.	Fluoride	LC		3			14.2	14[d,e]		3			8.6
		MLC											nr
15.	Malathion	LC						0.35[d,e]			4.4 (26)[g]		E4.4
		MLC											E0.35
16.	2,4-Dichlorophenoxy acetic acid	LC	416					600[d]					510
		MLC											nr
17.	Xylene	LC					E50				43 (66)		47
		MLC									11[h,j]		11
18.	Nicotine	LC								5			5
		MLC		10		10							10[f]
19.	Cyanide	LC									5		5
		MLC	2.5			3		3.1[d]					2.9
20.	Lithium	LC				69[f]		77[g]					72[k]
		MLC		24	24								24[k]
21.	Theophylline	LC					135	130[m]	150				150
		MLC	100[n]	183[f]		100	64						79
22.	Dextropropoxyphene	LC	6							50			8
		MLC					2			10			8
23.	Propranolol	LC	16	3.3[k]	2		3.3[k]	1.8[d]					1.9
		MLC		4.7		4[d]		3[g]					6.4
24.	Phenobarbital	LC	115										3.9
		MLC		80			120	117	110	200			136
25.	Paraquat	LC	2										100
		MLC		0.2				0.2	0.1				2[f]
													0.17
26.	Arsenic	LC			3.9[d]				1.5	2[o]			2.5
		MLC											nr
27.	Copper	LC							4.5	6			5.5
		MLC											nr

Table III: continued

No.	Chemical	LC/MLC	Concentrations (mg/l)										Other references	Mean concentration (mg/ml)
			References numbers											
			10	11	12	13	14	15	16	17	18	19		
28.	Mercury	LC								0.65[c]		2	14.3 (67)[d,e]	2.6[b]
		MLC	0.22		>0.1									0.22
29.	Thioridazine	LC								7.1[d]		20		14
		MLC												nr
30.	Thallium	LC								1.5[d,h]				1.5[b]
		MLC	0.3											0.3
31.	Warfarin	LC											110 (26)[d,e]	E110[f]
		MLC												107
32.	Lindane	LC									107[d]			E20.92
		MLC								0.92[c]				0.5
								0.5						400
33.	Chloroform	LC										400		180
		MLC								165[b]		200[g]		4.5[b]
34.	Carbon tetrachloride	LC								20[d,e]		1		1.8
		MLC								1.8[d,e]				E77
35.	Isoniazid	LC								77[c]				10
		MLC		10										
36.	Dichloromethane	LC										300[i]		300
		MLC												nr
37.	Barium	LC											97 (26)[d]	E97
		MLC								5.6[d]				5.6
38.	Hexachlorophene	LC	35.6[e]							52[c]				44
		MLC												nr
39.	Pentachlorophenol	LC								74[c]				74
		MLC	40											40

40.	Verapamil	LC	3[d,e]		*		4.1	4[d,e]				3.7
		MLC										nr
41.	Chloroquine	LC	10[d]		8		9	22[d]		4		11
		MLC										nr
42.	Orphenadrine	LC		6				3.6[e]				4.8
		MLC	6									6[f]
43.	Quinidine	LC		16.8[d]				14.6[d]		40		24
		MLC	14		10		9					11
44.	Diphenylhydantoin	LC	95					98		80		91
		MLC	60	50								55
45.	Chloramphenicol	LC						190[e]				E190
		MLC			75[i]		68[i]	68[i]				70
46.	Oxalate	LC						20[e]		20	20 (26)[e]	20
		MLC										nr
47.	Amphetamine	LC							4	2		3
		MLC										nr
48.	Caffeine	LC	150						160[d,e]	150		150
		MLC			150			135[d]				140
49.	Atropine	LC						0.13[d,e]				E0.13[c]
		MLC										nr
50.	Potassium	LC	397					364[d]	352			370
		MLC	293					313				300

[a]After 4 hours. [b]After 6 hours. [c]After 3 hours. [d]As judged from high survived concentrations. [e]SD analysis. [f]This value will substitute for the presented LC value in calculations based on LC values. [g]Based on one case only. [h]Geometrical mean value from a range of values with a quotient larger than ten. [i]TOMES® Information Services (ed. B.H. Rumack & D.G. Spoerke), Micromedex (Denver, CO, USA). [j]Also 69mg/l as judged from high survived concentrations in reference 16. [k]May include acute chronic dosage. [l]Peak concentration. [m]S/D: 90/170 = 130 mg/l (17). [n]Acute dosage. [o]In blood. [p]Represents acute on chronic dosage: no reports on single-dose lethal poisonings. [q]Plane 4 anaesthesia. [r]Value probably originating from forensic medicine data. [s]Reported value of 90mg/l, which seems too high. [t]Grey baby syndrome.

E = estimated/extrapolated; LC = mean lethal serum concentration; MLC = minimal lethal serum concentration; S/D = high survived and lethal concentrations from case reports, with a resulting mean value; nr = not reported.

Appendix VI: Post-Mortem Acute Lethal Concentrations in Humans

Table IV: Post-mortem acute lethal concentrations in humans

No.	Chemical	LC/MLC	Concentrations (mg/l) Reference numbers 17	20	21	22	23	24	25	Other references	Mean concentration (mg/ml)
1.	Paracetamol	LC	248		250	280[a]			160		230
		MLC		160	160		150	250			180
2.	Salicylic acid	LC	661	500	500	732			700		620
		MLC					450	450			450
3.	Iron	LC	9.0[b]	35							22
		MLC									nr
4.	Diazepam	LC	18							10 (68)	14
		MLC		20	5	50				50 (69)	31
5.	Amitriptyline	LC	3.7	6.32[a]	3.3[c]	5.58[a]			2		4.2
		MLC			0.55[d]		1.5	1.75			1.3
6.	Digoxin	LC	0.025	0.015	0.0103[c]				0.015		0.016
		MLC			0.0015[d]		0.005	0.005			0.0038
7.	Ethylene glycol	LC	2400	3000	2400						2600
		MLC			300						300
8.	Methanol	LC	1900		1900						1900
		MLC		890	200				900		660
9.	Ethanol	LC	5500		4000[c]				5000		4800
		MLC		3500	2250[d]	4000					3300
10.	Isopropanol	LC	1500								1500
		MLC			1000						1000
11.	1,1,1-Trichloroethane	LC	126						316[a]		170
		MLC			80[c]						15
12.	Phenol	LC	49	90	15[d]				90		76
		MLC									nr
13.	Sodium in sodium chloride	LC								13000 (26)[a]	13000
		MLC									nr

Source: Ekwall et al. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part V. ATLA 26:571-616. (reprinted with permission from the editor)

14.	Fluoride	LC	15	2	3			2		5.5
		MLC								nr
15.	Malathion	LC	281							280
		MLC								nr
16.	2,4-Dichlorophenoxy-acetic acid	LC	464			669				570
		MLC								nr
17.	Xylene	LC	43	10.9	13.4[a,b]			10.9		20
		MLC								nr
18.	Nicotine	LC	29	16[a]	25	17.7[a]				22
		MLC			5		13.6			9.3
19.	Cyanide	LC	24.7	3.7		7.6[a]		3.7		9.9[f]
		MLC			5					5[f]
20.	Lithium sulphate	LC	31.9[a]	34				35		34[g]
		MLC			13.9		14			14[g]
21.	Theophylline	LC	150	150				150		150
		MLC			50		50			50
22.	Dextropropoxyphene	LC	4.7	4.1[a]	15	7.7[a]				7.9
		MLC			2		1.5			1.8
23.	Propranolol	LC	14	10	9	16		7		11
		MLC			4		7			6
24.	Phenobarbital	LC	97		45[c]	210		125		120
		MLC		80	4.3[d]		55			35[e]
25.	Paraquat	LC	1.2[a,h]	35	1.2			2		3.2[e]
		MLC								nr
26.	Arsenic	LC	3.3	12	15	2.36[a]				8.2
		MLC								nr
27.	Copper	LC	36	12.5[a]						24
		MLC								nr
28.	Mercury	LC	4.2[i]			0.58				2.4
		MLC						0.6		0.6
29.	Thioridazine	LC	5.1	4.24[a]	5	7			3.3 (27)[c]	4.9
		MLC			2		10 11.5		2.4 (27)[d]	6.5
30.	Thallium	LC	4.0[j]	0.5						2.3
		MLC								nr

Table IV: continued

No.	Chemical	LC/MLC	Concentrations (mg/l) Reference numbers 17	20	21	22	23	24	25	Other references	Mean concentration (mg/ml)
31.	Warfarin	LC								100 (28)	100
		MLC			> 10		> 10	> 10	> 11		> 10
32.	Lindane	LC									nr
		MLC	0.02[a,b]								0.02
33.	Chloroform	LC	54	390	30[c]	29			390		97[a]
		MLC			74						7
34.	Carbon tetrachloride	LC	274[a]		260				150		230
		MLC									nr
35.	Isoniazid	LC	117[b]		150[a]		100	100			130
		MLC									100
36.	Dichloromethane	LC	364	280	395[b]	496			280		360
		MLC									nr
37.	Barium	LC								< 20[a,m]	nr
		MLC	L9[a,l]								1.9
38.	Hexachlorophene	LC	35[a]	35					35		35
		MLC									nr
39.	Pentachlorophenol	LC	107		99						100
		MLC		46	46				45		46
40.	Verapamil	LC	11	6.4					6		7.8
		MLC						2.5			2.5
41.	Chloroquine	LC	30.5	17.2[a]	10	11.2[a]			3		14
		MLC			3		4.5	3			3.5
42.	Orphenadrine	LC	20.6	6	9	16.7			6		12
		MLC			4		7	3.6			4.9
43.	Quinidine	LC	45[a]	40	55	40			40		44
		MLC			30		15				23
44.	Diphenylhydantoin	LC	54[a,n]		94				100		83
		MLC		100	70		50	50			68

Appendix VII: Human Kinetic Data

Table V: Human kinetic data[a]

No.	Chemical	Absorption in the gut[b]	Time to peak (ingestion)	Kinetics	T½	Vd l/kg	Passage of blood-brain barrier	Accumulation in vital organs	Blood protein binding	Reference[d]
1.	Paracetamol	Good	0.5–> 4 hours*	First-order[c]	> 12 hours*	0.9	Free?	Liver,[f] kidney[f]	20–50%*	[e]
2A.	Acetylsalicylic acid	Good		Zero-order	0.27 hours	0.2	Restricted	None	50–90%	
2B.	Salicylic acid	[h]	12–24 hours*	Zero-order	27 hours*	0.17	Restricted	None	< 80%	30
3.	Iron (II) sulphate	Good[i]	2–4 hours*	Biphasic	nr	nr[j]	Restricted	Blood, liver	100%	16, 30
4	Diazepam	Complete	1–3 hours	Biphasic	96 hours*	1.1	Free	CNS, liver,[f] kidney[k]	99%	
5.	Amitriptyline hydrochloride	Good[l]	20 hours*	Biphasic	8 and 27 hours*	15	Free	Liver,[f] kidney, lung, heart, CNS[k]	96%	
6.	Digoxin	Moderate	2–5 hours*	Biphasic	48 hours*	6	Restricted	Heart, kidney, liver[f,m]	29%	
7.	Ethylene glycol	Complete	1–4 hours	First-order?	8.4 hours*	0.65	Free	Liver,[f] kidney[f]	None	
8.	Methanol	Good	0.5–1.5 hours	Zero-order	27 hours*	0.65	Free	Kidney, liver[f]	None	"
9.	Ethanol	Good	0.5–> 3 hours*	Zero-order	4 hours*	0.6	Free	None	None	
10.	Isopropanol	Complete	1 hour	First-order	5.4 hours*	0.6	Free	None	nr	31
11.	1,1,1-Trichloroethane	Complete	1 hour?	Triphasic	0.7, 6 and 53 hours	> 1*	Free	CNS[k]	30–70%	32, 33
12	Phenol	Complete	E0.5 hours*	Biphasic?	2.8 hours	nr	Free	CNS	30–70%?	34, 35
13.	Sodium chloride	Complete	5 hours*	Zero-order	nr	0.64	Restricted	None	None	
14.	Sodium fluoride	Complete	> 1 hours*	Biphasic	5.5 hours	0.6	Restricted	None (bone only)	None	36
15.	Malathion	Good	1–5 hours*	Multiphasic	nr[n]	nr[o]	Free	Kidney, liver,[f] CNS[m]	nr[p]	37–39
16.	2,4-Dichlorophenoxy-acetic acid	Complete	7–24 hours*	First-order	58 hours*[q]	0.2*	Restricted	Liver, kidney	High	
17.	Xylene	Good	1.5 hours	Biphasic	1 and 26 hours	nr[r]	Free	Lipid-rich organs[k]	High	15
18.	Nicotine	Complete[s]	> 0.5 hours?	Biphasic	10 minutes and 2.2 hours	2	Free	CNS, liver,[f] kidneys[f,k]	High	
19.	Potassium cyanide	Complete	< 1 hour*	Biphasic	1 and 6–66 hours*	1	Free	Erythrocytes[k]	6%	15
20.	Lithium sulphate	Complete	nr	Biphasic	3–12 and 8–65 hours*	0.9	Restricted	Liver, kidney[k]	None	16

Source: Ekwall et al. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part V. ATLA 26:571-616. (reprinted with permission from the editor)

21.	Theophylline	Complete	2–8 hours[a]	Biphasic[a]	17 minutes and 6 hours[a]	0.5	Free	None	56%	63	
22.	Dextropropoxyphene hydrochloride	Complete[f]	1–2 hours	Biphasic[a]	5 and 15 hours[a]	16	Free	CNS, liver,[f] lung, kidney[g]	78%	*	
23.	Propranolol hydrochloride	Complete[f]	1–2 hours	Biphasic?	3.9 and 16 hours[a,?]	4.3	Free	CNS, liver,[f] kidney[g]	80–95%	26	
24.	Phenobarbital	Complete	nr	First-order	100 hours[a]	0.6	Free	Liver[f]	50%	16, 39	
25.	Paraquat	Moderate[a]	< 4 hours[a]	Biphasic	5 and 84 hours[a]	1.4[a]	Free[?]	Lung, liver, kidney	nr	40	
26.	Arsenic trioxide	Good	1 hour	Biphasic[a,?]	1–2 and 30 hours[a]	0.27	Restricted	Liver, kidney, heart, GIT[a]	nr		
27.	Copper (II) sulphate	Poor	nr	Biphasic	2–3 hours and 26 days[a]	2	Restricted	Blood, liver[f]	95%	16, 41	
28.	Mercury (II) chloride	Moderate[f]	nr	Biphasic	2 and 24–50 days[a]	> 1	Restricted	Blood, kidney, liver, heart	nr[a]	67[a]	
29.	Thioridazine hydrochloride	Good[f]	2–4 hours	Multiphasic	26 hours	18	Free	CNS, lung, liver,[f] kidney[g]	96–99%	16, 30, 42	
30.	Thallium sulphate	Good	2–4 hours	Biphasic[a]	48 and 96 hours[?,a]	4.6	Restricted	Kidney, heart, liver, CNS[a]	None	16, 30, 43	
31.	Warfarin	Good	3–9 hours	First-order	22–96 hours[a]	0.11[a]	Restricted	none	99%	15, 16, 44	
32.	Lindane	Good[a]	6 hours	Biphasic[a]	21 hours and 10 days[a,?]	nr[f]	Free	CNS, liver, kidney, (fat)	nr	16, 26	
33.	Chloroform	Complete	1 hour	First-order?	1.5 hours	2.6	Free	CNS, liver, kidney, (fat)[g]	nr	16	
34.	Carbon tetrachloride	Good	nr	Biphasic[a]	11 and 43 hours[a]	nr[f]	Free	Liver,[f] kidney,[f] (fat)	nr	16, 45	
35.	Isoniazid	Complete	1.5–3 hours[a]	First-order	2.4 and 5 hours[a,?]	0.6	Free	Liver,[f] kidney, lung, skin	10%	16	
36.	Dichloromethane	Complete	2 hours	First-order?	40 minutes	0.67	Free	None	nr	16	
37.	Barium nitrate	Good	> 2 hours[a]	Triphasic	3.6, 34 and 1033 days[a,?]	nr	Restricted	Muscle, lung, (bone)	54%	15, 26	
38.	Hexachlorophene	Good	3–6 hours?	Biphasic?[a]	24 hours[a]	nr	Restricted	Liver,[f] kidney	92%[a]	46, 47	
39.	Pentachlorophenol	Good	4 hours	First-order?	13 hours to 16 days[a]	0.35[a]	Restricted	Liver,[f] kidney	> 90%	16, 48	
40.	Verapamil hydrochloride	Good[f]	2 hours	Biphasic	23 minutes and 5 hours	5	Restricted?	Liver[f,h]	90%		

Table V: continued

No.	Chemical	Absorption in the gut[b]	Time to peak (ingestion)	Kinetics	T½[c]	Vd l/kg	Passage of blood-brain barrier	Accumulation in vital organs	Blood protein binding	References[d]
41.	Chloroquine phosphate	Good	1-3 hours*	Triphasic	2, 7 and 45 days*[e]	94	Free	Heart, liver, kidney, lung, erythrocytes[g]	55-61%	16, 49
42.	Orphenadrine hydrochloride	Good[f]	3 hours	First-order	15 hours	6	Free	CNS, liver, lung[h]	20-95%	16, 50, 51
				Biphasic?*	6 and 15 hours*					
43.	Quinidine sulphate	Good[f]	> 2 hours*	First-order?	> 7.8 hours*	2.7*	Restricted	Liver,[i] kidney, heart[h]	60-90%	15, 16
44.	Diphenyl-hydantoin	Poor/good	30-120 hours*	Zero-order and first-order*	24-230 hours*[e]	0.6*	Free	Liver,[i] kidney, CNS	60%*	52
45.	Chloramphenicol	Good	2-3 hours	First-order	2.5 hours	1.2	Free	Liver,[i] kidney	55%	
46.	Sodium oxalate	Poor	6 hours?	First-order?	4 hours?*	E0.4*	Restricted	Kidney, liver	nr	26, 64
47.	Amphetamine sulphate	Complete	1-4 hours*	First-order?	7-34 hours*[j]	3-6.1	Free	Liver, kidney	16%	15, 16
48.	Caffeine	Complete	1 hour	First-order?	9-16 hours*	0.6	Free	None (liver 2x)	35-60%	
49.	Atropine sulphate	Good	> 2 hours*	First-order?	3.5 hours	3	Free	Kidney, liver	50%	53, 54
50.	Potassium chloride	Complete	0.5 hours	Multiphasic	nr	nr	Free?	None	None	65[k]

[a]Data for the overdose situation are indicated by an asterisk. [b]Absorption: complete = 100% and rapid, good = 80%, moderate = 20-80%, and poor = 0-20%. [c]One value indicates T½ of the elimination phase. Successive values represent separate phases (alpha, beta, etc.). [d]Other than references 10, 11, 13, 14 and 17. [e]Non-linear in overdose? [f]Also a biotransforming organ. [g]POISINDEX®, Information Systems (ed. B.H. Rumack & D.G. Spoerke), Micromedex (Denver, CO, USA). [h]Absorbed as acetylsalicylic acid. [i]Due to corrosivity. [j]Probably large, i.e. around 5l/kg. [k]Early accumulation. [l]Documented first therapeutic doses, i.e. bioavailability is decreased by rapid binding in the liver of a large fraction of the absorbed dose (25-85%). For most such chemicals, passage of the intestinal mucosa is probably complete. However, the term "good" is often used in this table, based on literature reports on the total absorption (the sum of intestinal passage and first pass reduction of bioavailability). [m]Slow accumulation. [n]Alpha phase: 2.9 hours. [o]Probably large Vd and protein binding. [p]pH-dependent. [q]Dependent on formulation. [r]Biphasic up to 160 hours. [s]TOMES®, Information Systems (ed. B.H. Rumack & D.G. Spoerke), Micromedex (Denver, CO, USA). [t]Varies between rapid and slow acetylators. [u]Alpha-phase: 3 hours in overdose. [v]Dose-dependent.*

nr = non reported; CNS = central nervous system (brain); GIT = gastrointestinal tract (gut); T½ = plasma half-life; Vd = distribution volume.

Appendix VIII: Peaks from Approximate 50% Lethal Concentration (LC50) Curves

Table VI: Peaks from approximate 50% lethal concentration (LC50) curves[a]

No.	Chemical	Time to peak (hours)	Peak conc. mg/l	Type of curve	Case reports: Sub-lethal	Lethal (clinical)	Lethal (post-mortem)	Total
1.	Paracetamol	4	358	LC50	81	62	0	143
2.	Salicylic acid	20	1070	LC50	31	46	1	78
3.	Iron	4	43.5	LC50	15	12	0	27
4.	Diazepam	2	19.9	LC100	4	0	0	4
5.	Amitriptyline	6	1.69	LC50	8	6	10	24
6.	Digoxin	3	0.071	LC50	15	9	1	25
7.	Ethylene glycol	2.5	1550	LC50	28	12	9	49
8.	Methanol	2	3790	LC50	76	37	7	120
9.	Ethanol	1	8440	LC50	20	1	143	164
10.	Isopropanol	1	4960	LC50	13	2	2	17
11.	1,1,1-Trichloro-ethane	1	231	LC50	3	0	2	5
12.	Phenol	0.5	80	LC50	3	0	4	7
13.	Sodium in sodium chloride	5	11700	LC50	3	9	1	13
14.	Fluoride	3	19.4	LC0	3	3	7	13
15.	Malathion	5	1.88	LC0	2	1	11	14
16.	2,4-Dichloro-phenoxyacetic acid	14	1125	LC50	7	1	4	12
17.	Xylene	1	110	LC0	3	0	1	4
18.	Nicotine	0.5	13.5	LC0	1	1	3	5
19.	Cyanide	0.5	16.4	LC50	12	9	10	31
20.	Lithium	3	97.2	LC100	4[b]	0	0	4[b]
21.	Theophylline	12	180	LC50	57	18	1	76
22.	Dextropropoxy-phene	2	8	LC0	2	1	6	9
23.	Propranolol	4	3.11	LC50	6	2	1	9
24.	Phenobarbital	15	230	LC50	20	1	0	21
25.	Paraquat	2.5	12.6	LC50	23	66	16	105
26.	Arsenic	4	1.65	LC50	10	8	3	21
27.	Copper	11	15.9	LC50	10	5	1	16
28.	Mercury	12	40.1	LC50	12	2	4	18
29.	Thioridazine	4	4.08	LC50	1	1	4	6
30.	Thallium	24	7.35	LC50	25	5	2	32

[a] *From reference 26.*
[b] *Documented single-dose cases (not overdose on previous medication).*

Source: Ekwall et al. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part V. ATLA 26:571-616. (reprinted with permission from the editor)

Table VI: continued

No.	Chemical	Time to peak (hours)	Peak conc. mg/l	Type of curve	Case reports: Sub-lethal	Lethal (clinical)	Lethal (post-mortem)	Total
31.	Warfarin	6	200	LC0	3	0	0	3
32.	Lindane	6	1.3	LC0	5	2	1	8
33.	Chloroform	2	490	LC50	2	0	5	7
34.	Carbon tetrachloride	6	5.8	LC50	5	1	1	7
35.	Isoniazid	3	167	LC50	24	3	4	31
36.	Dichloromethane	3	344	LC0	0	0	9	9
37.	Barium	2	305	LC100	9	0	0	9
38.	Hexachlorophene	5	116	LC50	2	1	1	4
39.	Pentachlorophenol	10	79.1	LC50	1	0	3	4
40.	Verapamil	2	13.2	LC50	10	9	4	23
41.	Chloroquine	2	9.41	LC50	4	1	9	14
42.	Orphenadrine	2	11.3	LC50	6	1	8	15
43.	Quinidine	6	26	LC50	4	2	0	6
44.	Diphenylhydantoin	34	202	LC50	13	1	0	14
45.	Chloramphenicol	6	180	LC0	5	4	0	9
46.	Oxalate	6	110	LC0	1	1	0	2
47.	Amphetamine	2	15.5	LC50	1	1	5	7
48.	Caffeine	3	179	LC50	6	0	4	10
49.	Atropine	3	4.05	LC100	2	0	0	2
50.	Potassium	1	375	LC0	4	3	1	8

[b] *Documented single-dose cases (not overdose on previous medication).*

a few organs are routinely screened for chemicals, such as blood, heart, liver, kidney, brain and lung. Thus, the information on body distribution is often limited to these organs.

The qualitative human toxicity data

The human toxicity data presented in Table IX are the result of a study of references 10–17, in a few instances supplemented by data from other sources. In the same way as the kinetic data in Table V, the toxicity data represent the sum of the information from all the handbooks consulted. The classification of lethal symptoms into main causes and other causes of death, as well as the classification of lethal action into known, unknown and hypothetical mechanisms, represent judgements by the handbook authors. However, the lists of lethal symptoms in various handbooks have been extensively edited to provide uniform terminology. The handbook authors have used a plethora of terms for essentially the same type of event. To mention only one example, circulatory failure in Table IX stands for vascular collapse, vasomotor collapse, shock, circulatory shock, hypovolaemic shock, hypotensive shock, and so on.

Potentially the most controversial data in Table IX are those that are based on mecha-

Appendix IX: Human Acute, Single-Dose Toxicity Data

Table IX: Human acute, single-dose toxicity data

No.	Chemical	Lethal symptoms*	Mean time to death	Danger over	Target organs	Toxic metabolites*	Lethal mechanisms	References*
1.	Paracetamol	Hypoglycaemic coma Liver failure M Kidney failure	3-5 days	nr	Liver P Kidney P (CNS)	More toxic intracellular metabolites	Known: Covalent NAPQI binding and lipid peroxidation	
2.	Acetylsalicylic acid	Metabolic acidosis M Cerebral bleedings Pulmonary oedema Cardiovascular failure	48 hours	nr	Kidney P Liver P CNS P Lung P GIT P	Salicylic acid is the reactive metabolite of the parent compound	Known: General cell poison. Uncoupling of oxidative phosphorylation, inhibition of Kreb's cycle dehydrogenases	
3.	Iron (II) sulphate	Haemntemesis GIT perforation Pulmonary oedema CNS excitation/depression Circulatory failure Liver and kidney failure	6 or 48 hours	72 hours	GIT P Liver P Kidney CNS CVS Lung P	tp	Known: General cell poison. Inhibition of oxidative phosporylation and ATP; lipid peroxidation	
4.	Diazepam	CNS depression M	2 hours	3 hours	CNS	(Nordiazepam)	Unknown	
5.	Amitriptyline hydrochloride	CNS excitation/ depression Heart arrythmias/arrest M	< 12 hours	6 days	CNS Heart	(Nortriptyline)	Hypothetical: Blocks noradrenaline, 5-HT and dopamine presynaptic uptake; prevents reuptake of heart noradrenaline	
6.	Digoxin	Heart arrythmias/ arrest M Hyperkalaemia	7 hours	20 hours	Heart GIT CNS	(Metabolites)	Known: Impairing ion transport and increasing sarcoplasmic Ca by binding to Na/K ATPase, increasing automaticity of cells	

Source: Ekwall et al. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part V. ATLA 26:571-616. (reprinted with permission from the editor)

7.	Ethylene glycol	1–12 hours: CNS excitation/depression M 12–24 hours: heart failure 24–72 hours: kidney failure	17 hours	72 hours	CNS Heart P Kidney P	Glycolate Glycolate Oxalate	Hypothetical: Metabolites inhibit mitochondria, leading to metabolic acidosis. Oxalate decreases S-Ca		
8.	Methanol	CNS depression M Metabolic acidosis Cardiovascular failure	32 hours[d] 173 hours[d]	nr	CNS P* Pancreas P Liver P Kidney P Heart P	Formaldehyde Formic acid	Hypothetical: Accumulation of formic acid leads to metabolic acidosis. Lactate inhibits mitochondrial respiration		
9.	Ethanol	CNS depression M Cardiovascular failure	6 hours[d]	12 hours	CNS CVS	(Acetaldehyde)	Hypothetical: Interference with cell membrane fluidity, perturbing proteins, such as ion channels. Depression of postsynaptic potentials in CNS		
10.	Isopropanol	CNS depression M Cardiovascular failure Pneumonia	3 hours	48 hours	CNS CVS Lung P	tp	Unknown		60[a]
11.	1,1,1-Trichloroethane	CNS depression M Heart arrythmias Cardiovascular failure Pneumonia	3 hours	4 hours	CNS P CVS Lung P	tp	Unknown		
12.	Phenol	CNS excitation/depression M Heart arrest/pulmonary oedema Liver and kidney failure	1 hour	24 hours	CNS Heart Liver Kidney GIT P	tp	Known: General protoplasmic poison that denaturates proteins		18, 34
13.	Sodium chloride	CNS excitation/depression M Cerebral bleedings Cardiovascular failure Pulmonary oedema Vasculitis	20 hours	25 hours	CNS P Lungs Kidney VS P	tp	Known: Acute dehydration of brain cells caused by osmodic shift of water to the outside of the blood–brain barrier		

Table IX: continued

No.	Chemical	Lethal symptoms[a]	Mean time to death	Danger over	Target organs	Toxic metabolites[b]	Lethal mechanisms	References[c]
14.	Sodium fluoride	Cardiovascular failure CNS excitation/depression	2–4 hours	20 hours	Heart[b] CNS[b] Liver Kidney	tp	Hypothetical: Protoplasmic poison interfering with many enzymes. May lower S-Ca and induce potassium efflux from cells	
15.	Malathion	Early: Cholinergic crisis/ respiratory failure M Later: Heart failure Heart arrythmias/arrest	0.5–6 hours	24 hours	CNS Muscles Heart P	Malaxon	Known: Inhibition of acetylcholine esterase resulting in acetylcholine accumulation in CNS and effector organs	
16.	2,4-Dichloro-phenoxyacetic acid	Hyperthermia/myotonia CNS excitation/depression Metabolic acidosis Heart failure Liver failure	8–96 hours	48 hours	CNS P Liver P Kidney P Heart	tp	Hypothetical: Hypermetabolism due to uncoupling of oxidative phosphorylation. Direct toxin to striated muscle	
17.	Xylene	CNS depression M Heart arrythmias/arrest Heart failure Pulmonary oedema	1–2 hours?	72 hours	CNS P Heart Lung P Liver P	tp	Unknown: Heart failure caused by sensitisation of myocardium to endogenous catecholamines?	
18.	Nicotine	CNS excitation/depression M Cardiovascular failure	minutes –1 hour	4 hours	CNS PNS	tp	Known: Cholinergic block causing polarisation of CNS and PNS synapses	
19.	Potassium cyanide	CNS excitation/depression M Metabolic acidosis Circulatory failure	0.5–1 hour	4 hours	CNS P Heart VS	tp	Known: General enzyme inhibition. High affinity for ferric ion. Inhibits cytochrome oxidase and thereby cell respiration	

20.	Lithium sulphate	CNS depression Circulatory failure Kidney failure	1–7 days	7 days	CNS Heart Kidney	tp	Unknown: Partial substitution for normal cations of cells may disturb energy processes?
21.	Theophylline	CNS excitation M Metabolic acidosis Heart arrythmias Electrolyte disturbances GIT bleedings	1–5 days	nr	CNS Heart (GIT)	tp	Unknown: Inhibits prostaglandins and cGMP metabolism. Adenosine receptor antagonist
22.	Dextropropoxyphene hydrochloride	CNS excitation/depression Heart arrythmias/arrest Cardiovascular failure	0.5–2 hours	24 hours	CNS Heart	(Norpropoxyphene)	Hypothetical: Binds to morphine receptors. Stabilises cell membranes. Norpropoxyphene is a primary cardiotoxin
23.	Propranolol hydrochloride	CNS excitation/depression Cardiovascular failure Bronchospasm	0.5–2 hours	4–20 hours	CNS Heart VS	tp?	Unknown: Beta-adrenergic blockade?
24.	Phenobarbital	CNS depression M Circulatory failure	5 hours–7 days	10 days	CNS Heart	tp	Hypothetical: CNS depression through inhibition of GABA synapses? Inhibits hepatic NADH cytochrome oxidoreductase
25.	Paraquat	Early (24 hours): CNS excitation Pulmonary oedema Heart failure Kidney failure M Liver failure Later (48 hours–6 days): Pulmonary fibrosis M	3 hours–4 weeks	nr	Lung P Kidney P Heart P Liver P CNS P	tp	Hypothetical: Multisystem failure due to depletion of superoxide disputase, formation of free-radicals, and lipid peroxidation. Lung fibrosis due to accumulation of paraquat in this oxygen-rich organ

Table IX: continued

No.	Chemical	Lethal symptoms[a]	Mean time to death	Danger over	Target organs	Toxic metabolites[b]	Lethal mechanisms	References[c]
26.	Arsenic trioxide	Gastroenteritis Circulatory failure Heart failure Pulmonary oedema Intravascular haemolysis Kidney failure Liver failure CNS excitation/depression	1 hour–4 days	4 days	Kidney P Heart Liver P VS P CNS P GIT P	tp	Known: Cellular poison. Multisystem failure due to uncoupling of oxidative phosphorylation and inhibition of pyruvate and succinate oxidative pathways	
27.	Copper (II) sulphate	Liver failure Kidney failure Intravascular haemolysis Circulatory failure CNS excitation depression	3 hours–7 days	4 days	Liver P Kidney VS	tp	Hypothetical: Cupric copper is reduced to cuprous form by thiol groups in cell membranes. Superoxide is formed by reoxidation of cuprous copper, which induces lipid peroxidation	18
28.	Mercury (II) chloride	Gastroenteritis Circulatory failure Kidney failure	3 hours–14 days	14 days	Kidney P VS GIT P	tp	Hypothetical: Changes membrane potentials and blocks enzyme reactions in cells by targeting the sulphydryl part of active sites of some enzymes	
29.	Thioridazine hydrochloride	CNS depression Heart arrythmias/arrest M	2–10 hours	nr	CNS Heart	(Mesoridazine?)	Unknown	
30.	Thallium sulphate	Gastroenteritis Cardiovascular failure M Respiratory failure Kidney failure Liver failure CNS excitation depression	24 hours–3 weeks	4–5 weeks	Heart P VS Kidney P Liver P CNS P PNS	tp	Hypothetical: Enzyme inhibition by binding to sulphydryl groups of mitochondrial membranes. Interference with oxidative phosphorylation by inhibition Na/K ATPase	18

31.	Warfarin	Bleeding M[1]	36–48 hours	nr	Liver VS	(Metabolites?)	Known: Inhibition of liver synthesis of vitamin K-requiring clotting factors, notably prothrombin. Direct action on capillaries?
32.	Lindane	CNS excitation/depression M Pulmonary oedema Metabolic acidosis	1 hour–8 days	8 days	CNS Heart VS P Kidney P Muscle P	tp?	Unknown: CNS depression through inhibition of TBPS binding to the GABA receptor linked chloride channel, leading to blockade of chloride influx into neurons?
33.	Chloroform	CNS depression M Heart arrythmias/arrest Liver failure Kidney failure	10 minutes–5 days	5 days	CNS Heart P Liver P Kidney P	More toxic intracellular metabolites?	Hypothetical: Liver and/or kidney injury through covalent binding of toxic metabolites, for example, phosgene, to cell proteins and lipids
34.	Carbon tetrachloride	CNS depression[3] Kidney failure[3] Liver failure[3] Heart arrythmias/arrest	24 hours–7 days[3]	7 days	CNS P Heart Kidney P Liver P Pancreas	More toxic intracellular metabolites?	Hypothetical: Covalent binding of toxic intracellular metabolites (see above) Free-radicals inducing lipid peroxidation?
35.	Isoniazid	CNS excitation M Metabolic acidosis Circulatory failure CNS depression Liver failure	14 hours–3 days	nr	CNS Liver P	(Intracellular metabolites)	Hypothetical: Interference with metabolism of vitamin B6 reduces GABA and seizure threshold. Conversion of acetylhydrazine (ICM) to alkylating agent
36.	Dichloromethane	CNS depression M Heart arrythmias Pulmonary oedema Metabolic acidosis	2 hours	3 hours	CNS Heart	(Carbon monoxide?)	Unknown: Carbon monoxide–haemoglobin complex formation?

Table IX: continued

No.	Chemical	Lethal symptoms[a]	Mean time to death	Danger over	Target organs	Toxic metabolites[a]	Lethal mechanisms	Reference[a]
37.	Barium nitrate	Muscle paralysis/ respiratory failure Heart arrythmias/arrest High blood pressure Convulsions	2-3 hours or 2-3 days	24 hours	Muscle[m] Heart (Kidney)	tp	Hypothetical: Neuromuscular depolarisation. Potassium is forced into cells by an action on Na/K ATPase?	19
38.	Hexachlorophene	Early: Gastroenteritis Hyperthermin Circulatory failure 12-18 hours: CNS excitation/depression 48-60 hours: Heart arrythmias/arrest	4-60 hours	3 days	GIT VS Heart CNS[a]	tp	Hypothetical: Uncoupling of oxidative phosphorylation in cells. Binding to proteins in cytoplasma membrane and cell organelles	47
39.	Pentachlorophenol	Hyperthermia CNS excitation/depression Circulatory failure Myotonia Metabolic acidosis	4-24 hours	24 hours	Heart P VS CNS Liver P Kidney P	tp	Hypothetical: Uncoupling of oxidative phosphorylation. Protein binding, including selective enzyme inhibition (liver/kidney P450)	
40.	Verapamil hydrochloride	Circulatory failure Heart arrythmias/arrest Metabolic acidosis CNS depression Hypoglycaemia	24 hours	36 hours	VS Heart	(Metabolites)	Known: Inhibition of transmembrane Ca flux in excitatory tissues. Also alpha-adrenergic blocking	
41.	Chloroquine phosphate	Cardiovascular failure Cardiac arrythmias/arrest M CNS excitation/depression Hypokalaemia	1-24 hours	24 hours	Heart VS CNS	tp	Hypothetical: Stabilisation of cell membranes leading to reduction of excitation and conduction in heart. Interference with mitochondria	

42.	Orphenadrine hydrochloride	CNS excitation/depression (max. 2–5 hours) M Heart arrythmias (max. 12–18 hours) Heart failure Liver failure	1–48 hours	24 hours	CNS Heart Liver P	tp?	Unknown
43.	Quinidine sulphate	Early: Heart failure Heart arrythmias/arrest M Later: CNS excitation/depression Kidney failure	6 hours?	nr	Heart VS CNS Kidney	tp?	Unknown: Decreased electrolyte permeability of cell membranes leading to depression of heart excitability, conduction velocity and contractility.
44.	Diphenyl-hydantoin	(Nystagmus/ataxia) CNS excitation/depression M Heart arrythmias/arrest"	30 hours–14 days	14 days	CNS (Cerebellum) Heart	tp	Unknown: Binds to specific receptors in neuronal cell membranes. Inhibits voltage-dependent sodium channels
45.	Chloramphenicol	Cardiovascular failure CNS excitation/depression Metabolic acidosis (Liver and kidney failure)	5 hours–2 days	nr	Heart VS CNS Liver Kidney	tp	Hypothetical: Binds to mitochondrial ribosomes and inhibits enzyme synthesis, for example, enzymes necessary for oxidative phosphorylation
46.	Sodium oxalate	Initially (minutes): Gastroenteritis Circulatory failure Later (hours): CNS excitation/depression Heart arrythmias/arrest Later (2 days): Kidney failure	3 hours	nr	GIT CNS[b] Heart[b] Kidney	tp	Hypothetical: Calcium-complexing action, depressing the level of ionized calcium in body fluids. The direct action on GIT, VS and kidney cannot explained that way. Corrosivity is not caused by acidity.
47.	Amphetamine sulphate	(Hyperthermia) Cardiac arrythmias/arrest CNS excitation/depression M Metabolic acidosis	2–4 hours	nr	CNS P[a] Heart P Liver P Kidney	tp	Hypothetical: Release of biogenic amines (dopamine, norepinephrine) from nerve terminal stores. Direct action as false tranamitter

Table IX: continued

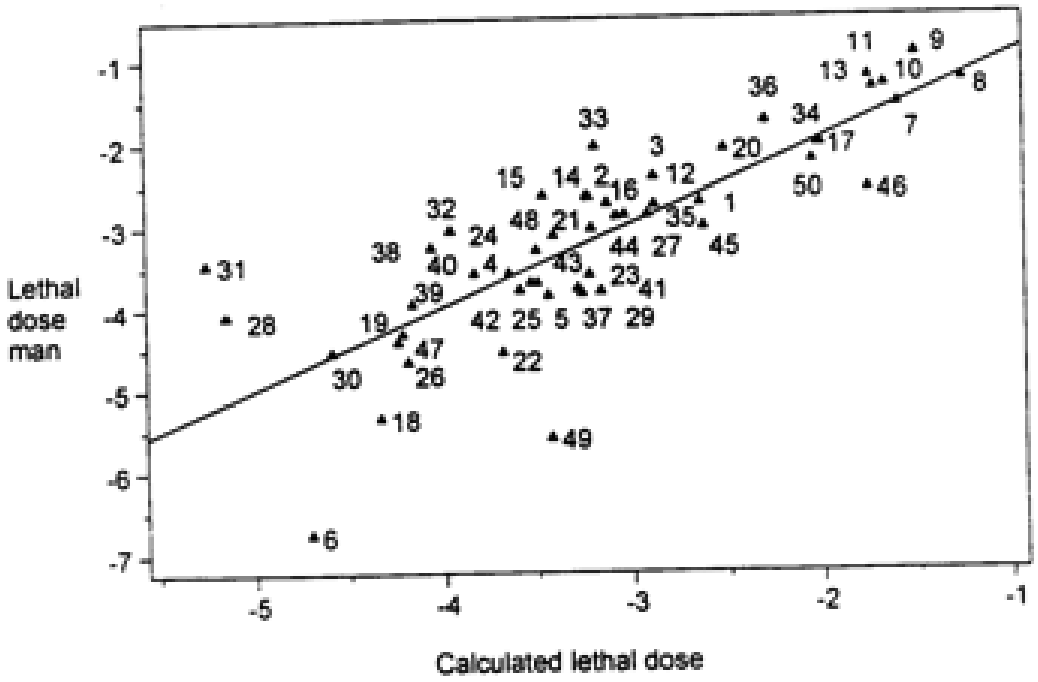
No. Chemical	Lethal symptoms[a]	Mean time to death	Danger over	Target organs	Toxic metabolites[b]	Lethal mechanisms	References[c]
48. Caffeine	Initially (3 hours): Heart arrythmias/arrest Pulmonary oedema Later (3 hours–3 days): CNS excitation/depression	3 hours–3 days	nr	Heart CNS	tp	Hypothetical: Inhibition of phosphodiesterase leading to AMP accumulation. Translocation of intracellular calcium? Adenosine receptor antagonism?	
49. Atropine sulphate	(Psychosis/hyperthermia) CNS excitation/depression Heart arrythmias/arrest M	15 hours	24–48 hours	CNS Heart PNS	tp	Known: Antimuscarinic, anticholinergic action. Competitive antagonism of acetylcholine at cardiac and CNS receptor sites	19
50. Potassium chloride	CNS excitation/depression Paralysis Heart arrythmias/arrest M	2 hours	nr	Heart CNS (Muscle)	tp	Known: Essential cellular electrolyte maintains normal transmembrane potential, necessary for heart conduction	-

[a]Arranged in order of appearance, when possible. Characteristic but non-lethal symptoms have generally been omitted. CNS excitation stands for seizures, and CNS depression stands for all phases of coma including final respiratory arrest. For chemicals with multisystem failure or a very rapid action, it is difficult to indicate the main cause of death. [b]Metabolites with higher toxicity than the parent compound. Metabolites with the same toxicity as the parent compound are bracketed. TP indicates toxicity from the parent compound, only. [c]Other than references 10–17. [d]Post-mortem cases. [e]Including the eye (blindness). [f]Clinical cases. [g]POISINDEX®, Information Systems (ed. B.H. Rumack & D.G. Spoerke), Micromedex (Denver, CO, USA). [h]Targets of a decreased blood calcium level? [i]POMES®, Information Systems (ed. B.H. Rumack & D.G. Spoerke), Micromedex (Denver, CO, USA). [j]Cerebral bleeding is most life-threatening. [k]Inhalation. [l]Ingestion. [m]Motor end-plates of muscles. [n]Repeated dermal exposure. [o]Intravenous administration. [p]Vasculitis, haemorrhages.

M = main causes of death; P = histopathological organ lesions; CNS = central nervous system (brain); CVS = cardiovascular system; VS = vascular system (blood vessels/capillaries); GIT = gastrointestinal tract (gut); PNS = peripheral nervous system; tp = toxicity of parent compound only; nr = not reported.

Appendix X: Plot of Acute Lethal Dosage in Humans Against Values Calculated by a PLS Model Based on Rat Oral LD50 and Mouse Oral LD50

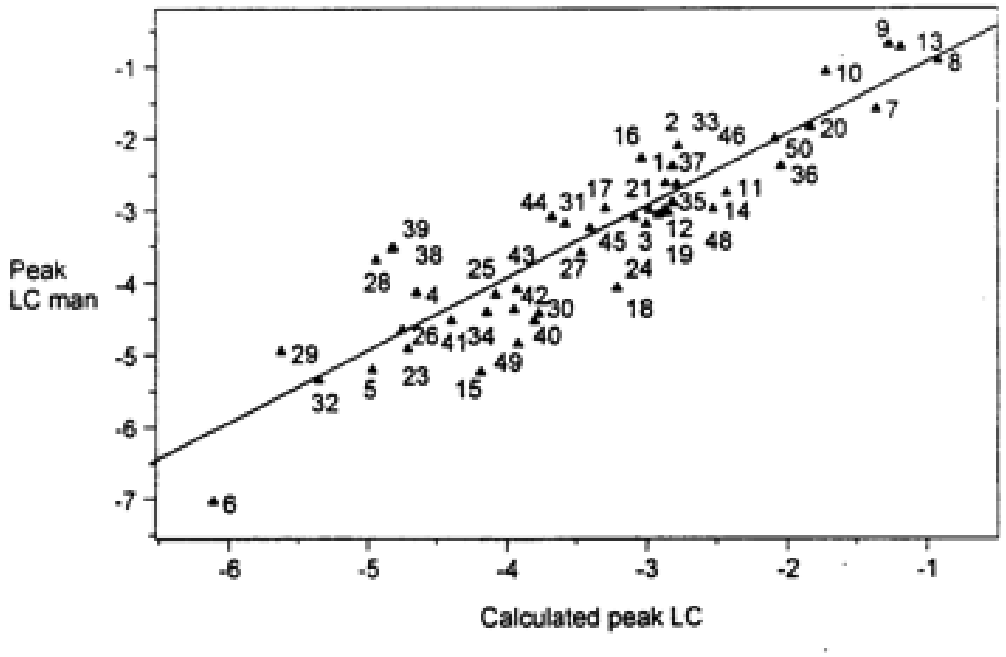
Figure 1: Plot of acute lethal dosage in humans against values calculated by a PLS model based on rat oral LD50 and mouse oral LD50.



Source: Ekwall et al. 1999. MEIC Evaluation of Acute Systemic Toxicity. Part VIII.
(reprinted with permission from the editor)

Appendix XI: Plot of Peak Lethal Blood Concentrations in Man Against IC50 Values

Figure 10: Plot of peak lethal blood concentrations in man against IC-50 values calculated by a PLS model based on peak lethal blood concentrations in man, all 50 chemicals, and "blood-brain barrier compensated results" from assays 1, 5, 9 and 16.



Source: Ekwall et al. 1999. MEIC Evaluation of Acute Systemic Toxicity. Part VIII.
(reprinted with permission from the editor)

Appendix XII: Priority Areas for Development and Evaluation of New *In Vitro* Tests

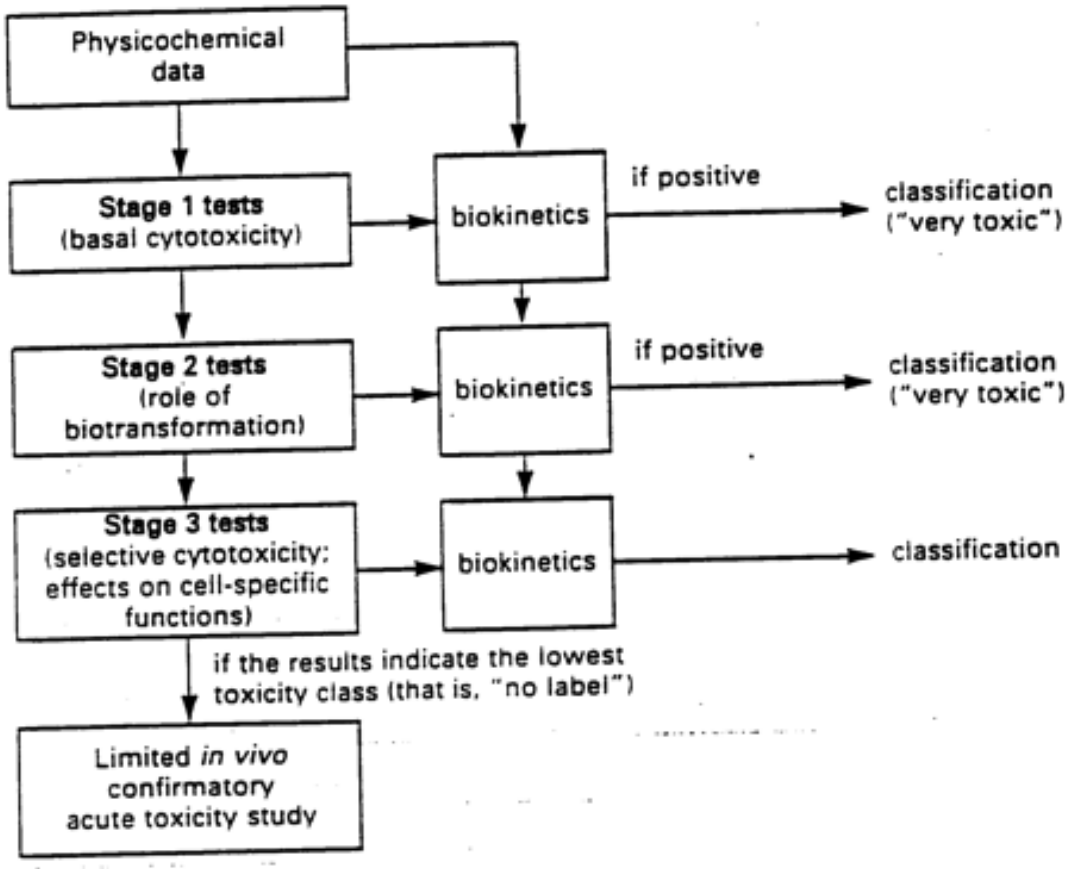
Table I: Priority areas for development and evaluation of new *in vitro* tests on systemic toxicity

No.	Subproject
1.	Repeat dose toxicity
2.	Mechanism studies:
	a) protein denaturation
	b) morphology of injury to cell lines
	c) differential cytotoxicity 30 minutes/24 hours
	d) toxicity to aerobic cells
	e) time-frames for cytotoxic effects
3.	Extracellular receptor toxicity
4.	Excitatory toxicity
5.	Reversibility of cytotoxicity
6.	Passage across blood–brain barrier
7.	Absorption in the gut
8.	Blood protein binding
9.	Distribution volumes (Vd)
10.	More-toxic metabolites

Source: Ekwall et al. 1999. EDIT: A new international multicentre programme to develop and evaluate batteries of *in vitro* tests for acute chronic systemic toxicity. ATLA 27:339-349. (reprinted with permission from the editor)

Appendix XIII: Proposed Testing Scheme for the Classification and Labelling of Chemicals

Figure 1: Proposed testing scheme for the classification and labelling of chemicals according to their potential acute toxicities



Source: Ekwall et al. 1999. EDIT: A new international multicentre programme to develop and evaluate batteries of *in vitro* tests for acute chronic systemic toxicity. ATLA 27:339-349. (reprinted with permission from the editor)

APPENDIX F

Federal Regulations on Acute Toxicity

Many of these materials are availble on the NICEATM website at https://ntp.niehs.nih.gov/pubhealth/evalatm/regs-guidelines/index.html

Refer to agency websites for other materials.

Table of Contents

(Appendices F.3, F.3.1, and F.3.2 were provided in the Background Materials and Supplemental Information Notebook for the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity [Section I, TAB 8]).

Appendix G
In Vitro Workshop Participants and Attendees

Fuad Amsyari
Environment Research Center Airlangga University Mulyorejo Camps-Surabaya Indonesia

Jim Antonini
National Institute for Occupational Safety & Health

Angela Auletta
U.S. EPA/OPPTS

Drew Badger
Procter & Gamble

Dan Bagley
Colgate-Palmolive Company

Michael Balls
ECVAM JRC Environment Institute Ispra (Va) Italy

Martin Barratt
Martin Consultancy United Kingdom

Bas Blaauboer
Utrecht University Research Institute of Toxicology, Utrecht, The Netherlands

Meta Bonner
U.S. FDA

Nancy Bordelon
NIEHS

June Bradlaw
International Foundation for Ethical Research

Ron Brown
FDA/DHHS/CDRH/OST/DLS

Nancy Brown-Kobil
Animal Legal Defense Fund

Peter Bullock
Purdue Pharma LP

Leon Bruner
The Gillette Company

Jennifer Burpee
American Society of Clinical Pathology

Nicole Cardello
Physicians Committee for Responsible Medicine

Kathleen Cater
The Dial Corporation

Jerry Chandler
George Mason University

Rajendra Chhabra
NIEHS

Larry Claxton
U.S. EPA

Harvey Clewell
KS Crump Group/ICF Kaiser

Richard Clothier
University of Nottingham School of Biomedical Sciences United Kingdom

Catherine Cohen
L'Oreal Aulnay-sousbois, France

Tom Collins
FDA/CFSAN

Robert Combes
FRAME United Kingdom

James Cone
CA Dept. of Health Services

Charles Crespi
Gentest Corp.

Mike Cunningham
NIEHS

Rodger Curren
Institute of Vitro Sciences Inc.

George Cushmac
Dept. of Transportation

Carol Eisenmann
Cosmetic, Toiletry and Fragrance Association

Joe Elder
U.S. EPA

Gene Elmore
University of California,Irvine

Julia Fentem
SEAC Unilever Research United Kingdom

Oliver Flint
Bristol-Meyers Squibb Company

Anna Forsby
Stockholm University Dept. of Neurochemistry & Neurotoxicology Stockholm, Sweden

John Frazier
U.S. Air Force DOD

Steve Galson
U.S. EPA

Roger Gardner
BPPD U.S. EPA

Elke Genschow
ZEBET at the BG VV Berlin, Germany

Benjamin Gerson
University Services, BIEX Clinical Laboratory Boston Clinical Laboratories

David Giampocaro
U.S. EPA

Martin Gilman
Celsis Laboratory Group

Alan Goldberg
Johns Hopkins University School of Hygiene & Public Health

Janet Gould
National Starch & Chemical Co.,

Sidney Green
Howard University College of Medicine

Stan Gross
US EPA

Karen Hamernik
U.S. EPA OPP

John Harbell
Institute of Vitro Sciences, Inc.

Masseh Hashim
U.S EPA

Kenneth Hastings
FDA/CDER

Gabrielle Hawksworth
University of Aberdeen
dept. of Medicine,
United Kingdom

A. Wallace Hayes
The Gillette Company

Jerry Heindel
NIEHS

Oscar Hernandez
U.S. EPA OPPTS

Richard Hill
U.S. EPA OPPTS

Stephen Hundley
FDA/CDER

Kim In Suk
USDA

Michael Ioannou
U.S. EPA OPP

Brian Jones
Avon Products

Peter Jurs
Pennsylvania State
University

Gregory Kedderis
Chemical Industry
Institute of Toxicolgy

Leonard Keifer
U.S. EPA

Abdullah Khasawinah
U.S. EPA OPP

Giles Klopman
Case Western Reserve
University

David Lester
FDA-
DHHS/CDER/OPS/
DAPR

Rich Leukroth
U.S. EPA

Marianne Lewis
U.S. EPA

Manfred Liebsch
ZEBET at the BG VV
Berlin, Germany

Elizabeth Margosches
U.S. EPA
RAD/OPPT/OPPTS

Irving Mauer
U.S. EPA

Eugenia McAndrew
U.S. EPA OPP/RD/TRB

Jill Merrill
Institute for In Vitro
Sciences, Inc.

David Monroe
U.S. EPA
OPPT/RAD/ECAB

Hari Mukhoty
U.S. EPA
PRB/SRRD/OPP

Tina Nelson
American
Antivivisection Society

David Nuber
L'Oreal

Alan Nugent
Midwest Research
Institute

Michael Pelekis
Exxon Mobile
Biomedical Sciences

Pat Phibbs
BNA's Chemical
Regulation Reporter

Kathleen Plotzke
Dow Corning
Corporation Health of
Environmental Sciences

Alberto Protzel
U.S. EPA OPP

John Redden
U.S. EPA

Mike Rexrode
U.S. EPA

Lennart Romert
Swedish National
Chemicals inspectorate,
Solna, Sweden

Karl Rozman
Kansas University
Medical Center

Harry Salem
USA Edgewood CB
Center Department of
Defense

Jessica Sandler
People for the Ethical
Treatment of Animals

Dalj Sawhney
Annandale, VA

Philip Sayre
U.S. EPA OPPT

Louis Scarano
U.S. EPA

Leonard Schechtman
FDA/CDER

Karen Schweikart
National Cancer
Institute

Troy Seidle
Canadian Federation of
Humane Societies

Suhair Shallal
U.S. EPA OPP

Valerie Stanley
Animal Legal Defense
Fund

Martin Stephens
Humane Society of the
U.S.

Kathy Stitzel
Proctor & Gamble

William Stokes
NIEHS

Mary Beth Sweetland
People for the Ethical
Treatment of Animals

Noriho Tanaka
Hatano Research
Institute - Jabagawa,
Japan

Regina Tihan
Institute for In Vitro
Sciences, Inc.

Joe Tomszewski
National Cancer
Institute

Charles Tyson
SRI International

Mary Ann Vasbinder
GlaxoSmith Kline

Bellina Veronesi
U.S. EPA

Erik Walum
Pharmacia & Upjohn
AB Biopharmaceuticals
Stockholm, Sweden

Jay Wang
NIH
DHHS/NIH/CC/CPD

Cal Willhite
State of California Dept.
of Toxic Substances
Control

Megan Wilson
EPA/OSCP

Marilyn Wind
U.S. CPSC

Anne Wolven
A.M. Wolven Inc.

Errol Zeiger
NIEHS

APPENDIX H

Federal Register Notices for International *In Vitro* Workshop

Federal Register notices are available at https://www.federalregister.gov/

ICCVAM Recommendations on *In Vitro* Methods for Assessing Acute Systemic Toxicity

An International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity was convened in Arlington, VA, on October 17-20, 2000. The Workshop was organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and was co-sponsored by the U.S. Environmental Protection Agency (EPA), the National Institute of Environmental Health Sciences (NIEHS), and the National Toxicology Program (NTP). The Workshop focused on reviewing the validation status and possible current uses of *in vitro* methods to assess acute oral lethality potential of chemicals. Workshop participants also recommended research, development, and validation efforts that would further advance the usefulness of *in vitro* methods. For a complete account of Workshop discussions and recommendations, please refer to the *Report of the International Workshop on In Vitro Methods for Assessing Acute Systemic Toxicity* (NIH Publication 01-4499). Based on a review of the Workshop Report, ICCVAM developed the following recommendations to forward to Federal agencies with the Report and Guidance Document.

Current Uses for *In Vitro* Methods

Workshop participants considered the merit of using *in vitro* cytotoxicity tests for predicting the acute oral lethality of chemicals in humans and animals, as suggested by previous studies (e.g., Clemedson and Ekwall, 1999; Halle and Goeres, 1988). They concluded that the available *in vitro* assays would require further development to accurately predict acute lethality (i.e., LD50). Workshop participants recommended that *in vitro* cytotoxicity data be included as one of the factors used to identify appropriate starting doses for *in vivo* acute lethality studies as described by Spielmann et al. (1999). In the approach developed by Spielmann, *in vitro* cytotoxicity tests are used to predict starting doses for acute *in vivo* lethality assays.

ICCVAM agrees with the Workshop Report that data from *in vitro* cytotoxicity assays can be useful as one of the tools (e.g., SAR or bridging from similar compounds or mixtures) in setting a starting dose for the *in vivo* assessment of acute oral toxicity. The attached *Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses for Acute Toxicity* (NIH Publication 01-4500) describes one method, the murine BALB/c 3T3 neutral red uptake assay, for which data for a number of chemicals supports its potential utility for estimating the starting dose. Starting doses are calculated using a regression formula based on an *in vitro-in vivo* correlation for 347 chemicals. Preliminary information suggests that use of this *in vitro* approach could reduce the number of animals currently used in *in vivo* acute toxicity tests. Additionally, new OECD Guidelines for *in vivo* acute toxicity testing recommend a starting dose below the estimated LD50 to minimize the number of animals that receive lethal doses and to avoid underestimating the hazard. ICCVAM recommends that Federal agencies consider making information about this *in vitro* approach available as one of the tools that can be used to select an appropriate starting dose for acute oral toxicity tests.

Research Directions

Workshop participants identified several areas for research and development activities to advance the use of *in vitro* methods for predicting acute oral toxicity in animals and humans. ICCVAM recognizes that there are many directions that such future research and testing might take. These include both near-term and long-term research activities.

- **Near-Term Research**

ICCVAM concurs with the Workshop recommendation that near-term validation studies should focus on two standard cytotoxicity assays: one using a human cell system and one using a rodent cell system. Since the murine BALB/c 3T3 cytotoxicity assay has been evaluated for only a limited number of chemical classes, there is

merit in determining its usefulness with a broader array of chemical classes. Cell lines established from the rat rather than the mouse might also be considered, as most acute oral toxicity testing is conducted in this species. Human cell lines should also be considered since one of the aims of toxicity testing is to make predictions of potential toxicity in humans. Future validation studies should therefore compare rodent and human *in vitro* data with one another, with rodent *in vivo* data, and with human *in vivo* data. Correlations between *in vitro* and *in vivo* data might help in selecting cytotoxicity assays for further evaluation.

The U.S. EPA and NIEHS are collaborating to further characterize the usefulness of *in vitro* methods for acute toxicity testing. ICCVAM recognizes that these activities may yield important information on the near-term and long-term application of *in vitro* tests. ICCVAM recommends the establishment of an interagency expert group under ICCVAM to advise on near-term activities such as assay selection, study design, and chemical selection.

➢ **Long-Term Research**

Longer-term research activities should be directed at improving *in vitro* systems that provide information on biokinetics, metabolism, and organ-specific toxicity. *In vitro* methodologies for gathering biokinetic and target organ specific effects data are needed to facilitate reasonably accurate predictions of LD50s, signs and symptoms associated with toxicity, and pathophysiological effects. Research efforts that might increase the predictive capability of *in vitro* assays include:

- Developing the use of quantitative structure-activity relationship (QSAR)/quantitative structure-property relationship (QSPR) models that predict kinetic parameters such as gut absorption and passage across the brain, kidney, and skin barrier systems.

- Developing efficient *in vitro* systems that provide accurate metabolic and biokinetic data.

- Developing accurate physiologically-based biokinetic models.

- Developing *in vitro* systems that accurately predict organ-specific toxicity.

- Investigating the mechanistic basis for "outlier" chemicals in *in vitro-in vivo* correlations and developing "exclusion" rules for identifying chemicals that cannot be accurately evaluated using *in vitro* methods.

- Investigating the utility of toxicogenomics/proteomics for the assessment of acute toxicity, especially the prediction of NOAELs/LOAELs for acute exposure.

ICCVAM appreciates that most of these long-term research activities will yield further improvements in the usefulness of *in vitro* methods for predicting acute systemic toxicity, but that significant resources would be required. ICCVAM concludes that such activities will warrant consideration along with other potential research efforts in establishing priorities.

Adopted by ICCVAM
April 23, 2001