Report on the ICCVAM-NICEATM/ECVAM/JaCVAM Scientific Workshop on Acute Chemical Safety Testing:

Advancing *In Vitro* Approaches and Humane Endpoints for Systemic Toxicity Evaluations

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

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

National Institute of Environmental Health Sciences
National Institutes of Health
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LIST OF ACRONYMS AND ABBREVIATIONS

ACToR	Aggregated Computational Toxicology Resource
ADME	Absorption, distribution, metabolism, excretion
ADE	Absorption, distribution, excretion
ASHP	American Society of Health-System Pharmacists
ASPCA	American Society for the Prevention of Cruelty to Animals
ATC	Acute Toxic Class method
ATP	Adenosine 5'-triphosphate
BDSM	Birth Defects Systems Manager
BUN	Blood urea nitrogen (test)
CDC	Centers for Disease Control and Prevention
CEBS	Chemical Effects in Biological Systems
CNS	Central nervous system
CPSC	U.S. Consumer Product Safety Commission
DMSO	Dimethylsulfoxide
DOT	U.S. Department of Transportation
DSSTox	Distributed Structure-Searchable Toxicity Database Network[5]
ECG/EKG	Electrocardiogram
ECVAM	European Centre for the Validation of Alternative Methods
EEG	Electroencephalogram
EOG	Electrooculogram
EPA	U.S. Environmental Protection Agency
EU	European Union
FDA	U.S. Food and Drug Administration
FDP	Fixed Dose Procedure
FOB	Functional observation battery
FR	*Federal Register*
GABA	Gamma aminobutyric acid
GCPR	G-coupled protein receptors
GHS	Globally Harmonized System (of Classification and Labeling of Chemicals)
GLP	Good Laboratory Practices
GM	Granulocyte/macrophage
GPM	General Poison Management
GST	Glutathione-S-transferase
HPV	High production volume
IC50	Test chemical concentration producing 50% inhibition of the endpoint measured (i.e., cell viability)
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
ILSI	International Life Sciences Institute
IOM	Institute of Medicine
JaCVAM	Japanese Center for the Validation of Alternative Methods

LC_{50}	The calculated concentration of the chemical in air expected to produce lethality in 50% of the test animals (rats and mice)
LD_{50}	The calculated oral dose expected to produce lethality in 50% of test animals (rats and mice)
LDH	Lactate dehydrogenase
MAOI	Monoamine oxidase inhibitor
MLI	National Institutes of Health (NIH) Molecular Libraries Institute
MOA	Mode of action
NAS	National Academies of Science
NCGC	National Institutes of Health (NIH) Chemical Genomics Center
NHK	Normal human epidermal keratinocytes
NICEATM	National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods
NIH	National Institutes of Health
NIEHS	National Institute of Environmental Health Sciences
NRC	National Research Council
NRU	Neutral Red Uptake
NSAID	Nonsteroidal anti-inflammatory drug
NTP	National Toxicology Program
-omics	Fields of study in biology ending in -omics (e.g., metabolomics, genomics, toxicogenomics, proteomics)
OECD	Organisation for Economic Co-operation and Development
ORD	Office of Research and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
OSHA	U.S. Occupational Safety and Health Administration
PBPK	Physiologically Based Pharmacokinetic
QRS	The deflections in the tracing of the electrocardiogram (ECG/EKG), comprising the Q, R, and S waves that represent the ventricular activity of the heart
QTc	QT intervals corrected for heart rate. The QT interval on the ECG, measured from the beginning of the QRS complex to the end of the T wave, represents the duration of activation and recovery of the ventricular myocardium.
RC	Registry of Cytotoxicity
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SAR	Structure-Activity Relationship
SGOT	Serum glutamic oxaloacetic transaminase
SPECT	Single-photon emission-computed tomography
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricylic antidepressant
3Rs	Replacement, Refinement, and Reduction
TG	Test Guideline
Tmax	Time of maximum toxicity
TNF	Tumor necrosis factor
ToxRefDB	Toxicological Reference Database
UDP	Up-and-Down Procedure
UK	United Kingdom
WP	Workpackage

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Preface

Poisoning is a serious public health issue. The Institute of Medicine (IOM) estimates that more than 4 million poisoning episodes occur annually in the United States (IOM 2004). In 2001, poisoning (30,800 deaths) was second only to automobile accidents (42,433 deaths) in the number of injury-related deaths caused (IOM 2004). Federal regulatory agencies require acute toxicity testing to provide the basis for accurate hazard labeling and risk management practices for chemicals and products. Worldwide, acute systemic toxicity testing is the most commonly required product safety test and it can result in significant pain and distress to test animals. Thus, the evaluation and promotion of alternative test methods for acute systemic toxicity testing is currently one of the four highest priorities of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)[1].

Despite decades of research, attempts to identify *in vitro* alternatives that correctly predict *in vivo* toxicity have made little progress. Recent initiatives have focused on identifying and using mechanistic data for the development of alternative methods for predicting toxicity. The National Toxicology Program (NTP) *Vision for the 21st Century*[1] supports the evolution of toxicology from a predominantly observational science at the level of disease-specific models to a predominantly predictive science focused on a broad range of target-specific, mechanism-based, biological observations in cell systems and short-term animal studies. Also, the National Research Council's (NRC) *Toxicity Testing in the Twenty-first Century: A Vision and a Strategy*[1] envisions the significant reduction and replacement of animal use with batteries of predictive *in vitro* assays to evaluate alterations to key toxicity pathways that can be elucidated using a systems biology approach. The development of predictive pathway-based methods for acute systemic toxicity testing can provide a proof-of-concept for application of the NRC vision to regulatory testing.

To advance the development and validation of *in vitro* methods for acute toxicity, the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and ICCVAM convened a Scientific Workshop on Acute Chemical Safety Testing: Advancing *In Vitro* Approaches and Humane Endpoints for Systemic Toxicity Evaluations. Workshop participants were charged to identify: 1) standardized procedures for collecting mechanistic information from *in vivo* acute oral toxicity testing to aid in developing batteries of predictive *in vitro* test methods that can further reduce and eventually replace animals; and 2) more predictive humane endpoints that may be used to terminate *in vivo* studies earlier in order to further reduce pain and distress.

During the workshop, participants recommended the *in vivo* key pathway information that is necessary to identify and develop the *in vitro* methods needed for accurate predictions. They also identified *in vivo* mechanistic information that may identify predictive biomarkers of systemic toxicity that could be used as earlier, more humane endpoints during *in vivo* tests to further reduce pain and distress.

The workshop, which was held on February 6 and 7, 2008 at the National Institutes of Health in Bethesda, MD included scientists from leading government and academic institutions,

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[1] http://iccvam.niehs.nih.gov/docs/5yearplan.htm (ICCVAM 2008)
[1] http://books.nap.edu/catalog/11970.html

national and global regulatory authorities, private industry, and the animal protection community. The efforts of many individuals who contributed to the organization of this workshop and the preparation, review, and revision of this report are gratefully acknowledged. We especially recognize all of the individuals who served as speakers and panelists at the workshop for their generous contributions of time and effort.

The ICCVAM Acute Toxicity Working Group (ATWG) was instrumental in both organizing and participating in the workshop. This included the active support of the international liaisons to the ATWG, Dr. Pilar Prieto (European Centre for the Validation of Alternative Methods [ECVAM]) and Dr. Hajime Kojima (Japanese Center for the Validation of Alternative Methods [JaCVAM]). ECVAM and JaCVAM also cosponsored the workshop.

The efforts of the NICEATM staff in organizing and preparing the workshop materials, administering the workshop, and preparing this final report are greatly appreciated. We especially acknowledge Dr. David Allen, Mr. Michael Paris, Ms. Linda Litchfield, Ms. Catherine Sprankle, Dr. Judy Strickland, and Mr. Douglas Winters of Integrated Laboratory Systems, Inc., the NICEATM Support Contractor, for their efforts. We also want to thank Ms. Debbie McCarley, Special Assistant to the Director, and Dr. Raymond Tice, Deputy Director of NICEATM, for their contributions to this project.

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Executive Summary

To ensure accurate labeling of hazards and to reduce the risk of accidental poisonings, regulatory agencies in the United States (e.g., the Environmental Protection Agency [EPA] and the Consumer Products Safety Commission [CPSC], Department of Transportation [DOT]) require that certain products and chemicals be tested to determine their potential to cause life-threatening or fatal acute systemic toxicity. This testing currently involves exposing a small number of rats to the product or chemical by applicable routes (oral, dermal, and/or inhalation) and monitoring whether animals die or exhibit any clinical signs of toxicity. The evaluation and promotion of alternatives for acute systemic toxicity testing is one of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) four highest priorities because (1) acute toxicity testing is the most commonly required product safety test worldwide, thus large numbers of animals are used, and (2) it can result in significant pain and distress to test animals.

A greater understanding of critical toxicity pathways is needed to facilitate development of alternative test methods and subsequent replacement of animals in acute oral toxicity testing. Both ICCVAM and an independent expert peer review panel recently recommended that future *in vivo* rat acute oral toxicity studies include standardized procedures to collect information pertinent to an understanding of the mechanisms of lethality (ICCVAM 2006a, b). The Scientific Workshop on Acute Chemical Safety Testing: Advancing *In Vitro* Approaches and Humane Endpoints for Systemic Toxicity Evaluations contributed to this proof-of-concept by developing approaches to identify the key toxicity pathways for acute systemic toxicity. This mechanistic information can then be used to develop predictive *in vitro* alternative test methods. The workshop suggested that this mechanistic information on acute systemic toxicity might also help identify predictive biomarkers of systemic toxicity for use as earlier, more humane endpoints during *in vivo* tests, thereby reducing pain and distress.

The workshop was organized by the ICCVAM Acute Toxicity Working Group (ATWG) and cosponsored by the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM). The workshop was held on February 6 and 7, 2008, at the National Institutes of Health (NIH) Natcher Conference Center in Bethesda, MD. More than 120 participants from six countries participated.

Workshop Goals

The goals of the Scientific Workshop on Acute Chemical Safety Testing: Advancing *In Vitro* Approaches and Humane Endpoints for Systemic Toxicity Evaluations were to

- Review the state-of-the science and identify knowledge gaps (at the whole organism, organ system, cellular, and/or molecular levels) regarding the key *in vivo* pathways involved in acute systemic toxicity.
- Recommend how these knowledge gaps can be addressed by collecting mechanistic biomarker data during currently required *in vivo* safety testing.

- Recommend how *in vivo* key pathway information can be used to develop more predictive mechanism-based *in vitro* test systems and to identify biomarkers that might serve as predictive earlier, more humane endpoints for *in vivo* test methods.
- Recommend how mechanism-based *in vitro* test systems and earlier, more humane endpoints can be used to further reduce, refine, and eventually replace animal use for acute systemic toxicity testing, while ensuring the protection of human and animal health.

Workshop Objectives

Specific workshop objectives included:

- Discuss the current understanding of key pathways for *in vivo* acute systemic toxicity and identify the knowledge gaps that exist, especially for (1) *in vivo* pathways and (2) chemicals and products tested for acute systemic toxicity.
- Identify and prioritize future research initiatives that would address these knowledge gaps and that are considered necessary to advance the development and validation of *in vitro* methods for assessing acute systemic toxicity.
- Review molecular, cellular, tissue, or other physiological and clinical biomarkers that are or could be measured or observed during *in vivo* acute systemic toxicity testing and discuss their potential usefulness for indicating key pathways of acute systemic toxicity.
- Discuss how the key toxicity pathways indicated by these *in vivo* measurements and observations might be modeled using alternative *in vitro* test methods.
- Discuss and identify observations and quantitative, objective measurements that could or should be included in the current *in vivo* acute systemic toxicity tests to elucidate key toxicity pathways that would support the future development and validation of predictive *in vitro* methods.
- Identify and prioritize research, development, and validation activities for *in vitro* test methods that model the key *in vivo* toxicity pathways and more accurately predict acute systemic toxicity hazard categories.
- Discuss what *in vivo* data collected to elucidate key toxicity pathways might lead to the identification and validation of more humane endpoints for acute systemic toxicity testing, and what data should be a priority for collection to aid in identifying earlier, more humane endpoints.
- Discuss how to promote the collection and submission of *in vitro* and *in vivo* toxicity test data to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in order to advance the development and validation of more predictive *in vitro* test methods and earlier, more humane endpoints for acute systemic toxicity testing.

Breakout group discussions, held after plenary speaker presentations, addressed questions posed by the workshop organizers, considering the information presented by the speakers. Recommendations from the breakout groups were then discussed in plenary session, with the opportunity for comments from all of the workshop participants. A summary of the major conclusions and recommendations from each of the five breakout groups follows.

Key Pathways for Acute Systemic Toxicity

The breakout group for this subject was charged with determining the key toxicity pathways associated with acute human poisonings. This group was to identify which *in vivo* test observations/measurements and data have might be most helpful for diagnosis and treatment of human poisonings. Knowledge gaps associated with these diagnoses and treatments were to be identified and specific toxicological observations and measurements needed to address these gaps and improve the information available were to be established. Recommendations for research and development activities were requested.

Although not entirely analogous to an animal acute systemic toxicity study, an examination of the diagnosis and treatment of acute chemical poisoning in humans may provide a better understanding and rationale for developing alternative *in vitro* acute toxicity testing systems. The following key pathways should be studied to better understand and treat acute human poisonings:

- General cellular function
- Neuronal transmission, both central and peripheral
- Sodium/potassium ATP-ase pump
- Xenobiotic and aerobic metabolism
- Cardiac conduction
- Oxidative stress
- Receptor activity
- Immune response and function

Definitive identification of the class of toxicant ingested by the patient is perhaps the most important information that could improve the diagnosis and treatment of poisoning because it would allow focused therapy to (1) prevent systemic toxicity by minimizing absorption and uptake, or (2) minimize organ damage when absorption to the specific target has already occurred. The following information could also inform diagnosis and/or treatment:

- Toxicant serum concentration vs. time of exposure data
- Accurate patient history reports
- Laboratory confirmation of known toxicant from reported cases
- Time course of acute life-threatening poisonings
- Chemical interactions (e.g., mixtures, polypharmacy, food additives)

The following are three high-priority areas of research and development:

1) Mode of action (MOA)-based test methods
2) Human cell-based systems as screening models (the human condition is the desired reference)
3) Cell models for assessing affected cellular pathways to assess the likelihood of interactions among these pathways

Future research and development activities should also include (1) methods to evaluate recovery and/or reversibility of an effect, (2) methods to address chemicals that are typically physicochemically incompatible with conventional *in vitro* cell systems (e.g., hydrophobic chemicals), and (3) tools for determining *in vitro* and *in vivo* toxicokinetics for dose-response assessments and various associated extrapolations (e.g., *in vivo* to *in vitro*, interspecies).

Current Acute Systemic Toxicity Injury and Toxicity Assessments

The workshop charge given to this group was to review clinical observations and quantitative measurements that should be included in acute systemic toxicity tests to support development of predictive *in vitro* methods. Toxicity pathways that could be modeled by using *in vitro* test methods were to be identified. The group was also asked to identify biomarkers that might be used to provide more information on *in vivo* pathophysiological effects and mechanisms of acute systemic toxicity along with how the timing of these measurements/observations might affect their interpretation. Optimal ways to measure suggested biomarkers were also to be explored as part of the current acute systemic toxicity tests.

Understanding key response pathways is critical to identifying the MOA for developing alternative test methods. Information about key toxicity pathways would be useful to both poison control centers and emergency departments, and the initial information on dosimetry and target organ toxicity could be used for longer-term studies. Hazard classification based on rodent LD_{50}[2] values (for both pure chemicals and mixtures) is the primary regulatory purpose of the acute systemic toxicity test methods. Therefore, nonanimal alternative test methods should accurately predict the rodent LD_{50}, but the prediction of acute human poisoning is the ultimate goal.

The following categories of key pathways need to be modeled using alternative test systems:

- Animal systems: absorption, distribution, metabolism, and excretion (ADME) of chemicals that can be mimicked *in vitro*; information (bioavailability, structure–activity relationship) available prior to testing; and human toxicokinetic information when available
- Whole organs: prioritized for pulmonary, renal, hepatic, cardiovascular, neurological (e.g., neurochemical, behavioral, brain swelling), gastrointestinal (e.g., production of endotoxin as a marker for sepsis), and hematopoietic (including hemorrhaging)
- Cellular systems: chemical toxicity (key issue is whether it is greater for dividing or nondividing cells)

The breakout group identified biomarkers expected to provide more information on pathophysiological effects and modes/mechanisms of acute systemic toxicity. Current biomarker methods should be adapted (e.g., appropriate sample volume, instrumentation of appropriate size and sensitivity for telemetry) to allow a better understanding of acute systemic toxicity in the rodent model. Noninvasive or minimally invasive methods should be developed to collect biomarkers that maximize the use of the limited number of animals currently required for acute toxicity tests. Early timepoints (less than 24 hours after dosing) were suggested for biomarker measurements.

[2] Lethal Dose 50: The calculated value of the oral dose that produces lethality in 50% of test animals (rats and mice).

The breakout group recommended the following research and development activities.

- Short-term activities
  - Noninvasive telemetry systems for real-time monitoring of physiological parameters in rodents
  - Automated systems for collecting behavioral information
  - Noninvasive analytical devices for analyzing small blood/urine volumes
  - Bioinformatics tools
- Long-term activities
  - "-omics" technologies to identify biomarkers
  - Noninvasive imaging techniques
  - Nanotechnology development for biomarker measurements.

Identifying Earlier Humane Endpoints for Acute Systemic Toxicity Testing

The charge to this group was to determine objective biomarkers that are sufficiently predictive of lethality and that could be used as routine humane endpoints along with clinical signs and observations for pain and distress. It is important to determine whether the use of humane endpoints would interfere with the collection and interpretation of mechanistic data (or other data) and, conversely, to what extent might the collection of additional data lead to incorporating more humane endpoints. Accordingly, this group proposed additional endpoints that could be used for identifying earlier humane endpoints for acute systemic toxicity testing. Research, development, and validation efforts were suggested that would address the identified knowledge gaps associated with predictive early humane endpoints.

In vivo data that could elucidate key toxicity pathways and lead to the identification and validation of more humane endpoints for acute systemic toxicity testing should include the following:

- Data that are currently (or should be) routinely collected and used as humane endpoints during *in vivo* acute toxicity testing
- Data that might-be routinely collected and could aid in identifying additional humane endpoints that occur sooner post-exposure
- Data that might be useful as predictive endpoints prior to the onset of overt toxicity and, therefore, warrant additional investigation and development

The breakout group discussed the concept of *evident toxicity* for the Fixed Dose Procedure (FDP; OECD TG 420; OECD 2001) in relation to biomarkers that might be routinely collected as humane endpoints. Clinical signs and observations of pain and distress should be routinely recorded, but objective measurements are needed instead of adding extra traditional subjective evaluations. Biomarkers sufficiently predictive of evident toxicity should be used routinely during acute toxicity testing. Importantly, consideration of humane endpoints should not interfere with the collection and interpretation of mechanistic data, and the group anticipated that such objective measurements might actually facilitate the collection and interpretation of better mechanistic data.

The group also considered the impact of potential dermal (whole body) vs. nose-only exposures that could compromise endpoints. They noted that investigators should make routine assessments of pulmonary function (i.e., respiratory rate and tidal respiratory volume) along with pulmonary histopathology, the latter of which would provide an assessment of both toxicity and any background infection. With regard to studies conducted with nose-only

exposure, animals should be acclimated to nose-only restraint devices prior to exposure, the duration of exposure should be minimized, and vital signs should be routinely collected.

In the context of the workshop charge given to this breakout group (i.e., more humane endpoints for acute systemic toxicity testing), the group recommended (although not unanimously) that the FDP become the preferred routine acute oral toxicity testing method. The group also recommended using a fixed-dose/concentration approach for acute toxicity testing by the dermal and inhalation routes, respectively. However, U.S. regulatory agency representatives at the workshop did not agree that the FDP should be the preferred method for acute systemic toxicity testing because it does not satisfy the regulatory needs for an LD_{50} estimate.

Research, development, and validation efforts should address knowledge gaps currently associated with predictive early humane endpoints. Development of objective criteria to characterize evident toxicity and publication of internationally harmonized guidance to detail these criteria are vital before initiating routine use of the FDP. Biomarkers (e.g., behavioral observations, body temperature, body weight, feed and water consumption) were identified that could be measured and observed in a standardized or systematic way during future animal studies to identify earlier, more humane endpoints for acute toxicity testing.

The breakout group agreed that dedicated funding to identify the most effective ways to implement the recommended activities is necessary and recognized the need for other incentives to motivate stakeholders to commit to these recommendations. Well-defined strategies for standardization among the international community should be generated, with existing guidelines improved. Data mining and sharing of existing and newly generated data among international stakeholders should be encouraged. Additional training in application of the recommended measurements and observations, as well as interpretation of their results, is essential to significant advancement in the application of more humane endpoints for acute toxicity testing.

Application of *In Vivo* Mode of Action and Mechanistic Information to the Development and Validation of *In Vitro* Methods for Assessing Acute Systemic Toxicity

This group was asked to determine knowledge gaps for and the extent of applicability of current and proposed *in vitro* test methods to adequately model the key toxicity pathways associated with acute systemic toxicity. The application of *in vivo* mode of action and mechanistic information was to be considered to further improve *in vitro* testing. Discussions were to include how the timing of observations might be adjusted to differentiate the initial pathway effects from downstream effects. The group also considered how *in vitro* tests might be incorporated into testing currently being conducted to meet regulatory testing requirements.

Different levels of biological organization (i.e., cellular signaling pathways, intercellular interactions, and organ level responses) define toxicity pathways, and all three levels contribute to acute systemic toxicity. *In vitro* test methods can evaluate a vast array of toxicity pathways to access both specific endpoints and dose–response characteristics (e.g., neuronal transmission, immunology and inflammation, cellular respiration). Other test methods focus on interaction with cellular targets, such as over-expression of transporters in cell lines and examination of uptake rates of chemical into these cells. However, the test method selection depends on knowledge of cellular, tissue, and organ-specific targets for chemicals.

The major knowledge gap is in understanding all *in vivo* mechanisms of the acute toxic action of chemicals. Approaches to assess mechanisms of action include "-omic" evaluations of tissues from rats in the acute toxicity screens. The proposed short-term targeted *in vivo* animal bioassay could be used to ascertain differential responses in tissues and help determine possible toxicity pathways. Correlations between LD_{50} and integrated cellular responses are limited for other than *in vitro* basal cytotoxicity test methods. Such evaluations might determine if other cellular response measures provide better predictive power. From the mechanistic perspective, no quantitative procedures have been developed to describe cascades of responses and predict LD_{50}. Research like this could potentially predict LD_{50} values as well as measures of chronic toxicity from pathway studies.

The identification or development of tissue-specific cellular models will be essential for assessing critical toxicity pathways, and these models will need to incorporate and allow for genetic variability if possible. Standardized testing protocols should be developed for cellular response assays for individual toxicity pathways and for identification of the necessary controls prior to initial evaluation of each cellular response pathway as a predictor of acute systemic toxicity. Chemicals active in the toxic response pathway as well as negative controls should be examined in the test methods. Statistical analyses can determine which cellular response pathways are best associated with acute systemic toxicity.

Validation of *in vitro* models requires a wider variety of data (e.g., ADME) than simply acute toxicity. Routine collection of blood levels and pharmacokinetic data could be useful if a goal of predicting human acute systemic toxicity requires estimating the human blood time course necessary to equal those that occur in the rat after a single lethal dose. Animal data obtained from acute dosing and from studies with other forms of dosing need standardization for use in validation studies. Access to stakeholders' study results for tests on chemicals and any associated data would be helpful in assessing possible toxicity pathways.

The following are research priorities:

- Apply a broad array of *in vitro* test methods to screen for MOAs.
- Collect as much data as possible from those animal studies that are conducted to better understand modes of action, and use this information to guide selection of *in vitro* test methods for these modes of action.
- Develop databases of genomic changes, and assess affected tissue-level pathways in animals used for acute systemic toxicity testing.
- Broaden the association between LD_{50} and *in vitro* measures by completing studies with larger numbers of chemicals, assaying more integrated measures of cellular function.
- Develop computational systems biology approaches to predict *in vivo* acute toxicity from sequential activation of specific cellular pathways.

Implementation strategies for relating *in vitro* test results with acute toxicity will vary for those approaches that attempt to establish correlations between outcome and *in vitro* test results (i.e., correlative approaches) and for those that attempt to mimic the sequential cellular and tissue responses that lead to toxicity (i.e., mechanistic approaches). Inclusion of tissue- or system-specific effects is advantageous because it potentially enhances accuracy in predicting rodent LD_{50} when combined with *in vitro* basal cytotoxicity test methods. Implementation and completion of this program will require the application of currently

available methods as well as the development of new methods. Substantial investment is needed for development of tissue-specific human cellular models that use normal human cells. Stem cell sources from human cord blood should be developed to provide sufficient access to cells on a broad scale and provide the standardization necessary for validation studies. The ultimate goal is to increase the accuracy of *in vitro* test methods' prediction of LD_{50} values from the acute toxicity test methods to better predict human LD_{50} values.

Implementation of *in vitro* testing strategies to predict acute *in vivo* toxicity will require the following activities:

- Collect standardized data from animal studies to aid in pathway determination.
- Identify model cellular systems for assessing chemical activity in the pathway.
- Identify agents that relate to toxicity in the model cellular systems.
- Develop model test systems including methods and endpoints.
- Interpret results using standardized test panels to compare with rodent LD_{50}.
- Use statistical tools, currently being developed and implemented, to facilitate interpretation for association between potency in specific pathway test methods and the rodent LD_{50}.
- Determine the effectiveness of each system to predict *in vivo* toxicity alone and in combination.
- Convene expert panels to address development of cell lines, design and use of appropriate biomarkers, test method implementation, and data analysis procedures.
- Consider incorporating individual *in vitro* test methods into the assessment of acute toxicity in parallel with the *in vitro* basal cytotoxicity test method.
- Develop appropriate procedures to compare the performance of new test methods with that of the *in vitro* basal cytotoxicity test methods in predicting the rodent LD_{50}.
- Consider how multiple measures of cellular toxicity pathways might be used to predict acute systemic toxicity.

Industry Involvement in Test Method Development, Validation, and Use

The focus of this breakout group was to determine how industry can be effectively encouraged to collect and submit (1) mechanistic observations and measurements from animals used in acute systemic toxicity studies and (2) concurrent *in vitro*/*in vivo* toxicity test data to ICCVAM to advance the development and validation of alternative *in vitro* test methods. The group was to examine how industry can increase the use of adequately validated *in vitro* cytotoxicity test methods for reducing the use of animals in acute systemic toxicity tests and how impediments to data collection can be overcome.

Private-sector participants at this workshop stated that collecting data from parallel *in vitro* and *in vivo* toxicity testing would require a significant monetary and staff commitment by a company, while the impact of *in vitro* test methods on further animal reduction would be limited at best. *In vitro* test methods could replace the *in vivo* acute toxicity test methods if a full battery of *in vitro* tests were available that accounted for the many mechanisms and MOAs of acute toxicity. At present, because of poor accuracy, *in vitro* cytotoxicity

predictions of acute oral toxicity are useful only in the complete absence of information for a particular chemical, which workshop participants say is rare.

The cost-benefit ratio does not justify using the validated *in vitro* methods to set starting doses for acute oral toxicity tests because the number of animals used is already at a minimum. Larger organizations might voluntarily use *in vitro* test methods in their acute toxicity testing program for public relations value. The availability of a validated *in vitro* test method for acute toxicity and the inclusion of the test in a formal testing guideline would facilitate its widespread use.

Breakout group members indicated that acute toxicity data constitute valuable proprietary information that companies are not likely to share. Additionally, industry is reluctant to submit *in vitro* cytotoxicity test data due to a fear that regulators may adversely interpret the data. Certain guarantees (e.g., "safe harbor" agreements with assurance that unfavorable *in vitro* data in the presence of favorable *in vivo* data would not be used in any regulatory action) and incentives (e.g., grants for development of methods, tax incentives, expedited regulatory review) would likely be necessary to encourage industry to share data. The creation of a public/private consortium was suggested to facilitate data collection and submission (e.g., Predictive Safety Testing Consortium formed under the Food and Drug Administration [FDA] Critical Path Initiative).

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1.0 Introduction

1.1 Background on Advancing In Vitro Approaches and Humane Endpoints for Systemic Toxicity Evaluations

Poisoning is a more serious public health problem than generally recognized. The Institute of Medicine (IOM) estimates that more than 4 million poisoning episodes occur annually in the United States (IOM 2004). In 2001, poisoning (30,800 deaths) placed second behind automobile accidents (42,433 deaths) as the leading cause of injury-related death (IOM 2004). To ensure accurate labeling of hazards and to reduce the risk of accidental poisonings, regulatory agencies in the United States (e.g., the Environmental Protection Agency [EPA] and the Consumer Products Safety Commission [CPSC], Department of Transportation [DOT]) require that certain products and chemicals be tested to determine their potential to cause life-threatening or fatal acute systemic toxicity. This testing currently involves exposing a small number of rats to the product or chemical by applicable routes (oral, dermal, and/or inhalation) and monitoring whether animals die or exhibit any clinical signs of toxicity.

Increasing societal concerns about animal use have led to the development and evaluation of alternative *in vivo* test methods that significantly reduce animal use for acute systemic toxicity testing[3]. Additionally, *in vitro* methods have been developed and recommended that can help further reduce the number of animals needed for each *in vivo* test (ICCVAM 2006a, b). Nevertheless, despite decades of research, attempts to identify *in vitro* alternatives that correctly predict *in vivo* toxicity have made little progress. Since an important goal of acute toxicity testing for regulatory purposes is to determine hazard classification and labeling, it produces information about the relative toxicity/lethality of a substance. Currently, the primary purpose of these studies is not to provide information about the mode or mechanism that causes toxicity or death. Current studies may generate some relevant data, but such data varies from study to study and is generally limited. Without such information it is difficult to develop mechanism-based *in vitro* test methods that can adequately model and predict *in vivo* toxicity.

A greater understanding of critical toxicity pathways is therefore needed to facilitate development of alternative test methods and subsequent replacement of animals in acute oral toxicity testing. Both the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and an independent expert peer-review panel recently recommended that future *in vivo* rat acute oral toxicity studies include standardized procedures to collect information pertinent to an understanding of the mechanisms of lethality (ICCVAM 2006a, b). Such information is considered necessary to support further development of predictive mechanism-based *in vitro* methods. The National Institute for Environmental Health Sciences (NIEHS) National Toxicology Program (NTP) published the *Vision for the 21st Century*[4], which supports the evolution of toxicology from a predominantly observational science at the level of disease-specific models to a predominantly predictive science focused on a broad range of target-specific, mechanism-

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

[4] http://ntp.niehs.nih.gov/index.cfm?objectid=EE4AED80-F1F6-975E-7317D7CB17625A15

based, biological observations in cell systems and short-term animal studies. The EPA has a similar initiative within its ToxCast Program[5]. Likewise, the National Research Council's (NRC) recently published *Toxicity Testing in the Twenty-first Century: A Vision and a Strategy*[6] envisions the significant reduction and replacement of animal use with batteries of predictive *in vitro* assays to evaluate alterations to key toxicity pathways[7] that can be elucidated using a systems biology approach.

Acute systemic toxicity testing provides an excellent opportunity to assess the feasibility (i.e., proof-of-concept) of the NTP/EPA/NRC approaches and to determine if these proposed nonanimal approaches can be sufficiently predictive to totally replace animal testing. These studies typically evaluate the adverse effects of a single dose of test chemical followed by a short observation period (up to 14 days), compared to other systemic toxicity testing that involves repeated daily dosing and observation for 14 days to 2 years.

The Scientific Workshop on Acute Chemical Safety Testing: Advancing *In Vitro* Approaches and Humane Endpoints for Systemic Toxicity Evaluations contributed to this proof-of-concept by developing approaches to identify the key toxicity pathways for acute systemic toxicity. This mechanistic information can then be used to develop predictive *in vitro* alternative test methods. The workshop suggested that this mechanistic information on acute systemic toxicity might also help identify predictive biomarkers of systemic toxicity for use as earlier, more humane endpoints during *in vivo* tests, thereby reducing pain and distress. The workshop was organized by the ICCVAM Acute Toxicity Working Group (ATWG) and cosponsored by the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM). The workshop was held on February 6 and 7, 2008, at the National Institutes of Health (NIH) Natcher Conference Center in Bethesda, MD. More than 120 participants from six countries participated.

The evaluation and promotion of alternatives for acute systemic toxicity testing[8,9] is one of ICCVAM's four highest priorities because (1) acute toxicity testing is the most commonly required product safety test worldwide, thus large numbers of animals are used, and (2) it can result in significant pain and distress to test animals. The international workshop also implemented one aspect of the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)–ICCVAM *Five-Year Plan (2008-2012)*[10] to identify approaches that would further reduce the potential pain and distress associated with acute toxicity testing by seeking to identify more humane acute toxicity endpoints.

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[5] http://www.epa.gov/ncct/practice_community/category_priority.html.

[6] http://books.nap.edu/catalog/11970.html

[7] Cellular response pathways that when sufficiently perturbed are expected to result in adverse health effects are termed *toxicity pathways*. (NRC 2007)

[8] EPA Health Effects Test Guidelines OPPTS 870.1100 Acute Oral Toxicity http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Revised/870r-1100.pdf (EPA 2002)

[9] OECD Series on Testing and Assessment Number 24: Guidance Document on Acute Oral Toxicity Testing http://www.olis.oecd.org/olis/2001doc.nsf/43bb6130e5e86e5fc12569fa005d004c/c1256985004c66e3c1256a92005087fe/$FILE/JT00111082.PDF. (OECD 2001)

[10] http://iccvam.niehs.nih.gov/docs/5yearplan.htm (ICCVAM 2008)

1.2 Workshop Goals

The goals of the workshop were to:

- Review the state-of-the-science and identify knowledge gaps (at the whole organism, organ system, cellular, and/or molecular levels) regarding the key *in vivo* pathways[11] involved in acute systemic toxicity
- Recommend how these knowledge gaps can be addressed by collecting mechanistic biomarker data during currently required *in vivo* safety testing
- Recommend how *in vivo* key pathway information can be used to develop more predictive mechanism-based *in vitro* test systems and to identify biomarkers that may serve as predictive earlier, more humane endpoints for *in vivo* test methods
- Recommend how mechanism-based *in vitro* test systems and earlier, more humane endpoints can be used to further reduce, refine, and eventually replace animal use for acute systemic toxicity testing, while ensuring the protection of human and animal health.

1.3 Workshop Objectives

The objectives of the workshop were to:

- Discuss the current understanding of key pathways for *in vivo* acute systemic toxicity and identify the knowledge gaps that exist, especially for
  (1) *In vivo* pathways, and
  (2) Chemicals and products tested for acute systemic toxicity
- Identify and prioritize future research initiatives that would address these knowledge gaps and that are considered necessary to advance the development and validation of *in vitro* methods for assessing acute systemic toxicity
- Review molecular, cellular, tissue, or other physiological, and clinical biomarkers that are or could be measured or observed during *in vivo* acute systemic toxicity testing and discuss their potential usefulness for indicating key pathways of acute systemic toxicity
- Discuss how the key toxicity pathways indicated by these *in vivo* measurements and observations might be modeled using alternative *in vitro* test methods
- Discuss and identify observations and quantitative, objective measurements that could or should be included in the current *in vivo* acute systemic toxicity tests to elucidate key toxicity pathways that would support the future development and validation of predictive *in vitro* methods
- Identify and prioritize research, development, and validation activities for *in vitro* test methods that model the key *in vivo* toxicity pathways and more accurately predict acute systemic toxicity hazard categories
- Discuss what *in vivo* data collected to elucidate key toxicity pathways might lead to the identification and validation of more humane endpoints for acute

[11] Cellular response pathways that when sufficiently perturbed are expected to result in adverse health effects are termed *toxicity pathways* (NRC 2007).

systemic toxicity testing, and what data should be a priority for collection to aid in identifying earlier, more humane endpoints
- Discuss how to promote the collection and submission of *in vitro* and *in vivo* toxicity test data to ICCVAM in order to advance the development and validation of more predictive *in vitro* test methods and earlier, more humane endpoints for acute systemic toxicity testing

1.4 Workshop Structure

The workshop was comprised of four sessions:

- Session 1 – Acute Systemic Toxicity: Public Health Significance and Regulatory Testing Needs
- Session 2 – Acute Systemic Toxicity: Human and Animal Assessments, Biomarkers, and Key Pathways
- Session 3 – Humane Endpoints
- Session 4 – State of the Science: Using *In Vitro* Methods to Predict Acute Systemic Toxicity

Workshop attendees participated in the following breakout groups:

- Breakout Group 1 – Key Pathways for Acute Systemic Toxicity
- Breakout Group 2 – Current Acute Systemic Toxicity Injury and Toxicity Assessments
- Breakout Group 3 – Identifying Earlier Humane Endpoints for Acute Systemic Toxicity Testing
- Breakout Group 4 – Application of *In Vivo* Mode of Action and Mechanistic Information to the Development and Validation of *In Vitro* Methods for Assessing Acute Systemic Toxicity
- Breakout Group 5 – Industry Involvement in Test Method Development, Validation, and Use

The four plenary sessions consisted entirely of presentations by invited speakers on subjects related to the session topic. Speaker presentations are viewable on the NICEATM-ICCVAM website at http://iccvam.niehs.nih.gov/methods/acutetox/toxwksp-present.htm . Following the presentations, workshop participants met in breakout groups to discuss the information provided in the presentations and to answer questions presented by the workshop organizers. The co-chairs of the breakout groups then presented summaries of breakout group discussions and recommendations during plenary sessions to all of the workshop participants.

Sections 2.0 through **5.0** of this report summarize the content of each of the four sessions. **Sections 6.0** through **10.0** summarize the breakout group discussions, and **Section 11.0** summarizes the overall recommendations and conclusions from the workshop. The individual breakout group discussions are summarized under the breakout group sessions during which they occurred and are edited to account for any relevant comments provided during the workshop.

2.0 Session 1 — Acute Systemic Toxicity: Public Health Significance and Regulatory Testing Needs

Co-chairs: William Stokes, D.V.M., D.A.C.L.A.M. (NIEHS/NICEATM), Marilyn Wind, Ph.D. (CPSC)

Session 1 reviewed the public health problem of poisoning from acute chemical exposures. The information presented included the incidence of acute poisonings for various demographic groups, the types of chemicals involved in acute poisonings, likely causes of death, and the methodology for clinical assessments (i.e., diagnosis and treatment) of acute poisoning cases. The session also provided an overview of the regulatory requirements and uses for acute systemic toxicity test data, the type of data collected during testing, and how the information is used for classification and labeling of chemicals (i.e., the Globally Harmonized System for Classification and Labelling of Chemicals [GHS system]; United Nations [UN] 2005), and risk management.

2.1 Public Health Perspective of Acute Poisoning

Presenter: Daniel J. Cobaugh, Pharm.D., D.A.B.A.T., FAACT – American Society of Health-System Pharmacists (ASHP) Research and Education Foundation
http://iccvam.niehs.nih.gov/methods/acutetox/workshop-docs/present/Cobaugh.pdf

This presentation provided a review of the public health problem of poisoning from acute chemical exposures, acute poisoning (types, incidence, causes of death), clinical assessments (i.e., diagnosis and treatment) of acute poisoning incidents, and likely causes of death. Data were provided on poisoning incidents in the United States as reported by the American Association of Poison Control Centers (AAPCC; Bronstein et al. 2007)[12], the Institute of Medicine (IOM 2004), and the Centers for Disease Control and Prevention (CDC) National Center for Health Statistics[13]. Workshop participants learned of the frequency and rate of human poisoning, the chemicals that (1) were most frequently implicated in calls to U.S. poison centers, (2) most often implicated in deaths in the population, and (3) most often implicated in major effects and deaths in older people.

According to the IOM (2004), there were 530 poisonings with 8.5 deaths per 100,000 persons in the United States in 2001. Poisoning and toxic effect hospitalizations peeked from 1985 to 1989 at approximately 240,000 per year and declined to approximately 200,000 per year in 2001, the most recent year for which these data are available[13]. Poisoning deaths (unintentional and suicide) have steadily increased from 1999 to 2002 from approximately 19,000 per year to approximately 26,000 per year[13]. While the suicide rate remained the same at approximately 5,000 per year, unintentional poisonings increased from approximately 12,000 per year to approximately 17,000 per year[13]. In 2002, most unintentional poisoning deaths occurred in the 35- to 44-year-old age group (6007 deaths), while most suicides occurred in the 35- to 44- and 45- to 54-year-old age groups (1569 and 1571, respectively)[13]. Most of the exposure reports received by the AAPCC in 2006 (Bronstein et al. 2007) were for children 1 year (380,000) to 2 years (400,000) of age[12]. The agents most frequently responsible for poisoning exposures reported to poison control centers in 2006 were

[12] http://www.aapcc.org/ (Bronstein et al. 2007)
[13] http://www.cdc.gov/nchs/

analgesics (284,906), cosmetics and personal care products (214,780), and household cleaning products (214,091). Most poisoning deaths, however, were caused by drugs (382 for sedatives/hypnotics/antipsychotics, 307 for opiods, and 252 for cardiovascular drugs).

2.2 Regulatory Needs and Uses for Acute Systemic Toxicity Data

Presenter: Deborah McCall, B.S., U.S. Environmental Protection Agency
http://iccvam.niehs.nih.gov/methods/acutetox/workshop-docs/present/McCall.pdf

This presentation provided a brief overview of the history of regulatory requirements for acute systemic toxicity testing and the impact the regulatory requirements have had on public health (i.e., EPA, DOT, Occupational Safety and Health Administration [OSHA]). It also covered the type of data collected during testing and how the information is used for classification and labeling of chemicals (i.e., the GHS system) and risk management. The current test guidelines (TG) for acute systemic toxicity testing are the EPA Office of Prevention, Pesticides and Toxic Substances (OPPTS) 870 Series Health Effects Test Guidelines and the Organisation for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals for acute oral, dermal, and inhalation toxicity. The data from acute systemic toxicity tests include lethality ranges, point estimates of the LD_{50}[14], confidence intervals around the point estimate, and toxic signs. The DOT (Packing Group Categories), CPSC, EPA (Hazard Labeling Statements for Acute Toxicity), OSHA, and GHS use LD_{50} (LC_{50}[15] values for acute inhalation toxicity tests) values for the oral, dermal, and inhalation exposure routes for various regulatory hazard classification systems[16].

Each hazard category has hazard statements and associated symbols that are used to warn workers, consumers, and other handlers of the potential safety hazards associated with transporting, handling, or using a particular substance.

Other uses of acute toxicity data include the following:

- Establishing dosing levels for repeated dose toxicity studies
- Aiding in the diagnosis and treatment of toxic reactions and in the standardization of biological products
- Serving as a standard for evaluating alternatives to animal tests
- Poison prevention packaging
- Aiding in judging the consequences of single, high accidental exposures in the workplace or home or from accidental release
- Identifying specific organs affected and mode of toxic action

[14] Lethal Dose 50: The calculated value of the oral dose that produces lethality in 50% of test animals (rats and mice).
[15] Lethal Concentration 50: The concentration of the chemical in air that kills 50% of the test animals in a given time.
[16] See PDF for tables of hazard classification criteria. http://iccvam.niehs.nih.gov/methods/acutetox/workshop-docs/present/McCall.pdf.

3.0 Session 2 —Acute Systemic Toxicity: Human and Animal Assessments, Biomarkers, and Key Pathways

Co-chairs: Deborah McCall (EPA) and Thomas Hartung, M.D., Ph.D. (ECVAM)

This session reviewed the state of the science and understanding of the key pathways of acute systemic toxicity and covered qualitative and quantitative objective biomarkers (i.e., measurements and observations) that could be considered for inclusion in the current acute systemic toxicity tests to elucidate key toxicity pathways. Presentations reviewed (1) the general conduct of oral, dermal, and inhalation acute systemic toxicity tests and the data currently collected; and (2) specifically the Fixed Dose Procedure (FDP; OECD 2001a) for acute oral toxicity, the data currently collected, and how it is interpreted to identify evident toxicity.

Clinical and physiologic measurements and observations used to diagnose and treat acute poisoning in humans and animals were discussed. The presentations described the types of clinical and physiological information derived from animal studies that could improve the diagnosis and/or treatment of acute systemic chemical exposures.

Biomarker information (i.e., measurements and observations) routinely collected during short-term repeated dose toxicity studies (i.e., 14- and 28-day tests) or safety pharmacology studies were discussed for potential inclusion in the current acute systemic toxicity tests to assist in identifying mechanisms of toxicity. Additionally, a review of noninvasive and minimally invasive techniques for measuring physiological parameters in laboratory rodents relevant to acute toxicity perturbations was presented.

3.1 Data Currently Collected During Acute Systemic Toxicity Tests

Presenter: Gary Wnorowski, B.A., M.B.A., Eurofins | Product Safety Labs
http://iccvam.niehs.nih.gov/methods/acutetox/workshop-docs/present/Wnorowski.pdf

This presentation provided a review of the conduct of acute systemic toxicity tests and the data currently collected. From a regulatory perspective, the primary reason for conducting these studies is to allow for the proper labeling of pesticides and antimicrobials. Current regulatory needs are met following the technical guidance provided by the EPA OPPTS 870 Series TG and the OECD Guidelines for the Testing of Chemicals. The common rat acute toxicity studies that continue to be conducted reguarly test oral, dermal, and inhalation toxicity (see **Table 3-1**).

Table 3-1 Acute Systemic Toxicity Test Methods

Test Method	Exposure	Observations/Measurements
Oral	Oral gavage	Following administration, and for the next 14 days, the animals are closely monitored for clinical signs. Animals are weighed at study initiation, day 7 and day 14. At the end of 14 days, the surviving animals are sacrificed and gross necropsies performed.
Dermal	Applied dermally; test site covered with gauze and tape; patches removed and sites cleaned after 24 hours of exposure	Same as above
Inhalation	Exposure to aerosolized test chemical for 1 to 4 hours	Same as above

Although current OPPTS and OECD TG emphasize the importance of reducing the numbers of animals used for these studies, the criteria for assessing toxicity has remained largely unchanged (see **Table 3-2**). However, the key endpoint for U.S. regulatory agencies is death, because deaths are used to determine the LD_{50}, which is then used for classification and labeling.

Table 3-2 Endpoints Assessed in Acute Systemic Toxicity Tests

Abdominal distention	Desquamation	Hunched posture	Piloerection
Aggressive	Diarrhea	Hyperactivity	Prolapsed penis
Ano-genital staining	Dyspnea	Hyperkeratosis	Prolapsed uterus
Alopecia	Edema	Hypoactivity	Prone
Ataxia	Emaciation	Hypothermia	Prostrate
Blanching	Erythema	Irregular respiration	Rales – Moist
Convulsions – Clonic	Eschar	Moribund	Rales – Dry
Convulsions – Tonic	Exophthalmos	Mouth discharge	Reduced fecal volume
Corneal opacity	Facial stains	Nasal discharge	Reduced food consumption
Cyanosis	Fissuring	Normal	Soft feces
Dead	Necrosis	Ocular discharge	Tremors
Enophthalmos	Gasping	Pannus	Unthrifty

3.2 Quantifying Evident Toxicity for the Fixed Dose Procedure

Presenter: Robert Guest, B.Sc., SafePharm Laboratories
http://iccvam.niehs.nih.gov/methods/acutetox/workshop-docs/present/Guest.pdf

This presentation reviewed the conduct of the FDP for acute oral toxicity, the data currently collected, and how it is interpreted to identify evident toxicity. The principles of the FDP (OECD TG 420; OECD 2001a) include the following:

1) Assessment of acute oral toxicity upon the observation of evident toxicity at one of four fixed dose levels
2) Administration of only moderately toxic doses
3) Determination of endpoints other than lethality/moribundity
4) Use of data for GHS classification.

Use of the FDP is driven by:

- *Directive 86/609/EEC (*regulates the use of animals for experimental and other scientific purposes in the European Union [EU])[17]
- United Kingdom (UK) Animals (Scientific Procedures) Act 1986[18]
- Standard Condition of UK Project Licenses, Appendix D; Animals (Scientific Procedures) Act 1986[19]

The Up-and-Down Procedure (UDP; OECD 2001c) and the Acute Toxic Class method (ATC; OECD 2001b) can be used in the UK when scientifically justified, but the FDP, which is considered the most humane, is the preferred method. The FDP consists of a sighting study using one animal and a main study using four animals. Clinical observations, measurement of body weights, and gross pathology methods are similar to those of the other acute oral toxicity methods. Clinical parameters such as body temperature, heart rate, respiratory rate, clinical chemistry, hematology, and food/water consumption are not routinely collected.

OECD TG 420 defines *evident toxicity* as "a general term describing **clear signs of toxicity** following the administration of test substance such that at the next highest fixed dose either severe pain and enduring signs of severe distress, moribund status, or probable mortality in most animals can be expected." Currently, there are no globally agreed-upon systems to quantify evident toxicity. It is based on the nature, severity, and duration of the clinical signs of toxicity (including body weight effects). Evident toxicity is identified by expert professional judgment of animal technicians, scientists, veterinarians, and animal care and welfare officers. Although guidance documents are useful, familiarity with the species and strain is important.

3.3 Clinical Biomarkers Used to Diagnose and Treat Acute Poisoning in Humans

Presenter: Frank Paloucek, PharmD., D.A.B.A.T., College of Pharmacy, University of Illinois-Chicago

This presentation covered the clinical and physiologic measurements and observations used to diagnose and treat acute poisoning in humans. It also included a description of the types of clinical and physiologic information derived from animal studies, which could improve the diagnosis and/or treatment of acute systemic chemical exposures, and an overview of key pathways for acute systemic toxicity.

Clinicians in emergency room settings generally use whatever data are available as markers at the time of the poisoning incident because time and opportunity are limited. General Poison Management (GPM) in the clinical setting was explained using the acronym ABCDEFG:

- Airway
- Breathing
- Circulation
- Decontaminate
- Enhance elimination
- Focused therapy antidotes and/or supportive care
- Get toxicological consult

[17] http://eur-lex.europa.eu/smartapi/cgi/sga_doc?smartapi!celexapi!prod!CELEXnumdoc&lg=EN&numdoc=31986L0609&model=guichett

[18] http://www.archive.official-documents.co.uk/document/hoc/321/321-xa.htm

[19] http://www.archive.official-documents.co.uk/document/hoc/321/321-xd.htm

Evaluation and assessment is a continuous process throughout these steps. The general approach in GPM is to evaluate mental status, vital signs (e.g., blood pressure, pulse respiratory rate, O_2 saturation), physical appearance (to identify toxidromes; see below), objective measures, (e.g., arterial blood gas, electrolytes, electrocardiograms, urine findings, radiologic tests), follow-up diagnostic procedures, and response to interventions.

A *toxidrome* (portmanteau of *toxic* and *syndrome*) is a set of clinical signs/syndromes caused by a dangerous level of toxins/poisons in the body that may suggest specific classes of poisoning. Important toxidromes for the clinician are opioid/narcotic, sedative/hypnotic, stimulant, anticholinergic, and cholinergic.

The anion and osmolar gap is one of the most specific biomarkers in clinical toxicology. The mnemonic "ME DIE" (methanol, ethylene glycol, diuretics, isopropyl alcohol [acetone], ethanol) produces such a gap. Acetaminophen serum concentration is the best example of a clinical biomarker frequently used in clinical toxicology.

Often only common drugs of abuse are screened in blood or serum, and positive results do not change empiric therapy. Results are usually independent of the acuity of the exposure. Urine toxicology screens are seldom helpful because they lack appropriate temporal correlation to time of poisoning and presentation. Miscellaneous evaluation measures include fingerstick glucose, blood gases, pregnancy testing, and measures to assess pH. Therapies focus on toxidromes and laboratory determinations, and are refined based on the response.

A *biomarker* was defined as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention[20]. Dr. Paloucek suggested that more biomarkers are not available because turnaround times for most laboratory tests are too long for them to be clinically useful, the incidence of poisonings is insufficient to prepare the clinician for all possibilities, and the development of markers is expensive. The clinician needs additional information to

1) Understand acetaminophen poisoning
2) Develop tools for predicting the need for liver transplants
3) Assess chronic exposure
4) Understand the effect of psychiatric medications
5) Assess serum concentrations
6) Understand the cause of bradycardia/hypertension

In addition, the clinician needs a better understanding (and biomarkers) of

- Drug interactions involving oxidative species
- Chemical transporters and assessing their function clinically in an acute care setting
- Idiosyncratic metabolic hypersensitivity reactions.

[20] Biomarker Definitions Working Group - 1998

3.4 Clinical Biomarkers Used to Diagnose and Treat Acute Poisoning in Animals

Presenter: Steven Hansen, D.V.M., D.A.B.T., D.A.B.V.T., American Society for the Prevention of Cruelty to Animals (ASPCA) Animal Poison Control Center, University of Illinois at Urbana-Champaign

http://iccvam.niehs.nih.gov/methods/acutetox/workshop-docs/present/Hansen.pdf

The purpose of this presentation was to examine the clinical and physiologic measurements and observations used to diagnose and treat acute chemical poisoning in animals and to describe what physiologic information derived from laboratory animal studies could improve the diagnosis and treatment of acute systemic chemical exposures (e.g., melamine in pet food).

The ASPCA Animal Poison Control Center animal exposure statistics for 2007 included poisoning data for approximately 70,000 dogs and approximately 12,000 cats. The most common substances identified in poison exposures were pharmaceuticals (89,076), pesticides (39,974), foods (11,200), biologicals (9,594), cleaning products (7,267), and other chemicals (7,090). **Table 3-3** lists the clinical and physiological information a veterinarian needs to make accurate poisoning diagnoses and the additional information from acute animal studies that would assist in evaluating animal poisoning.

Table 3-3 Information Requirements of the Veterinary Clinician

Clinical Measurements	Physiologic Measurements	Information Needed from Acute Animal Studies
Hematology	General (activity level, appetite, body condition)	Species, sex, age
Coagulation profile	Integument (wounds, alopecia, pruritus)	Route of exposure
Serum electrolytes	Gastrointestinal (vomit, diarrhea)	Delivery system/carrier
Blood gas	Neurologic (depression, tremors, seizures)	Mechanism of action
Renal, liver function	Cardiovascular	Metabolic pathways
Cardiac, respiratory function	Respiratory	Clinical sign chronology
Blood cholinesterase		Organ systems affected
Blood lead, iron, etc.		Organ system pathology
Specific agents and metabolites		Outcome

A difficult aspect of identifying biomarkers for animals is extrapolation of acute toxicity data from one species (e.g., rat) to other species. Challenges include identifying biomarkers for

- Absorption (monogastric vs. ruminant)
- Distribution (low body fat [e.g., lean dog breeds] vs. high body fat)
- Metabolism/excretion (carnivore vs. omnivore/herbivore, specific pathway differences)

Table 3-4 lists the various known mechanisms of toxic action for effects on the liver, kidney, and nervous system and example toxicants that act by these mechanisms.

Dr. Hansen discussed the melamine pet food poisoning incidents of 2007, which included the U.S. Food and Drug Administration (FDA) and ASPCA responses to the crisis. The

poisonings resulted from the combination of melamine and cyanuric acid. Though toxicity data for melamine ingestion by animals were available, there were no reports on the combination of the chemicals and the acute effects thereof. Additional acute toxicity data on the individual chemicals would not have helped in the investigation of the crisis.

Table 3-4 Mechanisms of Toxic Action and Example Toxicants for Major Organ Systems

Liver	Kidney	Nervous System
Free radical production (acetaminophen, iron, carbon tetrachloride)	Crystalluric tubular damage (ethylene glycol, sulfonamides, oxalates, melamine/cyanuric acid	Neurotransmission alterations (serotonin [SSRIs, MAOIs], glycine [strychnine, tetanus], GABA [avermectins, benzodiazepines], norepinephrine [albuterol, yohimbine, TCAs])
Disruption of calcium homeostasis (acetaminophen, quinines, cadmium)	Ischemic tubular damage (NSAIDs, salicylates, amphotericin B)	Alteration of ion channels (sodium [saxitoxin, tetrodotoxin, pyrethroids], potassium [4-aminopyridine, quinidine, bee venom], chloride [benzodiazepines, barbiturates, potassium bromide])
Mitochondrial injury (ethanol)	Direct tubular damage (heavy metals, *Amaranthus*, oak)	Interference with neuronal respiration/ energy production (5-fluorouracil)
Cytoskeletal disruption (microcystin [blue-green algae], Amanitin [hepatotoxic mushrooms]	Renal mineralization (vitamin D and analogues)	Uncoupling of oxidative phosphorylation (bromethalin, salicylic acid)
Cholestasis (sporodesmin [mycotoxin], sapogenic chemicals [e.g., *Tribulus terrestris*])	Glomerular damage (snake venom, mercury)	
Immune-mediated (suspected) (NSAID hepatopathy, sulfonamides, phenytoin, halothane [i.e., many idiosyncratic drug reactions])		

Abbreviations: GABA=Gamma aminobutyric acid; MAOI=Monoamine oxidase inhibitor; NSAID=Nonsteroidal anti-inflammatory drug; SSRI=Selective serotonin reuptake inhibitor; TCA=Tricylic antidepressant

3.5 Methodologies for Collecting Clinical Biomarker Data in Laboratory Animals

Presenter: Karen Steinmetz, Ph.D., D.A.B.T., SRI International

This presentation evaluated biomarker information (i.e., measurements and observations) collected during short-term repeated dose toxicity studies (i.e., 14- and 28-day tests) that could be considered for inclusion in the current acute systemic toxicity tests as well as the data requirements for safety pharmacology, which included quantitative objective endpoints (e.g., blood pressure, body temperature, heart rate, respiratory rate) that may be used to assess less-than-lethal acute systemic toxicity

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) addresses guidelines for safety pharmacology[21] in the following objectives from *Safety Pharmacology Studies for Human Pharmaceuticals (S7A)*[22]:

[21] http://www.ich.org/cache/compo/276-254-1.html
[22] http://www.ich.org/LOB/media/MEDIA504.pdf

1) Identify undesirable pharmacodynamic properties of a substance that may have relevance to its human safety
2) Evaluate adverse pharmacodynamic and/or pathophysiological effects of a substance observed in toxicology and/or clinical studies
3) Investigate the mechanism(s) of the adverse pharmacodynamic effects observed and/or suspected

Safety pharmacology includes a specific battery of tests required for FDA submissions[23]. Described in the ICH S7A guidelines, the test battery has two parts. The core battery includes:

- Cardiovascular system: monitor blood pressure, heart rate, electrocardiogram, methods for repolarization and conductance abnormalities
- Central nervous dystem (CNS): evaluate using the functional observational battery (FOB)[24] or the Irwin test; monitor motor activity, behavioral changes, coordination, sensory/motor reflex responses, and body temperature
- Respiratory system: measure blood oxygen saturation; monitor rats under plethysmograph-restraint to measure respiratory rate and tidal volume

Included in the second part of the battery are supplementary studies that evaluate potential effects on organ systems that are not addressed in the core battery or in repeated dose toxicity studies. These systems include:

- Renal/urinary system
- Autonomic nervous system
- Gastrointestinal system
- Other systems to evaluate dependency potential, immune function, skeletal muscle, and endocrine function

Dr. Steinmetz listed a number of possible ways to reduce animal use for toxicity testing:

- Include the CNS evaluation in the 28-day repeated dose Good Laboratory Practices (GLP) study
  - Conduct FOB on Day 1 or 28 at the T_{max} (time of maximum toxicity) of the chemical
- Include respiratory and cardiovascular function in a GLP definitive study designed for other purposes
  - Apply new sensor technologies for noninvasive (e.g., VivoMetric LifeShirt®)[25] measurements of heart rate, electrocardiogram (ECG), electroencephalogram (EEG), electrooculogram (EOG), periodic leg movement, body temperature, respiratory tidal volume, end tidal CO_2, blood oxygen saturation, blood pressure, cough/bronchial spasm, polysomnography (sleep)
  - Use monitoring sensors that can be worn continuously (similar to ambulatory patients)
- Screen chemicals against *in vitro* receptor and pharmacology panel(s)

[23] http://www.fda.gov/cder/guidance/index.htm
[24] 40CFR 798.6050 Functional Observational Battery http://www.gpoaccess.gov/cfr/index.html
[25] http://www.vivometrics.com/

  - Kinases, G-coupled protein receptors (GCPRs), nuclear receptors, neurotransmitter receptors, ion channels, enzymes, etc.
- Expand the evaluation of biological fluids/tissues
  - Emphasize the collection of potential clinical biomarkers from a variety of fluids or tissues (e.g., blood, urine, buccal cells)

3.6 Noninvasive and Minimally Invasive Techniques for Measuring Physiological Parameters in Laboratory Rodents

Presenter: Kathleen Murray, D.V.M., M.S., D.A.C.L.A.M., Charles River Laboratories
http://iccvam.niehs.nih.gov/methods/acutetox/workshop-docs/present/MurrayCRL.pdf

This presentation provided a review of noninvasive and minimally invasive techniques for measuring physiological parameters in laboratory rodents relevant to acute toxicity perturbations, such as alterations in body temperature and the cardiovascular and respiratory systems. Blood collection and relevant biomarkers that could be measured from blood samples were also addressed. **Table 3-5** lists historical procedures for measuring the various physiological parameters, along with recent advances in technology applied to these types of measurements. Data for these parameters are not routinely collected during acute toxicological studies.

Using the historical methods for body temperature measurement (colonic or rectal temperature probes) when studying the effects of a toxicant on thermoregulatory effects is accurate for a single fixed timepoint. However, multiple measurements can potentially be stressful to the animal. Minimally invasive and noninvasive instruments such as infrared thermometers and implantable microchip transponders are less stressful for multiple measurements. They are capable of monitoring decreasing body temperature, which may be used as a humane endpoint biomarker. Radiotelemetry can provide continuous monitoring of body temperature but requires costly surgical implantation.

Cardiovascular measurements, specifically blood pressure and heart rate, have been collected using surgical procedures on anesthetized animals for direct access to different vessels. More recent methods include the tail cuff, an indirect measurement for a fixed timepoint, and radiotelemetry, which requires surgical implantation but allows continuous monitoring. ECGenie™ and e-MOUSE™[26] noninvasively record ECGs in conscious ambulatory lab animals.

Oxygen saturation (respiratory system parameter) can be determined either by collecting arterial blood and measuring oxygen saturation or through pulse oximetry, which is a noninvasive method that measures absorbance of red and infrared light through a sensor attached to the animal's anatomy.

A plethysmograph (used to measure respiratory rate and volume) can also be used on conscious unrestrained animals to measure variations in the volume or size of an organ, limb, or whole body (usually resulting from variations in the amount of blood or air it contains).

Blood is commonly collected from the orbital sinus or the jugular vein in mice and rats, usually under anesthesia. Blood can also be collected from the saphenous, submandibular, or facial veins in the absence of anesthesia without inducing unrelieved pain and distress.

[26] Mouse Specifics, Inc. http://www.mousespecifics.com/

Recent advances allow the measurement of multiple analytes as biomarkers with much smaller blood samples (e.g., 50 µL) (e.g., RodentMap™ from Rules-Based Medicine, Inc.).

Table 3-5 Historical Methods of Physiological Measurements and Recent Advances

Physiological Parameter	Historic Method	Recent Advances
Body temperature[1]	Colonic or rectal temperature with probe	• Infrared thermometer[2] • Transponders for animal identification and temperature (implantable microchips – subcutaneous implant) • Radiotelemetry (continuous monitoring)
Cardiovascular system: blood pressure	Anesthetized animals with surgical procedures for direct access to vessels	• Tail cuff – indirect measurement[3] • Radiotelemetry[4]
Cardiovascular system: heart rate	Anesthetized animals with traditional ECG leads	ECGenie™ and e-MOUSE™, from Mouse Specifics,[5] Inc., provide noninvasive measurements of cardiac function in conscious ambulatory lab animals
Respiratory system: oxygen saturation	Arterial blood gas	Pulse oximetry at the base of the tail, thigh, foot
Respiratory system: respiratory function	Intubated, anesthetized	Whole body plethysmograph (conscious and unrestrained) – inspiratory and expiratory time, peak inspiratory and expiratory flow, tidal volume, relaxation time, minute volume, frequency of breathing rate, end-inspiratory and end-expiratory pause and enhanced pause
Respiratory system: oxygen consumption and metabolic rate	Respirometer	Respiratory gas analyzer for measuring[6] • Oxygen consumption rate • Carbon dioxide elimination rate • Respiratory quotient • Metabolic rate
Blood collection	• Orbital Sinus • Jugular vein • Saphenous vein	Submandibular vein or facial vein[7]
Biomarkers	Traditional assays	RodentMap™, a multi-analyte profile from Rules-Based Medicine, Inc.[8] measures more than 60 analytes from 50 µL plasma

[1] Gordon 2005
[2] Warn 2003
[3] Lorenz 2002
[4] Whitesall 2004
[5] Mouse Specifics, Inc., http://www.mousespecifics.com/
[6] CLAMS – Comprehensive Laboratory Animal Monitoring System (Columbus Instruments) http://www.colinst.com/brief.php?id=61
[7] http://www.medipoint.com
[8] Rules-Based Medicine http://www.rulesbasedmedicine.com/product_services.asp

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4.0 Session 3 — Humane Endpoints

Co-chairs: Masih Hashim, Ph.D. (EPA) and Cassandra Prioleau, Ph.D. (CPSC)

This session discussed the humane endpoints used for acute toxicity testing and the potential for information on key toxicity pathways to yield earlier, more humane endpoints. Descriptions of methods to identify and monitor pain and distress in experimental animals were provided.

4.1 The Concept of Humane Endpoints and Their Identification: Application for Acute Systemic Toxicity Testing

Presenter: William Stokes, D.V.M., D.A.C.L.A.M., NIEHS/NICEATM
http://iccvam.niehs.nih.gov/methods/acutetox/workshop-docs/present/StokesWkshp03Feb08FD.pdf

This presentation presented a discussion of current guidance for the use of humane endpoints for acute toxicity testing and how information on key toxicity pathways may yield earlier, more humane endpoints.

Safety testing invariably includes pain and distress to test animals because endpoints needed to identify potential toxicity often involve pain and/or distress when toxic effects occur. To minimize or avoid pain and distress, U.S. regulations and policies state that more than momentary or slight pain or distress (1) must be limited to that which is unavoidable for the conduct of scientifically valuable research, (2) must be conducted with appropriate sedatives, analgesics, or anesthetics unless withholding such agents is justified for scientific reasons in writing, and (3) will continue for only the necessary period of time to attain scientific objectives. Animals that would otherwise suffer severe or chronic pain or distress that cannot be relieved should be painlessly killed at the end of the procedure, or if appropriate, during the procedure. Analgesics and tranquilizers can be used in GLP studies only if they do not interfere with the study. However, nearly all testing regulations allow humane euthanasia if there is severe pain and distress or a moribund condition. Death is not a required endpoint for toxicity testing.

OECD Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals used in Safety Evaluations (OECD 2000) is applicable to all OECD test guidelines and largely provides guidance and criteria for humane euthanasia to avoid spontaneous deaths. Demers et al. (2006) provide the following international principles for establishment of humane endpoints.

- There is strong evidence that animals experience pain and distress in situations comparable to those that cause pain and distress for humans.
- Death or severe pain and distress should be avoided as endpoints.
- The earliest possible endpoint consistent with the scientific objectives should be used.
- Studies should be designed to minimize any pain or distress likely to be experienced by the animals while meeting the scientific objectives.
- The duration of studies involving pain and distress should be kept to a minimum.

- Pilot studies should be encouraged as means of determining morbidity, time course of events, and frequency of observations required to set an earlier endpoint.
- Before commencing the experiment, agreement should be reached on the appropriate endpoints for the study and the person or persons responsible for making the judgment that the endpoint has been reached.
- A team approach should be used, employing the professional judgment of the scientist, veterinarian, animal care staff, and ethics committee to agree on the appropriate endpoint for the study.
- Research and animal care staff must be adequately trained and competent in recognition of species-specific behavior and, in particular, species-specific signs of pain, distress, and moribundity.
- Animals should be monitored by means of behavioral, physiological, and/or clinical signs at an appropriate frequency to permit timely termination of the experiment once the endpoint has been reached.

Humane endpoints must be consistent with attainment of research or testing objectives. Biomarkers that may serve as earlier, more humane endpoints in acute toxicity testing include:

- Clinical signs
  - Abnormal behavior
  - Abnormal appearance
- Changes in objective clinical measurements
  - Body temperature
  - Body weight
  - Blood pressure
  - Heart rate; heart rhythm
  - Respiratory rate
  - Transcutaneous oxygen saturation (e.g., using pulse oximeter)
- Serum biomarkers
  - Hematology
  - Serum chemistry
- Urinary biomarkers of renal damage
- Molecular biomarkers in serum or tissues
- Imaging biomarkers

Researchers should move forward in developing and applying humane endpoints for animal research and testing. They should identify and collect potential biomarker data during *in vivo* studies that involve pain and distress. At a minimum this should include collection of detailed clinical signs and data for objective biomarkers that are candidates for earlier humane endpoints. They should periodically analyze data to determine if any biomarkers are sufficiently predictive to use as earlier, more humane endpoints and should routinely consider humane endpoints prior to the use of animals and whenever unrelieved pain and distress is anticipated or expected. Investigators should consider and use new science and technology such as sensitive biomarkers, remote sensing devices, and telemetry. Humane endpoints can reduce the duration and severity of pain and distress experienced by animals and can coexist with research and toxicology studies.

4.2 Opportunities for Detecting Pain and Distress Associated with Acute Systemic Toxicity

Presenter: Steven Niemi, D.V.M., D.A.C.L.A.M., Center for Comparative Medicine, Massachusetts General Hospital
http://iccvam.niehs.nih.gov/methods/acutetox/workshop-docs/present/Niemi.pdf

This presentation discussed methods to identify and monitor pain and distress in experimental animals. The development of alternatives and/or changes to animal models that are used in understanding acute human poisonings requires that a fourth "R" (Relevance) be considered in the context of the 3Rs (Replacement, Reduction, and Refinement). Diagnosing poisoning in humans as soon as possible is important to provide enough time for medical intervention to avoid serious illness or death. The physician must be cognizant of *early* clinical signs and symptoms as well as later stage changes presented by the patient. Fortunately, there are many clinical signs associated with poisoning besides coma and death that may inform the physician about the type, dose, and duration of exposure (**Table 4-1**).

It may be useful to focus on initial clinical signs in animal models as well**,** rather than morbidity and mortality, in order to provide physicians with more relevant indicators of acute toxicity and determine in the most humane way possible the sequence and severity of organs and tissues affected. By contrast, waiting for the animal subject to reach death or near-death after test article administration may miss clinical endpoints of value to physicians. And the relevance of extreme (lethal) toxicity in animals is questionable with respect to situations involving potential or emergency poisonings of humans.

Consequently, in order to reframe the objectives to detection and assessment of initial clinical signs, a laboratory animal should be viewed as a poisoning victim, not a test subject, when used in acute toxicity assays.

Malaise[27] is a common early symptom in human poisonings. Because symptoms are defined as changes felt only by the patient rather than observed by someone else, we cannot detect malaise, per se, in animals. However, malaise in animals can present via the same behavioral cues we see in humans, such as decreases in general activity, appetite, and libido, or perhaps an increase in restlessness and fighting. There are tools for measuring these and other behavioral changes in animals:

- Video and associated software to monitor and interpret an animal's movements (http://www.cleversysinc.com)
- Implantable chips monitored by transponders that can determine general activity and eating and drinking behavior of an animal (http://www.newbehavior.com)
- Gantries of infrared cameras mounted on ventilated cage racks for around-the-clock monitoring of animals; programmed schedule and web controlled (MyMice®—in development at Massachusetts General Hospital)

[27] A feeling of general discomfort or uneasiness, an 'out-of-sorts' feeling, often the first indication of an infection or other disease (http://www.stedmans.com)

Table 4-1 Common Clinical Signs of Poisoning[28]

Abnormal breath odor	Cough	Hyperpyrexia	Ocular, facial palsy	Skin vesicles
Abnormal urine odor	Cyanosis	Hypertension	Oliguria	Smoky urine
Abortion	Deafness	Hyperthermia	Oxaluria	Sneezing
Alopecia	Death	Hyperventilation	Pallor	Spasticity
Angioedema	Decreased respiration	Hypotension	Paralysis	Stained lips
Anorexia	Dehydration	Hypothermia	Parkinsonism	Stimulation
Anuria	Diaphoresis	Incoordination	Perspiration	Stomatitis
Areflexia	Diarrhea	Increased activity	Pinpoint pupils	Stupor
Asphxia	Dilated pupils	Insomnia	Pneumonia	Sweating
Ataxia	Dry mouth	Iridocyclitis	Prostration	Tachyarrythmia
Bloody diarrhea	Dry skin	Jaundice	Pulmonary congestion	Tachycardia
Bradycardia	Dysphagia	Lacrimation	Pulmonary edema	Tachypnea
Bright red venous blood	Dyspnea	Laryngeal edema	QT prolongation	Tetany
Brown urine	Emphysema	Loss of corneal reflex	Rales	Throat constriction
Brown mucous membranes	Exaggerated reflexes	Menorrhagia	Rashes	Torticollis
Burns	Fever	Miosis	Restlessness	Tremors
Cardiac arrest	Flushing	Muscle fasciculations	Retching	Unconsciousness
Cardiac arrhythmias	Frothing at the mouth	Muscle spasms	Retinal injury	Urinary retention
Carpopedal spasm	Gangrene of feet	Muscular rigidity	Rhinorrhea	Urticaria
Cataracts	Glottal edema	Mydriasis	Salivation	Violent behavior
CNS depression	Glottal spasm	Myodystonia	Sedation	Vomiting
CNS excitation	Hematamesis	Narcosis	Seizures	Wakefulness
Coma	Hematuria	Nystagmus	Shallow respirations	Weakness
Convulsive movements	Hepatomegaly	Obtundation	Skin irritation	

Furthermore, neuroanatomical and neurochemical phenomena associated with anxiety, fear and depression appear to be identical among the rat, monkey, and man (Phelps and Ledoux 2005). Single-photon emission-computed tomography (SPECT) is a new imaging modality that provides 3-dimensional "snapshots" of cerebral blood flow to indicate areas of normal vs. abnormal activity. Brain SPECT imaging is used to diagnose depression, anxiety, and

[28] Merck Manual of Diagnosis and Therapy .http://www.merck.com/mmpe/index.html.

other abnormal mental states in patients[29]. Because of the homology, it may be informative to apply SPECT imaging to animals to confirm that early changes in behavior interpreted as indicators of malaise are extrapolated accurately. The subject has to be conscious at the time of radioactive tracer injection but not necessarily at the time of imaging.

By considering the test animal as a patient, a tiered approach to detecting changes associated with toxicity can be designed at increasing levels of intervention as follows:

- Observations without any animal handling involved (e.g., video or ultrasonic monitoring for changes in behavior (Holy and Guo 2005)
- Noninvasive collection of biological samples (e.g., voided urine and feces, expired air)
- Painless/non-distressful collection of biological samples (e.g., cortisol levels measured in saliva[30] or hair (Davenport et al. 2006)
- Temporarily stressful procedures (e.g., blood collection, anesthesia, euthanasia)

[29] http://www.cancer.gov/templates/db_alpha.aspx?CdrID=306519
[30] http://www.research.yerkes.emory.edu/biomarkers_core/assay/index.html

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5.0 Session 4 — State of the Science: Using *In Vitro* Methods to Predict Acute Systemic Toxicity

Co-chairs: Steve Reynolds, Ph.D. (National Institute for Occupational Safety and Health [NIOSH]) and Hajime Kojima, Ph.D. (JaCVAM)

This session provided a summary of a previous workshop, including major conclusions, recommendations, and initiatives, as well as the status of ongoing activities resulting from the workshop. ICCVAM's current recommendations for the use of *in vitro* methods for assessing acute systemic toxicity were discussed. A presentation on the future of toxicology as a predictive science emphasized focusing on understanding and applying the key toxicity pathways to facilitate the development of *in vitro* models of acute systemic toxicity. The use of quantitative high-throughput screening (HTS) methods to establish predictive *in vitro* biomarkers for acute systemic toxicity was also discussed.

5.1 The Future of Toxicology as a Predictive Science

Presenter: Melvin Andersen, Ph.D., D.A.B.T., Division of Computational Biology, The Hamner Institutes for Health Sciences
http://iccvam.niehs.nih.gov/methods/acutetox/workshop-docs/present/Andersen.pdf

This presentation examined the future of toxicology as a predictive science with emphasis on alternative methods for acute toxicity assessment. It focused on developing and applying new understanding of key toxicity pathways to the development of *in vitro* models of acute systemic toxicity.

The presentation was based on the report titled *Toxicity Testing in the Twenty-first Century: A Vision and a Strategy* from the National Research Council (NRC 2007) Committee on Toxicity Testing and Assessment of Environmental Agents of the National Academy of Sciences (NAS). The committee report aimed to design a toxicity testing program to quantitatively assess potential human health risks posed by exposure to environmental agents over a broad range of doses and chemicals. The development of predictive pathway-based methods for acute systemic toxicity testing can provide a proof-of-concept for application of the NRC vision to regulatory testing. The design criteria for this modern toxicity testing program were:

- A more robust scientific basis for assessing health effects of environmental agents (mechanistic data)
- Broad coverage of chemicals, chemical mixtures, outcomes, and life stages
- Reduced cost and time for testing
- Decisions based on human rather than rodent biology and focused on dose levels more relevant to humans

The current paradigm for toxicity testing (i.e., the exposure response continuum) is to test high doses, find responses, and try to understand them in some context across a paradigm that includes exposure, dose, early response, late response, and pathology. The new paradigm is based on an understanding of biological function and evaluates the activation of toxicity pathways. A *toxicity pathway* is a cellular response pathway that when sufficiently perturbed is expected to result in an adverse health effect. Chemical exposure is a perturbation of biology caused by exposure, tissue dose, and biologic interaction. While low doses do not

disturb normal biologic function, higher doses produce early cellular changes that can cause adaptive stress responses; and even higher doses produce cell injury, morbidity, and mortality.

The committee discussed different options for the future of toxicity testing, as shown in **Table 5-1,** with Option I being the status quo. In the committee's vision, the optimal approach is based primarily on human biology and understanding of perturbations in human cells and in human tissues, where a broad range of doses, from high doses that might be relevant for acute toxicity to very small doses consistent with environmental exposures, can be studied. The dose-response assessment includes understanding the toxicity pathways and the effects of different doses by both empirical and mechanistic descriptions to understand the homeostatic processes and the negative feedback control. The dose-response assessment evaluates how the external dose of a chemical is related to the internal doses and effects for various human organs, tissues, and cells. Toxicity testing should be based on perturbations of toxicity pathways while taking advantage of computational tools for *in silico* approaches. The optimal approach, indicated by Options III and IV, primarily uses high-throughput and medium-throughput cell-based test methods for specific pathways and other test methods for specific cell behaviors like proliferation and apoptosis, cell death, or abrupt death. Substantially fewer animals would be used if animal testing were based on perturbations of toxicity pathways identified using nonanimal methods.

Table 5-1 Options for Future Toxicity Testing Strategies

Option I ***In Vivo***	**Option II** **Tiered *In Vivo***	**Option III** ***In Vitro/In Vivo***	**Option IV** ***In Vitro***
Animal biology	Animal biology	Primarily human biology	Primarily human biology
High doses	High doses	Broad range of doses	Broad range of doses
Low throughput	Improved throughput	High and medium throughput	High throughput
Expensive	Less expensive	Less expensive	Less expensive
Time-consuming	Less time-consuming	Less time-consuming	Less time-consuming
Relatively large number of animals	Fewer animals	Substantially fewer animals	Virtually no animals
Apical endpoints	Apical endpoints	Perturbations of toxicity pathways	Perturbations of toxicity pathways
	Some *in silico* and *in vitro* screens	*In silico* screens possible	*In silico* screens

To study perturbations of toxicity pathways, rather than high-dose responses in animals, one examines the perturbations that occur at lower doses and develops approaches to ensure that these perturbations do not occur in human populations. Assessing perturbations at various doses requires the following:

- Empirical dose-response models developed on the basis of data from *in vitro* mechanistically based assays
- Physiologically based pharmacokinetic (PBPK) models to equate tissue-media concentrations from toxicity tests with tissue doses expected in humans

- Dose-response models for toxicity pathways to reliably predict concentrations expected to cause measurable precursor-effect responses
- PBPK and toxicity-pathway models to identify biomarkers of susceptibility for sensitive subpopulations

Implementation of a strategy to assess the perturbations of toxicity pathways requires:

- A comprehensive suite of *in vitro* tests, preferably based on human cells, cell lines, or components
- Computational models of signal transduction in toxicity pathways to support application of *in vitro* test results in risk assessments
- PBPK models to assist *in vitro*-to-*in vivo* extrapolations
- Validation of toxicity pathway tests and test strategies

Toxicity testing in the regulatory context should shift in focus away from apical outcomes in experimental animals. It should focus on important perturbations of toxicity pathways relevant to human biology and interpret them in a dose-response context. Risk assessment practices should be developed based on pathway perturbations, and the regulatory statutes under which risk assessments are conducted should be re-interpreted or possibly rewritten.

5.2 Recommendations from the ICCVAM-NICEATM International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity

Presenter: A. Wallace Hayes, Ph.D., D.A.B.T., FATS, FlBiol, Harvard School of Public Health
http://iccvam.niehs.nih.gov/methods/acutetox/workshop-docs/present/Hayes.pdf

This presentation summarized and reviewed the recommendations of participants in the October 2000 International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity (ICCVAM 2001)**,** which was sponsored by NIEHS, NTP, and EPA. Among the recommendations was the need to gather *in vitro* data for gut absorption, passage of the blood-brain barrier, biokinetics, ADME (absorption, distribution, metabolism, excretion), and organ-specific toxicity in order to produce an *in vitro* test battery to accurately predict acute toxicity.

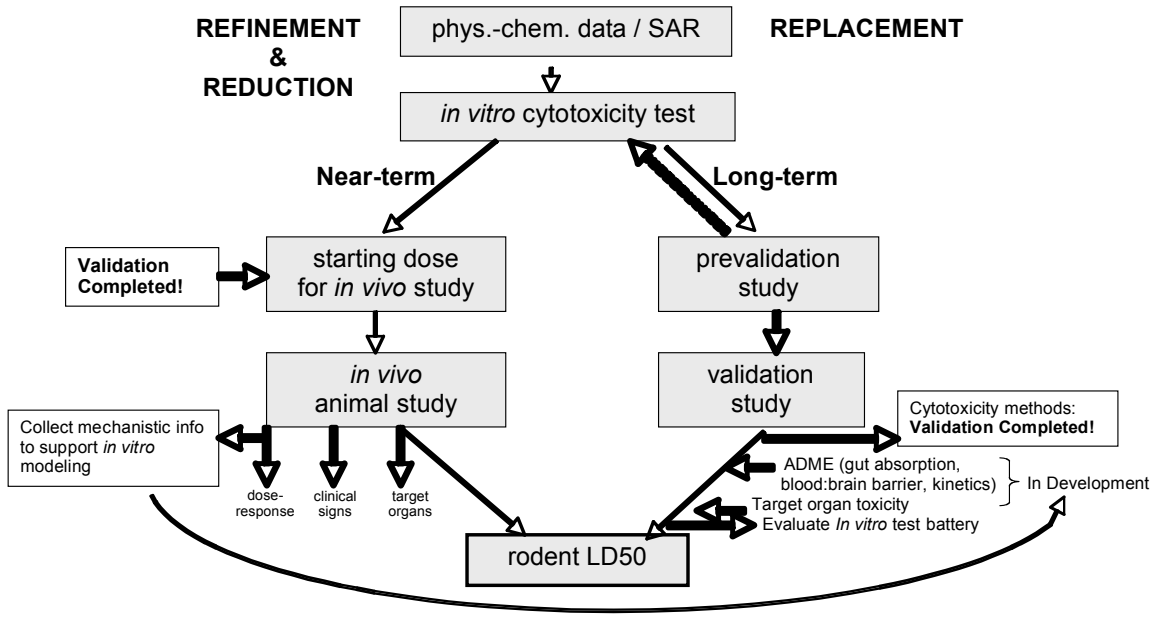
The workshop participants evaluated *in vitro* screening methods for assessing acute toxicity, biokinetic determinations, and organ-specific toxicity, and discussed chemical data sets for validation of *in vitro* acute toxicity test methods. The goals of the workshop were to:

- Review validation status of *in vitro* methods
- Recommend priority *in vitro* methods for further evaluation and appropriate validation studies
- Identify reference chemicals for validation studies
- Identify priority research and development efforts

Figure 5-1 displays the workshop participants' recommended strategy for the reduction, refinement, and replacement of animals in acute LD_{50} testing. They concluded that initially a prevalidation study should be undertaken for several potential *in vitro* cytotoxicity tests (e.g., the 3T3[31] Neutral Red Uptake [NRU] test method). Meanwhile, to potentially reduce the number of animals used in acute oral toxicity tests, *in vitro* cytotoxicity data should be generated to help establish the starting dose for *in vivo* testing of new chemical substances.

[31] BALB/c 3T3 clone A31 mouse fibroblasts developed in 1968 from disaggregated 14- to 17-day-old BALB/c mouse embryos (American Type Culture Collection [ATCC]; # CCL-163)

Figure 5-1 Strategy for the Reduction, Refinement, and Replacement of Animals in Acute LD_{50} Testing[1]



Abbreviations: ADME=Absorption, distribution, metabolism, excretion; LD_{50}=The calculated value of the oral dose that produces lethality in 50% of test animals (rats and mice); SAR=Structure-Activity Relationship.

[1]Adapted/updated from ICCVAM 2001a. Available: http://iccvam.niehs.nih.gov/

Table 5-2 lists recommendations to advance the development of alternative test methods for acute systemic toxicity and the current status of these recommendations, several of which have been completed. Validation is complete for using i*n vitro* cytotoxicity tests to determine the near-term starting dose for *in vivo* studies, and cytotoxicity test methods have been validated under the long-term recommendations (ICCVAM 2006a, b). Additionally, evaluation of ADME (gut absorption, blood-brain barrier, kinetics), target organ toxicity, and mechanistic information to support *in vitro* modeling is currently being investigated.

5.3 Current ICCVAM Recommendations for the Use of *In Vitro* Test Methods to Estimate Acute Systemic Toxicity

Presenter: Marilyn Wind, U.S. Consumer Product Safety Commission (CPSC)
http://iccvam.niehs.nih.gov/methods/acutetox/workshop-docs/present/Wind.pdf

This presentation provided the results of the NICEATM/ECVAM validation study of two NRU test methods and presented ICCVAM recommendations for use of the test methods to estimate relative toxicity.

Table 5-2 Recommendations from the ICCVAM-NICEATM International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity

Recommendation	Status
Data from *in vitro* cytotoxicity assays can be useful as one of the tools in setting a starting dose for the *in vivo* assessment of acute oral toxicity.	ICCVAM published a Guidance Document on how to use the *in vitro* cytotoxicity methods to estimate starting doses.
Federal agencies should 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.	In 2001, EPA sent letters to 1200 companies recommending that they consider *in vitro* cytotoxicity methods and provide the data to ICCVAM. (Note: No data received yet)
Near-term validation studies should focus on two standard cytotoxicity assays: one using a human cell system and one using a rodent cell system.	Validation study completed by NICEATM/ECVAM and NIEHS with EPA support
Establish an interagency expert group under ICCVAM to advise on near-term activities such as assay selection, study design, and chemical selection.	Established the ICCVAM Acute Toxicity Working Group
Validate and implement the use of *in vitro* cytotoxicity methods to estimate starting doses to reduce animal use.	Complete (ICCVAM 2006a, b, c)
• Develop and improve *in vitro* methods to gather biokinetic and target organ data needed for accurate LD_{50} predictions, signs and symptoms associated with toxicity, and pathophysiological effects. • Investigate the mechanistic basis for "outlier" chemicals in *in vitro-in vivo* correlations and develop "exclusion" rules for identifying chemicals that cannot be accurately evaluated using *in vitro* methods. • Investigate the utility of toxicogenomics/proteomics for the assessment of acute toxicity, especially the prediction of no-observed-adverse-effect levels (NOAEL)/lowest-observed-adverse-effect levels (LOAEL) for acute exposure.	ICCVAM and NICEATM collaborating with the European Commission/ECVAM ACuteTox Initiative to address these recommendations

A NICEATM/ECVAM-sponsored validation study (ICCVAM 2006a, b) evaluated two *in vitro* NRU basal cytotoxicity assays[32] by testing 72 substances with the following objectives:

- Determine the extent that NRU test methods could estimate rodent acute oral LD_{50} values to set the starting doses for *in vivo* acute oral toxicity tests
- Develop high-quality *in vivo* acute oral lethality and *in vitro* NRU cytotoxicity databases
- Further standardize and optimize the *in vitro* NRU basal cytotoxicity protocols to maximize test-method reliability (intralaboratory repeatability, intra- and interlaboratory reproducibility)

An independent peer review panel was convened in May 2006 to evaluate the validation study results and associated draft ICCVAM test method recommendations (ICCVAM 2006b). ICCVAM considered the peer review panel report (ICCVAM 2006c) and public

[32] BALB/c 3T3 (clone A31) mouse fibroblast NRU test method
http://iccvam.niehs.nih.gov/methods/acutetox/invidocs/phIIIprot/3t3phIII.pdf
Normal human keratinocyte (NHK) NRU test method
http://iccvam.niehs.nih.gov/methods/acutetox/invidocs/phIIIprot/nhkphIII.pdf

comments to finalize recommendations for the use of the *in vitro* NRU test methods. The test methods cannot be used as stand-alone tests for regulatory hazard classification purposes. ICCVAM recommended that *in vitro* basal cytotoxicity test methods be used as part of a weight-of-evidence approach to determine starting doses for the acute oral toxicity tests. This approach will reduce the number of animals needed and may also reduce the number of animals that die or need to be humanely killed. **Figure 5-2** shows the IC_{50}[33]-LD_{50} regression formula used to determine starting doses for mixtures, test substances with low or unknown purity, or test substances with unknown molecular weights[34].

The performance of other *in vitro* basal cytotoxicity test methods that are based on similar scientific principles and that measure or predict the same biological response (i.e., basal cytotoxicity and, therefore, the rat acute oral LD_{50} value) should be demonstrated to meet or exceed the accuracy and reliability of the 3T3 and the normal human keratinocyte (NHK) NRU test methods.

The 3T3 NRU test method, which is less labor intensive and less expensive to conduct than the NHK NRU test method, is recommended for general use. Although the 3T3 NRU test method was less reproducible than the NHK NRU test method, it produced slightly higher animal savings and accuracy in predicting GHS (UN 2005) acute oral toxicity categories using the IC_{50} and the regressions evaluated for the prediction of LD_{50}.

The following example illustrates animal savings using *in vitro* cytotoxicity testing to estimate starting doses for the UDP. In this example, a standard UDP has a default starting dose of 175 mg/kg for a new chemical and no basis for an estimated LD_{50} with which to select another dose. Assuming the actual LD_{50} is > 5000 mg/kg, six animals would be tested in the UDP in 12 days. However, if the cytotoxicity test is performed first and *in vitro* data predicts an LD_{50} > 5000 mg/kg, then the UDP would proceed with a starting dose of 5000 mg/kg rather than the default starting dose of 175 mg/kg. Three animals would be used in six days to determine that the LD_{50} is greater than 5000 mg/kg. Thus, the use of the *in vitro* cytotoxicity test method would result in 50% reduction in animal use (3 vs. 6) and 50% reduction in time (6 days vs. 12 days).

The *in vitro* approach will save animals, because most substances have LD_{50} >2000 mg/kg values. Spielmann et al. (1999) stated that, since 1982, 75% of 1115 new industrial chemicals in the EU had an oral LD_{50} > 2000 mg/kg and need not be classified in the EU.

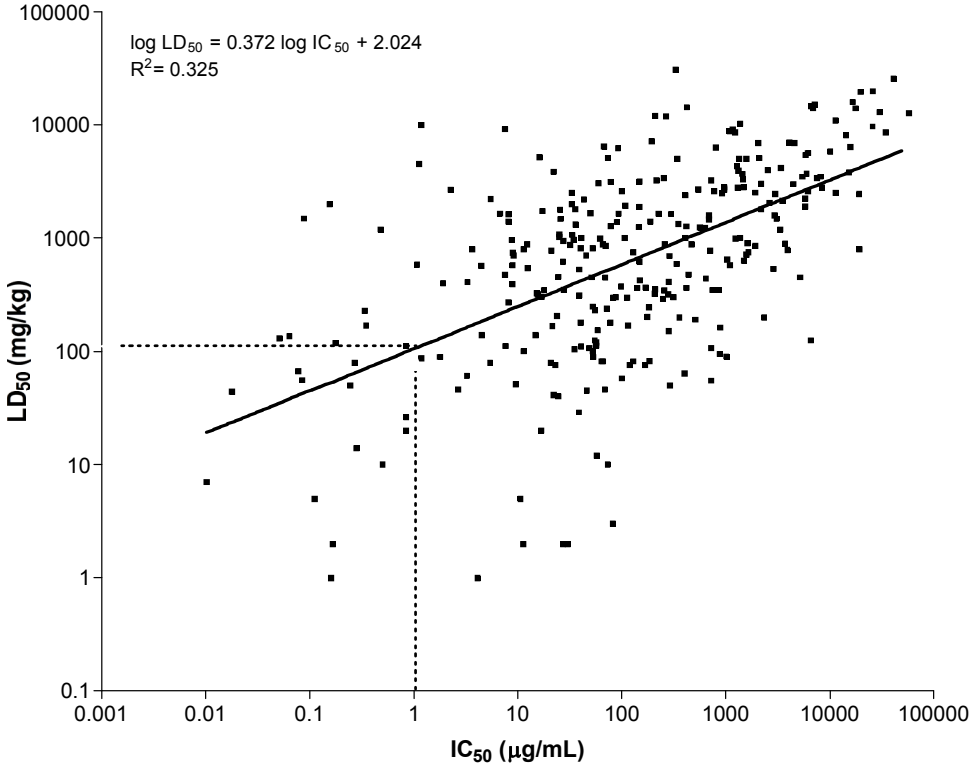
5.4 The ACuteTox Project: Optimization and Prevalidation of an *In Vitro* Test Strategy for Predicting Human Toxicity

Presenter: Thomas Hartung, M.D., Ph.D., ECVAM
http://iccvam.niehs.nih.gov/methods/acutetox/workshop-docs/present/Hartung.pdf

This presentation provided a review of the ACuteTox approach and the models proposed for replacing *in vivo* acute oral toxicity testing with an *in vitro* test battery strategy examining parameters such as ADME and organ specificity (nervous system, kidney and liver) to predict human oral toxicity.

[33] Inhibitory Concentration 50: Test chemical concentration producing 50% inhibition of the endpoint measured (i.e., cell viability).
[34] $\log LD_{50}$ (mg/kg) = 0.372 $\log IC_{50}$ (µg/mL) + 2.024

Figure 5-2 Prediction of LD_{50}



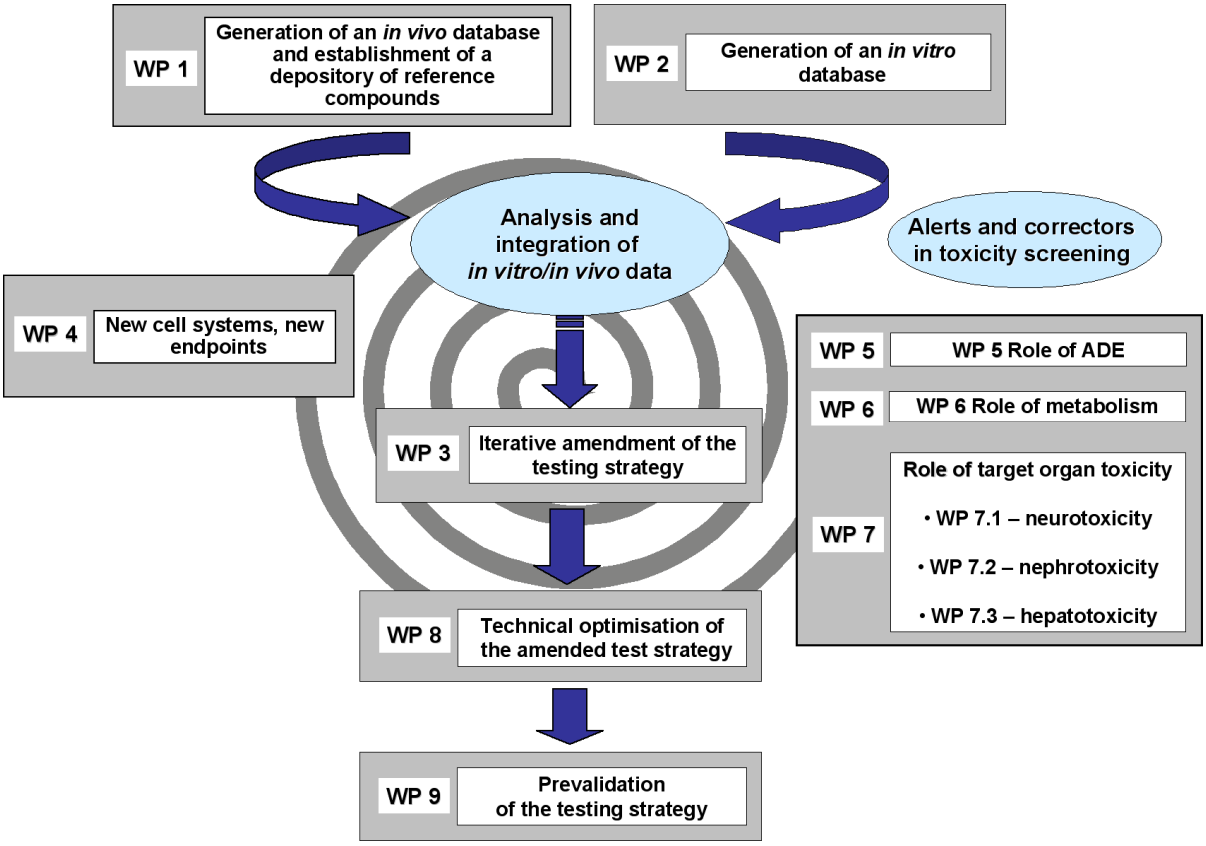
The ACuteTox Project's (http://www.acutetox.org) overall objective is to develop an *in vitro* test strategy sufficiently robust and powerful to completely replace *in vivo* testing to determine the acute toxicity of chemicals. The project is sponsored under the EU 6th Framework Programme and coordinated by ECVAM. Thirty-five partners from 13 European countries are involved, and the project is scheduled for 2005-2010.

ACuteTox builds upon information from an ECVAM workshop (Gennari et al. 2004) and data collected in previous studies that attempted to correlate *in vitro* cytotoxicity data with animal LD_{50} data and human lethal blood concentration data. These studies, the Registry of Cytotoxicity (RC; Halle 1998, 2003), the Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC)[35], and the ICCVAM/ECVAM *in vitro* validation study (ICCVAM 2006a, b, c) show approximately 70% correlation of *in vitro* to *in vivo* data. Further research is needed to improve the *in vitro/in vivo* correlation by evaluating existing outliers in order to introduce more parameters (e.g., ADME, organ specificity).

The project is divided into nine workpackages (WP) illustrated in **Figure 5-3**.

[35] http://www.cctoxconsulting.a.se/meic.htm

Figure 5-3 ACuteTox Project Workpackages



Abbreviations: ADE=Absorption, distribution, excretion; WP=Workpackage.

The 97 reference chemicals in WP 1 were selected within a wide range of acute toxicity. They represent all six GHS classification categories and various use categories. These chemicals have kinetic information, *in vivo* data (nearly 2000 LD_{50} values), and human acute toxicity data (blood concentrations from poisonings used to calculate lethal concentration values [i.e., LC_{50}]) obtained from poison control centers.

WP 2 has produced an internet-based database (AcuBase[36]) that contains information on the 97 test chemicals proposed for the project, animal *in vivo* data, human poisoning case data, *in vitro* data, and project reports. It is available to the project's partners and will be publicly available in the future.

Six cell systems (human, rat, mouse) were tested in WP 3 using different measures of cytotoxicity that yielded essentially the same results as the basal cytotoxicity tests. No system was more advantageous than the previously validated 3T3 NRU test system. WP 4 examines new test systems such as peripheral blood monocyte testing that has been validated as a cytokine secretion system (84% correlation) and the granulocyte/macrophage (GM) colony assay that was validated to assess myelotoxicity (bone marrow toxicity) of chemotherapeutic agents (87% correlation).

[36] http://www.acutetox.org/docs/Publication/WP1/WEB_Kolman_abstract_SSCT_2007.pdf

WP 5 is using *in vitro* models and neuronal networks to measure transport across the intestinal (72% predictability) and the blood–brain barrier (73% predictability). Protein binding, microsomal stability, and lipophilicity were measured too. WP 5 is evaluating measurement and modeling of free concentrations of chemicals in the *in vitro* systems and generic biokinetic modeling for the interpretation of *in vitro* toxic concentrations in relation to the *in vivo* acute toxic dose. WP 6 addresses the role of metabolism by using primary hepatocytes and hepatoma cell lines that are either transfected with p450 or not. Transfection should lead to a shift in the concentration–response curve as an indication of the role of metabolism in toxic effects.

WP 7 is investigating other target organs such as those involved in neurotoxicity, nephrotoxicity, and hepatotoxicity. Neurotoxicity research includes basal cytotoxicity, cell physiology (e.g., energy status, glycolytic activity, Ca^{+2} homeostatsis, membrane potential, oxidative stress), and neurochemistry (e.g., receptor function, neurotransmitter synthesis/degradation and uptake/release, electrical activity, ion channels). Nephrotoxicity is evaluated using transepithelial resistance as an indicator for loss of barrier function. Metabolism is being researched by using basal cytotoxicity to compare IC_{50} values of hepatocytes, hepatoma cells, and nonhepatic cells.

WP 8 will pursue the technical optimization of the amended testing strategy and then proceed to WP 9, which is the prevalidation of the testing strategy. ECVAM will maintain close ties with NICEATM and ICCVAM throughout the project.

5.5 Using HTS to Identify Predictive *In Vitro* Biomarkers for Acute Systemic Toxicity

Presenter: Raymond Tice, Ph.D., NIEHS/NICEATM
http://iccvam.niehs.nih.gov/methods/acutetox/workshop-docs/present/Tice.pdf

This presentation evaluated the use of quantitative HTS to establish predictive *in vitro* biomarkers for acute systemic toxicity. An overview of the NTP/NIH Chemical Genomics Center screening program, which incorporates a variety of *in vitro* cell systems and associated endpoints to assess the toxicity of chemicals, was provided.

The NTP Roadmap[37] includes a major initiative to develop an HTS program to meet the challenges of 21st century toxicology with the following main goals:

- Prioritize chemicals for further in-depth toxicological evaluation
- Identify mechanisms of action
- Develop predictive models for *in vivo* biological response

HTS methods are being used to identify small molecules that can be optimized as chemical probes to study the functions of genes, cells, and biochemical pathways. In mid-2005, NTP became a formal participant in the NIH Molecular Libraries Institute (MLI)[38] by collaborating with the NIH Chemical Genomics Center (NCGC). As a result, the NTP gained the opportunity to link data generated from HTS assays for biological activity to toxicity data produced by the NTP testing program.

[37] http://ntp.niehs.nih.gov/?objectid=EE4AED80-F1F6-975E-7317D7CB17625A15
[38] http://nihroadmap.nih.gov/molecularlibraries/

The NTP generated a list of 1408 chemicals and provided those chemicals as samples to the NCGC. All were evaluated in one or more toxicological tests. There are 1353 unique chemicals and 55 duplicates (to evaluate replication within a plate). NTP test data were available for 1206 chemicals; and the other 147 chemicals were identified from various ICCVAM reference substance lists that had been recommended for the validation of alternative *in vitro* methods, including dermal corrosion, acute toxicity, and endocrine activity. Criteria for selection included availability, solubility in dimethylsulfoxide (DMSO), and lack of excessive volatility. The largest class of chemicals in the list is industrial, but natural products, food constituents, pesticides, pollutants, and dyes are also included, as are many substructures.

The NCGC performed assays in a 1536-well plate format. The assays included two cytotoxicity assays (measurement of adenosine triphosphate [ATP] and lactate dehydrogenase [LDH]), three apoptosis assays, and a p-glycoprotein ATP-ase assay. Cell types used in the assays included human (9), rat (2), and mouse (2). **Table 5-3** lists a set of databases that will be publicly available for mining of *in vitro* and or *in vivo* toxicity data to establish mechanistic relationships.

Current NTP activities for the HTS program include:

- Within the next set of 1408 chemicals, include duplicates and focus on the following:
  - Chemicals of specific interest for cancer and immunotoxicity
  - Structurally related chemicals that have a range of activities
  - Chemicals that require metabolic activation
- Focus on assays that are representative of key steps in pathways important to cancer and immunotoxicity
- Expand the use of human primary cells (metabolically competent)
- Establish protocols for water-soluble chemicals
- Consider current concentration limits
- Evaluate differential responses among cell types
- Evaluate relationship between HTS and mid-throughput screening assay data (*C. elegans*, zebrafish embryos) and *in vivo* adverse health responses (e.g., acute toxicity, immunotoxicity, cancer)
- Incorporate various measures of chemical space (log p, molecular weight, number of rotatable bonds, number of hydrogen acceptors and donors) into the analysis
- Evaluate genetic differences in sensitivity using human HapMap cell lines and mouse strain cell lines
- Evaluate assay calls, reliability, and relevance

Other efforts to use mechanistic toxicity information include NIEHS genetics, genomics, and bioinformatics projects. These use knowledge about genes associated with human disease to find the pathways that link the genes to those diseases, identify the chemicals that interact with those disease pathways, and analyze the data to determine the critical proteins, genes, and connection points between pathways (Ball 2008). NIEHS will evaluate recommendations made in *Toxicity Testing in the Twenty-first Century: A Vision and a Strategy* (NRC 2007), including assessment of key exposures and toxicity outcomes, state-of-the-science testing

and assessment procedures, efficient experimental design and reduced use of laboratory animals, new and alternative test methods, and computational and molecular techniques in risk assessment. Additionally, NIEHS is drafting a memorandum of understanding on "High-Throughput Screening, Toxicity Pathway Profiling and Biological Interpretation of Findings" for interagency cooperation between NTP/NIEHS, NCGC/National Human Genome Research Institute, and the Office of Research and Development/EPA. The purposes are to coordinate assays; test chemicals; analyze and interpret data (within and across assays).

Table 5-3 Publicly Available *In Vivo* and *In Vitro* Toxicity Databases

Organization	Database	Description
NIEHS/NTP	CEBS – Chemical Effects in Biological Systems[1]	Integrates study design, clinical pathology, and histopathology data with microarray data and enables discrimination of critical study factors.
EPA	ToxCast™ Program[2]	Uses data from state-of-the-art HTS bioassays developed in the pharmaceutical industry to build computational models to forecast the potential human toxicity of chemicals.
EPA	ACToR – Aggregated Computational Toxicology Resource[3]	Centralizes many types and sources of data on environmental chemicals derived from more than 150 sources.
EPA	ToxRefDB– Toxicological Reference Database[4]	Compiles *in vivo* toxicology data for ToxCast™ with current focus on all relevant data from data evaluation records on 280 food-use pesticides from EPA OPPTS.
EPA	DSSTox – Distributed Structure-Searchable Toxicity Database Network[5]	Curates chemical structure and related assay data with its web site, providing a publicly available forum for publishing downloadable chemical structure files.
EPA	Genomics Data Management ArrayTrack[6]	Relies on ArrayTrack to house genomics data from Office of Research and Development (ORD) labs.
EPA	BDSM – Birth Defects Systems Manager[7]	Provides a reference collection of gene-expression data for modeling animal development.

[1] http://cebs.niehs.nih.gov/cebs-browser/cebsHome.do
[2] http://www.epa.gov/comptox/toxcast/
[3] http://www.epa.gov/comptox//pdf/Judson_et_al_ACToR_2008_TAAP.pdf
[4] http://epa.gov/comptox/forum/abstracts/informatics/martin_etal_ToxRefDBposter_ISFCT_may2007.pdf
[5] http://www.epa.gov/ncct/dsstox/
[6] http://www.epa.gov/NCCT/bosc_review/2006/files/06_Dix_genomics.pdf
[7] http://epa.gov/ncct/bosc_review/2007/files/BOSC_2007_Knudsen_Virtual_Embryo.pdf

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6.0 Breakout Group 1 — Key Pathways for Acute Systemic Toxicity

Co-chairs: Daniel Acosta, Ph.D. (University of Cincinnati, U.S.) and Frank P. Paloucek, Pharm.D., D.A.B.A.T., FASHP (University of Illinois College of Pharmacy, U.S.)

Panelists: Melvin E. Andersen, Ph.D., D.A.B.T. (The Hamner Institutes for Health Sciences, U.S.); Richard A. Becker, Ph.D., D.A.B.T. (American Chemistry Council [ACC], U.S.); Rajendra Chhabra, Ph.D.. D.A.B.T. (NIEHS, U.S.); Daniel J. Cobaugh, Pharm.D., D.A.B.A.T. (American Society of Health-System Pharmacists [ASHP] Research and Education Foundation, U.S.); Eugene L. Elmore, Ph.D. (University of California-Irvine, U.S.); Robert L. Guest, B.Sc. (SafePharm Laboratories Ltd., U.K.); Abigail Jacobs, Ph.D., D.A.B.T. (FDA, U.S.); Hajime Kojima, Ph.D. (Research Laboratories, Nippon Menard Cosmetic Co. Ltd.; JaCVAM, Japan); Steven Reynolds, Ph.D. (NIOSH, U.S.); Amy S. Rispin, Ph.D. (EPA, U.S.); Robert A. Scala, Ph.D., D.A.B.T., FATS (Consultant, U.S.); Raymond R. Tice, Ph.D. (NIEHS/NICEATM/ICCVAM, U.S.); Marilyn L. Wind, Ph.D. (CPSC/ICCVAM, U.S.)

Breakout Group 1 was charged with determining the key toxicity pathways associated with *in vivo* acute systemic toxicity and acute human poisonings. This group was asked to identify *in vivo* test observations/measurements and data that might be the most helpful for diagnosis and treatment of human poisonings and assessing acute systemic toxicity. A request was included in the charge to review molecular, cellular, tissue, or other physiological and clinical biomarkers that are or could be measured or observed during *in vivo* acute systemic toxicity testing. The group was asked to identify knowledge gaps associated with the diagnoses and treatments of poisoning and establish specific toxicological observations and measurements needed to address these gaps and improve the information available. The group was asked to provide recommendations for research and development activities for determining the key toxicity pathways and to suggest ways of implementing these activities.

This group reviewed several workshop presentations on the diagnosis and treatment of acute poisoning in humans and animals. The group focused on gaining a better understanding of how a clinician treats a patient who has presented with acute poisoning with a known or unknown chemical agent. Although not entirely analogous to an acute systemic toxicity study with experimental animals, an examination of human cases of acute chemical poisoning might provide better understanding and rationale for developing alternative *in vitro* acute toxicity testing systems. The key pathways that should be studied to better understand the toxic effects of chemicals and to better understand and treat acute human poisonings include:

- General cellular function
- Neuronal transmission, both central and peripheral
- Sodium/potassium ATP-ase pump
- Xenobiotic metabolism
- Cardiac conduction and aerobic metabolism
- Oxidative stress
- Receptor activity
- Immune response and function

6.1 Diagnosis of Human Poisoning

Given the compressed timeline that clinicians have in which to address poisonings, the *in vivo* test observations/measurements and data that have been most helpful for diagnosis and treatment of human poisonings are relatively nonspecific. An assessment of treatment for a human poisoning case typically starts with recording vital signs (blood pressure, heart rate, respiration rate, temperature, O_2 saturation). Measuring blood pressure and heart rate in combination with evaluating cognitive function helps address both cardiac and CNS functionality, while measuring respiratory rate addresses pulmonary function. Hyperthermia is associated with increased morbidity and mortality in human poisonings. Such bedside observations are typically used to generate a set of findings that would be classified into a toxic syndrome ("toxidromes"). A classic example is the use of these findings to distinguish sympathetic vs. parasympathetic poisonings.

These physical assessments/observations lead to recommendations for various laboratory determinations such as serum electrolytes and blood gases. Specifically, serum potassium assists in assessing potential risk or severity of cardiac conductivity. In addition, when coupled with blood gas measurements, these assessments are important in evaluating acid/base status with a focus on the use of the anion gap in generating a differential diagnosis. The QRS[39] and QTc[40] intervals on a 12-lead electrocardiogram are used in the generation of a differential diagnosis for cardiotoxicants affected by conductivity. These measurements provide indications for specific supportive therapies such as sodium bicarbonate and/or magnesium sulfate bolus injections. Measurement of serum creatinine and urine output helps assess renal function, although they are not always associated with acute renal damage and are associated with indications for hydration and vasopressor therapies.

The availability and timeliness of specific toxicant measurements in serum or urine typically limit their utility. Determination of acetaminophen concentration remains universally recommended and represents the sole marker that clinicians have to identify poisonings prior to the manifestation of signs and symptoms of toxicity. The presence of therapeutic agents (e.g., digoxin) that could be associated with toxicity is usually determined from patient history records. Nonmedicinal toxicants such as pesticides are often identified through measurement of cholinesterase activity. However, because such measurements often require referral to outside laboratories, their clinical utility might be minimal. This series of selected clinical evaluations, though the most useful for the diagnosis and treatment of acute human poisoning, does not include other clinical evaluations that may be used in common practice.

6.2 Knowledge Gaps Related to the Diagnosis and Treatment of Human Poisoning

A number of knowledge gaps persist related to the diagnosis and/or treatment of human poisoning. Definitive identification of the class of toxicant ingested by the patient is perhaps the most important information that could improve the diagnosis and treatment of poisoning, because it would minimize absorption and uptake, thereby allowing for focused therapy to prevent systemic toxicity. It could minimize organ damage when absorption to the specific target has already occurred. However, this approach requires rapid in-house analyses. The observations and measurements outlined in **Section 6.0** are nonspecific; therefore, earlier or

[39] Deflections in the tracing of the electrocardiogram (ECG or EKG), comprising the Q, R, and S waves.
[40] QT intervals measured from the beginning of the QRS complex to the end of the T wave on the ECG, corrected for heart rate.

more specific markers are necessary to refine a differential diagnosis and allow a narrowing of therapeutic interventions. Other knowledge gaps (listed below), if adequately filled, could inform diagnosis and/or treatment.

- More toxicant serum concentration vs. time of exposure data
- Accuracy of patient history reports
- Laboratory confirmation of known toxicant from reported cases
- Time course of acute life-threatening poisonings
- Chemical interactions (e.g., mixtures, polypharmacy, food additives)

The breakout group identified several toxicological observations and measurements to address these gaps and improve the information available to diagnose and/or treat human poisoning. Biomarkers of organ/system damage (e.g., cardiac troponin, acute renal damage [Kim-1]), and other renal biomarkers [beyond proximal tubule damage]) are useful. Certain other biomarkers could be useful if they could be assayed soon enough. For example, cholinesterase measurements could provide substantial clinical information. Markers of oxidative stress (e.g., glutathione, 8-oxoguanine) could be used to inform when to continue or discontinue anti-oxidant therapies. In general, serum/blood determinations are preferred to urine measurements because of the greater temporal association of serum/blood levels with acute toxicities. Clinicians' assessments could clearly benefit from dosimetry in humans during adverse events and consideration of dosage formulations (e.g., sustained release vs. immediate release formulations).

6.3 Recommended Research and Development Activities

Three areas of research and development that are not considered mutually exclusive share the highest priority. First, MOA-based test methods are considered vital because they can provide better understanding of the action of chemicals in living systems. They also increase understanding of the alteration of early obligatory pathways, which may not lead to toxicity (e.g., receptor binding) when perturbed individually, vs. alteration of various interconnected pathways that affect the quantitative relationship of mechanisms/MOA. Next, use of human cell-based systems (i.e., either primary or early passage normal human cells) as screening models is considered important since the human condition is the desired reference. The group recommended that cell models developed to assess affected cellular pathways should also receive attention to assess the likelihood of interactions among these pathways. It is important to remain committed to advancing high-throughput screening initiatives and applying computational toxicology, where possible, as well as to have associated data management capabilities when these methods are used. Lower-priority but still important research needs include developing tools for determining toxicokinetic (*in vitro* and *in vivo*) information and assuring consideration of ADME in model systems. Similarly, development of *in vitro* methods that, coupled with currently used basal cytotoxicity test methods, would form a screening battery for acute toxicity is a priority that would necessarily combine both target-specific and MOA-based test methods.

Future research and development priorities include (1) methods to evaluate recovery and/or reversibility of an effect and (2) methods to address chemicals that are typically physicochemically incompatible with conventional *in vitro* cell systems (e.g., hydrophobic chemicals). Successful implementation of any of these activities would require the

development and training of an adequate workforce, which necessitates a paradigm shift in traditional toxicology training.

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7.0 Breakout Group 2 — Current Acute Systemic Toxicity Injury and Toxicity Assessments

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The workshop charge given to Breakout Group 2 included reviewing clinical observations and quantitative measurements that could be included in current *in vivo* acute systemic toxicity tests to support development of predictive *in vitro* methods. The group was asked to identify toxicity pathways that could be modeled by using *in vitro* test methods as well as biomarkers that might provide more information on *in vivo* pathophysiological effects and mechanisms of acute systemic toxicity. The group was also asked to explore optimal ways to standardize measurements of the suggested biomarkers to be included in the current acute systemic toxicity tests. Additionally, the group was to suggest and prioritize research and development activities for obtaining more information on key toxicity pathways.

Understanding key response pathways is critical to identifying the MOA to develop alternative test methods which might vary depending on purpose (see **Section 9.3**). Information about key toxicity pathways would be useful to both poison control centers and emergency departments, and the initial information on dosimetry and target organ toxicity could be used for longer-term studies. Hazard classification based on rodent LD_{50} values (for both pure chemicals and mixtures) is the primary regulatory purpose of the acute systemic toxicity test methods. Therefore, nonanimal alternative test methods must be able to accurately predict the rodent LD_{50}. However, the group agreed that prediction of acute human poisoning is the ultimate goal.

7.1 Key Pathways to Be Modeled Using Alternative Test Methods

The breakout group first identified key pathways that need to be modeled using alternative test systems (see **Section 6.0**). These pathways encompass the following categories:

- Animal and human systems
  - ADME
  - Components of metabolism that can mimic *in vitro*
  - Information (bioavailability, Structure-Activity Relationship [SAR]) available before testing
  - Human toxicokinetic information, when available

- Whole organs (prioritized for the following)
  - Pulmonary
  - Renal
  - Hepatic
  - Cardiovascular
  - Neurological (e.g., neurochemical, behavioral, brain swelling)
  - Gastrointestinal (e.g., production of endotoxin as a marker for sepsis)
  - Hematopoietic (including hemorrhaging)
- Cellular systems
  - Chemical toxicity (key issue is whether it is greater for dividing or nondividing cells)

Observations at the genomic level would probably be limited to blood samples. An investigator must consider all available information (e.g., SAR) in choosing the most appropriate alternative method to meet a specific purpose. Some sampling measurements may be possible on moribund animals, but some group members questioned the relevance of those measurements. The group considered animal or human cadavers inadequate for collecting most of the suggested endpoint measurements.

7.2 *In Vivo* Biomarkers to Provide Mode/Mechanism of Action Information for Acute Systemic Toxicity

The group identified biomarkers (clinical observations and quantitative measurements; see **Table 7-1**) expected to provide more information and a better understanding of the pathophysiological effects and modes/mechanisms of action of acute systemic toxicity. In recommending additional biomarker information that should be collected from acute animal studies, the group assumed that (1) no additional animals would be used, and (2) cost was not a consideration. Methods currently exist to generate these types of information but may need to be adapted (e.g., appropriate sample volume, instrumentation of appropriate size and sensitivity for telemetry) for rodent models. For chemicals expected to require data sets broader than acute systemic toxicity information, the group suggested combining the acute study with a repeated dose study by making the acute study measurements at the first timepoint in the repeated dose study.

The group agreed that early timepoints (less than 24 hours after dosing) were better for biomarker measurements in animal studies. In addition, to maximize the use of the limited number of animals currently required for acute toxicity tests, the group recommended that, if not already available, noninvasive or minimally invasive methods be developed for additional biomarker measurements. While the optimal way to measure biomarkers may vary, standardized procedures for sample collection and processing, as well as biomarker detection and quantification, should be used. Such standardization will ensure consistency across laboratories.

Given the vast number of potential blood/serum biomarkers and the relatively small blood/serum volume available from a rat, continued protocol refinement is also needed to reduce the required sample volume and increase the number of tests that can be performed. Target tissues should be properly stored for future research and development studies related to new biomarkers (e.g., those identified using "-omics" technologies). Collecting the

biomarkers recommended in **Table 7-1**, with the possible exception of blood, should have minimal effect on the current protocols for acute systemic toxicity tests (i.e., FDP [OECD 2001a], ATC [OECD 2001b], UDP [OECD 2001c]).

Table 7-1 Biomarkers for Pathophysiological Effects and Modes/Mechanisms of Acute Systemic Toxicity

Level of Organization	Acute Systemic Toxicity Test Biomarker Observations and Measurements	Recommended Biomarker Observations and Measurements	Recommended Research and Development of Biomarkers
Whole Animal System	• Clinical observations • Body weight • Feed consumption • Water consumption	• Clinical observations • Body weight • Feed consumption • Water consumption • Functional observations (heart rate, electrocardiogram, respiratory rate, respiratory volume, body temperature, limited observations for neurotoxicity)	• Stress: corticosteroids • "-omics" technologies • Toxicokinetics • Metabolism
Organ/Cellular	• Gross pathology	• Gross pathology • Clinical pathology and urinalysis (early, mid, and late timepoints) • Serum and urine pH (anion gap, etc.) • Histopathology • Gastrointestinal: measure presence of cytokines (TNF) in body cavity as a measure of endotoxin	• Kidney: creatinine, tubular markers, protein (urine), BUN, GST (urine), n-acytl glucosamine • Liver: glutathione, SGOT • Heart: physiological measurements, body chemistry, blood pressure, heart rate and rhythm, serum troponin levels • Neurological: neurotrans-mitter levels (e.g., catecho-lamines), FOB [optional]) • Lungs: respiratory imbalance • Blood and other tissue concentrations of toxicant (early, mid, and late timepoints)

Abbreviations: BUN= Blood urea nitrogen test; FOB=Functional observation battery; GST=Glutathione-S-transferase; SGOT=Serum glutamic oxaloacetic transaminase; TNF=Tumor necrosis factor.

7.3 Recommended Research and Development Activities

The group identified both short- and long-term activities to obtain more information on key toxicity pathways from the current acute systemic toxicity tests. They include the following:

Short-Term Activities

- Noninvasive telemetry systems for real-time monitoring of physiological parameters in rodents
- Automated systems for collecting behavioral information

- Noninvasive analytical devices for analyzing small blood/urine volumes (e.g., glucose meters used by diabetics)
- Bioinformatics (a need for parallel development for handling the increased information)

Long-Term Activities

- "-omics" technologies to identify biomarkers (e.g., metabolomics)
- Imaging (pursue any noninvasive technique such as ultrasound or other imaging techniques [e.g., radionuclide cardiovascular test, brain swelling, internal hemorrhaging])
- Nanotechnology (e.g., for use in early detection of toxicity; noninvasive scanners have been used for physiological measurements)

8.0 Breakout Group 3 — Identifying Earlier, Humane Endpoints for Acute Systemic Toxicity Testing

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The charge to Breakout Group 3 was to determine objective biomarkers that elucidate key toxicity pathways that are sufficiently predictive of lethality and that could be validated and used along with clinical signs and observations for pain and distress as routine humane endpoints for acute systemic toxicity testing. The group was to determine whether the use of humane endpoints would interfere with the collection and interpretation of mechanistic data (or other data) and conversely, to what extent the collection of additional data might lead to incorporation of more humane endpoints in *in vivo* tests to further reduce pain and distress. The group was to suggest research, development, and validation efforts that would address any identified knowledge gaps associated with predictive early humane endpoints.

This group primarily focused on identifying and prioritizing *in vivo* data for collection to elucidate key toxicity pathways that might lead to the identification and validation of earlier, more humane endpoints for acute systemic toxicity testing. Such data collection includes:

- Data that currently are or should be routinely collected and used as humane endpoints during *in vivo* acute toxicity testing
- Data that might-be routinely collected and could aid in identifying additional humane endpoints that occur sooner after exposure
- Data that might eventually be useful as predictive endpoints before the onset of overt toxicity and which therefore warrant additional investigation and development

8.1 Use of Biomarkers to Identify Earlier, Humane Endpoints for Acute Toxicity Tests

The group discussed the use of evident toxicity as an earlier, more humane endpoint than moribund condition or death. Evident toxicity is the endpoint used in the FDP for acute oral toxicity testing. The FDP (approved OECD TG 420; OECD 2001a) is one of three test guidelines that can be used internationally for acute oral toxicity hazard classification and labeling purposes In the context of the charge given to this breakout group (i.e., more humane endpoints for acute systemic toxicity testing), the group recommended (although not unanimously) that the FDP become the preferred acute oral toxicity testing method to be used routinely instead of the UDP (OECD TG 425; OECD 2001c) or the ATC (OECD TG 423; OECD 2001b), unless adequate scientific justification and rationale can be provided to justify

that the UDP or ATC would be more appropriate.[41]. For example, the derivation of a point estimate of the LD_{50}, around which 95% confidence limits can be defined, was stated as necessary for certain regulatory requirements. In this case, the UDP would be the requisite acute toxicity test because the FDP and ATC do not generate such data.

To effectively implement this recommendation, the group recognized the need for two separate, globally standardized scoring systems that would allow for weighting of observations. One scoring system would describe evident toxicity (a term that has been accepted by regulators with the international adoption of OECD TG 420), and one would describe severe toxicity and lethality. The group also recommended using fixed-dose/concentration approaches for acute toxicity testing by the dermal and inhalation routes, respectively, in order to use evident toxicity as an earlier, more humane endpoint for such studies.[42].

Considering these recommendations, the group generated a list of biomarkers sufficiently predictive of evident toxicity that they should be used routinely during acute toxicity testing:

- Simple behavioral observations for evaluating level of activity
- Body temperature decreases
- Body weight and feed and water consumption, if appropriate (the group noted that consideration should be given to the potential impact of social housing versus individual housing on these measurements, and suggested that hydration status could be used as a surrogate for water consumption)

Although clinical signs and observations for pain and distress should be routinely recorded, biomarkers that can be measured and observed in a standardized or systematic way are needed instead of more traditional subjective evaluations. Employing humane endpoints should not interfere with the collection and interpretation of mechanistic data, and the group anticipated that such objective measurements might actually facilitate the collection and interpretation of better mechanistic data and avoid autolyzed tissues from dead animals.

There currently are insufficient data to support routine inclusion of the biomarkers listed below in acute toxicity testing, but the group identified several types of data to collect during future animal studies. These data could help identify earlier, more humane endpoints. In some cases, they are currently collected, but not in a standardized or systematic way; and the results are not communicated or consistently reviewed and assessed.

The recommendations for measurements and observations included:

- Clinical pathology data gathered shortly after exposure

[41] OECD TG 420 is not equivalent to OECD TG 423 and OECD TG 425. The decision criteria in the FDP test guideline include death along with evident toxicity. OECD TG 420 introduced an animal welfare override at each initial test dose, allowing for classification based on the outcome for a single animal. Some workshop participants stated that this component of the FDP was not validated.

[42] Because the FDP does not satisfy the regulatory needs for an LD_{50} estimate, some U.S. regulatory agency representatives at the workshop did not agree that the FDP should be the preferred method for any acute systemic toxicity testing, including potential applications to acute dermal toxicity and acute inhalation toxicity. Recommendations for using the FDP were made only in the context of identifying humane endpoints; there are scientific and regulatory reasons for using a method other than the FDP. The UDP for acute oral toxicity was developed to provide LD_{50} values to satisfy U.S. regulatory requirements.

- Functional measurements (e.g., heart rate, electrocardiogram, functional observational battery [FOB] for neurotoxicity)
- Measurement of toxicant levels in body fluids (with blood levels used for timepoints)
- Fecal occult blood to indicate gastrointestinal hemorrhage
- Fecal measurements of corticosteroids
- Measurement of catecholamine levels in blood or serum
- Measurement of cytokines (TNF) in the body cavity or detection of proteinuria in mice (Sever 2007) as a surrogate measurement of endotoxin levels in the body

The group addressed the influence of inhalation exposures on endpoints. It is important to consider the impact of potential dermal (whole body) vs. nose-only exposures that may compromise endpoints. In addition, investigators should make routine assessments of pulmonary function (i.e., respiratory rate and tidal respiratory volume) along with pulmonary histopathology, the latter of which would provide an assessment of both toxicity and any background infection. With regard to studies conducted with nose-only exposure, animals should be acclimated to nose-only restraint devices prior to exposure, the duration of exposure should be minimized, and vital signs should be routinely collected.

8.2 Recommended Research, Development, and Validation Activities

Research, development, and validation efforts should address knowledge gaps currently associated with predictive early humane endpoints. The group considered development of objective criteria to characterize evident toxicity and publication of internationally harmonized guidance to detail these criteria to be vital steps before initiating routine use of the FDP. A number of measurements warrant further evaluation for their usefulness in defining humane endpoints for acute toxicity testing. These include those discussed above, quantitative measures of activity/behavior, and use of saliva and exhaled air instead of blood for detection of potentially useful biomarkers.

8.3 Implementation of Recommended Activities

The final charge to this breakout group was to identify the most effective ways to implement the recommended activities. The group agreed that dedicated funding is necessary and recognized the need for other incentives to motivate stakeholders to commit to these recommendations. Well-defined strategies for standardization among the international community should be generated, with existing guidelines improved. Data mining and sharing of existing and newly generated data among international stakeholders should be encouraged, where possible. Finally, the group asserted that additional training in application of the recommended measurements and observations, as well as interpretation of their results, is essential to significant advancement in the application of more humane endpoints for acute toxicity testing.

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9.0 Breakout Group 4 — Application of *In Vivo* Mode of Action and Mechanistic Information to the Development and Validation of *In Vitro* Methods for Assessing Acute Systemic Toxicity

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Breakout Group 4 was asked to determine the extent of applicability of *in vitro* test methods to adequately model the key toxicity pathways indicated by *in vivo* measurements (molecular, cellular, tissue, or other physiological and clinical biomarkers) and observations associated with acute systemic toxicity, and to subsequently identify any relevant knowledge gaps. The charge to the group included prioritizing activities for developing and validating the *in vitro* methods that will more accurately predict acute systemic toxicity hazard categories. The group was also asked to consider the application of *in vivo* mode of action and mechanistic information to further improve *in vitro* testing. Discussions were to include how the timing of observations might be adjusted to differentiate the initial pathway effects from downstream effects. Finally, the group was to consider how *in vitro* tests might be incorporated into current testing to meet regulatory testing requirements.

This breakout group discussed how current and future *in vitro* test methods could model key toxicity pathways (see **Section 6.0**) for acute systemic toxicity identified by *in vivo* measurements (molecular, cellular, tissue, or other physiological and clinical biomarkers and observations). The group identified and prioritized research, development, and validation activities for use of *in vitro* test method models of key *in vivo* toxicity pathways to more accurately predict acute systemic toxicity hazard categories.

9.1 *In Vivo* Toxicity Pathways to be Modeled by *In Vitro* Systems

A key challenge to the group was providing a clear definition of *toxicity pathways*. Different levels of biological organization (i.e., cellular signaling pathways, intercellular interactions, and organ-level responses) define pathways, and pathways at all three levels contribute to acute systemic toxicity. Each level of organization encompasses different characteristics of the exposure-dose-toxicity continuum. While identification of the affected cellular signaling pathways may clarify the key initial interactions of a chemical with biological targets, the pathways alone are insufficient for understanding the subsequent processes involved in acute systemic toxicity. More integrated test methods will help identify the pathways. For instance, the *in vitro* basal cytotoxicity test methods (ICCVAM 2001a, b; 2006a, b, c) provide a measure of integrated cellular response that has been empirically associated with LD_{50} values in rodents. There is fair correlation of these two test methods given that IC_{50} predicted LD_{50} within an order of magnitude for about 70% of the chemicals evaluated (Halle 1998, 2003). The group noted opportunities to attempt more refined evaluations of cellular responses (e.g.,

direct cytotoxicity, apoptosis, cell proliferation), alone or in combination, to identify other integrated cellular measures that might be better correlated to LD_{50} than basal cytotoxicity.

Using *in vitro* tests to assess interaction among tissues presents an even greater challenge. Such interactions include (1) activation of inflammatory responses following toxicity in one organ (leading to enhanced target organ toxicity) or in remote tissue targets, or (2) interactions between initial target tissues and immune system components. These integrated tissue interactions may eventually be correlated to responses of a single target tissue. For example, cytokine release from an *in vitro* test on a defined cell type may predict subsequent inflammatory or immunologic responses.

The group also discussed identification of key cellular pathways. High-throughput test methods could be used to identify cellular targets of test chemicals, and *in vitro* cell models of these targets could be developed for routine application. Chemicals in families with known mechanisms could also be evaluated with specific targeted test methods. It may be possible to use genomic and other "-omic" approaches to infer both cellular and tissue-level pathways that are altered in tissues within animals undergoing acute systemic toxicity testing with chemicals for which knowledge of cellular targets is limited (Ball 2008). Such refinements to *in vivo* studies might facilitate identification of biomarkers that could subsequently be used to enhance the correlation with LD_{50} values for a broader proportion of test chemicals.

9.2 *In Vitro* Modeling of *In Vivo* Acute Systemic Toxicity

The National Research Council report titled *Toxicity Testing in the Twenty-first Century: A Vision and A Strategy* (NRC 2007), from the National Research Council Committee on Toxicity Testing and Assessment of Environmental Agents of the NAS, proposes a long-term goal in which high-throughput *in vitro* test methods will be used to assess key cellular toxicity pathways. Mechanistic models of these *in vitro* test systems, along with physiologically based pharmacokinetic models, would identify human exposure levels without significant effects on the pathways (NRC 2006, 2007). A related goal for predicting acute toxicity includes identification of initial cellular pathways (e.g., oxidative stress, loss of membrane function, specific interaction with key receptors) and quantitative modeling of the integrated cellular and tissue cascades that follow these initial interactions and lead to acute systemic toxicity. The long-term goal of these more quantitative, mechanistic approaches is development of dose-response models with which to predict acute systemic toxicity based on the patterns and dose-response characteristics of pathways perturbed by chemical treatment *in vitro*.

In vitro test methods can evaluate a vast array of toxicity pathways to access both specific endpoints and dose-response characteristics. Potential *in vitro* test methods include:

- Neuronal transmission
- Immunology/inflammation
- Cellular respiration
- Sodium/potassium pump
- Hepatic metabolism
- Cardiac conduction
- Cardiac aerobic metabolism
- Oxidative stress
- Overstimulation of receptors
- Basal cytotoxicity

- Specific organ sites (functional test methods)
- Cytotoxicity (measured as apoptosis and other non-necrotic pathways)
- Hepatocytes (associated pathways that trigger inflammatory response)
- Mitochondrial function/damage
- Cellular glutathione content
- Various ion channels
- Blood-brain barrier function
- Transporter protein function
- Cell arrhythmia
- Renal tubule cell (in test methods) for excretory function
- Neuronal cells (in test methods)

Many of these endpoints are accessible in various cell types that are available for *in vitro* evaluations. In general, these test methods are more related to integrated cell responses (e.g., cellular glutathione concentration, mitochondrial function, cytotoxicity, apoptosis, proliferation). Other test methods focus on interaction with cellular targets, such as overexpression of transporters in cell lines, and examination of uptake rates of chemical into these cells. Generally, it is now possible to model many *in vivo* measures with human cells *in vitro*. These test methods are not validated for replacement of current *in vivo* tests. However, test method selection depends on knowledge of cellular, tissue,- and organ-specific targets for chemicals. When the mechanisms are unknown or only partially known, broader screens might be required to obtain a better idea of the pathway to model.

9.3 Knowledge Gaps Related to *In Vitro* Modeling of *In Vivo* Acute Systemic Toxicity

The major knowledge gap is in understanding all of the chemicals' *in vivo* mechanisms of action that could help direct selection of *in vitro* test method systems. Among approaches to assess mechanisms of action are "-omic" evaluations of tissues from rats in the acute toxicity screens. If testing batteries envisioned by the NAS Toxicity Testing Panel were implemented, the proposed short-term targeted *in vivo* animal bioassay could be used to ascertain differential responses in tissues *in vivo* and could help determine possible toxicity pathways. Other than basal cytotoxicity test methods, there is little experience in assessing correlations between LD_{50} and integrated cellular responses; therefore, such analyses and the associated correlations might determine if other cellular response measures provide better predictive power. From the mechanistic perspective, no quantitative procedures have been developed to describe cascades of responses and predict LD_{50}. Research of this kind could predict LD_{50} values and measures of chronic toxicity from pathway studies.

The group discussed ways to address data requirements that would allow an *in vitro* approach to predicting target tissues and the LD_{50} for acute toxicity. Use of human-based *in vitro* cell systems is a priority. However, if human cell models are not available, then the use of relevant animal cell models would also be appropriate.

9.4 Recommended Research, Development, and Validation Activities

The identification or development of tissue-specific cellular models is essential for assessing critical toxicity pathways, and these models will need to incorporate and allow for genetic variability if possible. The use of human cord blood to isolate stem cells and direct their

differentiation to express biomarkers normally expressed in the tissue should be considered. This approach has previously proven useful (Buzańska et al. 2002, 2005, 2006a, 2006b; Sun et al. 2005) and is likely to become routine in the near future. It would allow the development of large repositories of relevant cells to support method validation for predicting acute systemic toxicity. The cells could then be available for use in routine testing. Production of adequate supplies of human cells is a critical step that will require allocation of funds to support the development of relevant test methods based on perturbations of toxicity pathways *in vitro*.

Standardized testing protocols need to be developed, and the necessary controls must be identified before initial evaluation of each cellular response pathway as a predictor of acute systemic toxicity. Chemicals active in the toxic response pathway, as well as negative controls, should be examined in the test methods. Once data are available from these studies, statistical analyses can determine which cellular response pathways are best associated with acute systemic toxicity. Addressing the development and validation of *in vitro* test methods to measure cellular response pathways underlying the toxicity pathways will require careful deliberation, as well as input from experts in each associated target tissue and its toxicity-related cellular pathways. These interactions would require a meeting with appropriate opportunity for discussion of and deliberation on specific test methods.

The group acknowledged that validation of *in vitro* models requires a wider variety of data (e.g., ADME) than simply acute toxicity. Acute toxicity tests do not include routine collection of blood levels and pharmacokinetic data that could be very helpful, especially if predicting human acute systemic toxicity requires estimating the human blood time course necessary to equal those that occur in the rat after a single lethal dose. Data from animal studies with acute dosing and other forms of dosing need to be standardized for use in validation studies. Identification and assessment of possible toxicity pathways would be facilitated if all stakeholders, including those from industry and government, provided access to study results and any associated data from tests on chemicals.

Once test methods are developed for the required cellular response pathways to identify chemicals that act via these mechanisms, validation can incorporate unknown chemicals, as well as those used in the *in vitro* basal cytotoxicity validation. The toxicity pathways associated with the test chemicals used in this validation effort should be defined based on *in vivo* studies, and prospective testing should be conducted with coded chemicals. Data from these new test methods and from the previous *in vitro* basal cytotoxicity validation can be used to determine how well they predict *in vivo* effects. All chemicals tested in the previous study should then be tested using the newly developed cellular models. Data analysis will require *in vivo* data from standardized test methods and various analytical tools that are currently under development, including but not limited to bioinformatics programs and pathway analysis methodologies.

The breakout group recommended the following research priorities:

- Apply a broad array of *in vitro* test methods to screen for modes of action as noted in **Section 9.2.**
- Collect as much data as possible from those animal studies that are conducted to better understand modes of action, and use this information to guide selection of *in vitro* test methods for these modes of action.

- Develop databases of "-omic" changes, and assess affected tissue-level pathways in animals being tested for acute systemic toxicity.
- Attempt to broaden the association between LD_{50} and *in vitro* measures by completing studies with larger numbers of chemicals, assaying more integrated measures of cellular function.
- Develop computational systems biology approaches to predict *in vivo* acute toxicity from sequential activation of specific cellular pathways.

9.5 Implementation of Recommended Activities

Implementation strategies for relating *in vitro* test results with acute toxicity will vary for those approaches attempting to establish correlations between outcome and *in vitro* test results (i.e., correlative approaches) and for those that attempt to mimic the sequential cellular and tissue responses that lead to toxicity (i.e., mechanistic approaches). The group attempted to identify key toxicity pathways for acute systemic toxicity by considering pathways identified in **Section 6.0** and the underlying cellular response pathways. Examples include immediate toxicity by inhibiting neuronal transmission and cardiac conduction, and delayed toxicity due to a strong inflammatory response in the liver or damage to the proximal tubules in the kidney. **Section 6.0** lists examples of toxicity pathways assessable by *in vitro* test methods. Many common cellular response pathways present in most cells were not directly considered because they are included in the currently validated *in vitro* basal cytotoxicity test methods (ICCVAM 2006a, b, c). The inclusion of tissue- or system-specific effects is advantageous because it potentially enhances accuracy in predicting rodent LD_{50} when combined with *in vitro* basal cytotoxicity test methods..

While the implementation and completion of this program will require the application of currently available methods as well as the development of new methods, a substantial investment will be required for the development of tissue-specific human cellular models that use normal human cells. Stem cell sources from human cord blood should be developed to provide sufficient access to cells on a broad scale and provide the standardization necessary for validation studies. The ultimate goal is to increase the accuracy of *in vitro* test methods' prediction of LD_{50} values from the acute toxicity test methods and, consequently, to better predict human LD_{50} values.

This implementation requires attention to multiple factors as described below:

- Collect any available standardized data from animal studies to aid in pathway determination.
- Identify model cellular systems for assessing chemical activity in the pathway.
- Identify agents that relate to toxicity in the model cellular systems.
- Develop model systems for testing, including methods and endpoints.
- Interpret results using standardized test panels to compare with the rodent LD_{50}.
- Use statistical tools, currently being developed and implemented, to facilitate interpretation for association between potency in specific pathway test methods and the rodent LD_{50}.
- Determine the effectiveness of each system alone and in combination.

- Convene expert panels to address development of cell lines, design and use of appropriate biomarkers, test method implementation, and data analysis procedures.
- Consider incorporating individual test methods into the assessment of acute toxicity in parallel with the *in vitro* basal cytotoxicity test method.
- Develop appropriate procedures to compare the performance of new test methods in relation to the *in vitro* basal cytotoxicity test methods for predicting the rodent LD_{50}.
- Consider how multiple measures of cellular toxicity pathways might be used to predict acute systemic toxicity.

10.0 Breakout Group 5 — Industry Involvement in Test Method Development, Validation, and Use

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The focus of Breakout Group 5 was to determine the most effective way to encourage industry to collect and submit to ICCVAM (1) mechanistic observations and measurements from animals used in acute systemic toxicity studies and (2) concurrent *in vitro*/*in vivo* toxicity test data to be used in the development and validation of alternative *in vitro* test methods. This included consideration of how industry can increase the use of adequately validated *in vitro* cytotoxicity test methods for reducing the use of animals in acute systemic toxicity tests and how impediments to data collection (*in vitro* and *in vivo*) can be overcome.

10.1 Current Uses of *In Vitro* Cytotoxicity Testing by Industry

In 2001, the EPA High Production Volume (HPV) Challenge Program sent a letter to 1200 organizations requesting data submitters to consider and use *in vitro* basal cytotoxicity test methods to set the starting doses when testing HPV chemicals for acute systemic toxicity. There has been only one submission of *in vitro* basal cytotoxicity and *in vivo* rodent LD_{50} data. Private-sector participants at this workshop stated that collecting data from parallel *in vitro* and *in vivo* toxicity testing would require a significant monetary and staff commitment by a company, while the impact of *in vitro* test methods on further animal reduction would be limited at best. This effort is in the face of an already considerable reduction in the number of animals used for acute oral testing. The numbers have scaled downward in many instances from the traditional 50 or more rodents per test to only six or eight. *In vitro* test methods could replace the *in vivo* acute toxicity test methods if a full battery of *in vitro* tests were available that accounted for the many mechanisms and modes of action of acute toxicity. At present, because of poor accuracy, *in vitro* cytotoxicity predictions of acute oral toxicity are useful only when there is a complete lack of information for a particular chemical, which workshop participants say is rare.

The *in vitro* basal cytotoxicity test methods (3T3 NRU and NHK NRU test methods) validated by NICEATM and ECVAM (ICCVAM 2006a, b, c) were most effective at predicting the toxicity (i.e., LD_{50}) of moderately toxic chemicals. However, because the default starting dose is also moderately toxic (175 mg/kg), no animal savings were realized for chemicals with similar toxicity. The number of animals needed based on the predicted starting dose was similar to the number needed when using the default starting dose. Some group participants noted that exposure needs to be factored into any testing strategies. Testing would not be needed if the potential exposures of humans, animals, or the environment were judged *de minimis* (i.e., too small for concern).

Moreover, the cost and time required for *in vitro* testing may become a practical issue for industry when such test methods do not provide for substantial animal savings. The observation was made regarding what was described as "the size of the prize." Alternative *in vivo* protocols have already reduced and refined animal use in acute toxicity testing. Longer-term *in vivo* test methods use far more animals and have far greater opportunities for reduction and refinement of animal usage.

The group recognized that larger organizations might voluntarily use *in vitro* test methods in their acute toxicity testing program for the public relations value. Contract laboratories will implement the procedures for their competitive value in approaching clients. Whereas suppliers of consumer products may do little or no *in vivo* testing, their raw material suppliers have probably provided them with the *in vivo* data needed to satisfy regulatory requirements. Pharmaceutical firms also perform some *in vitro* testing as part of acute toxicity data development.

The group consensus was that the availability of a validated *in vitro* test method for acute toxicity and the inclusion of such a test in a formal testing guideline would facilitate its widespread use. Group participants suggested, from their experience in the United States and abroad, that regulatory agencies will accept for consideration data from a well-developed and well-thought-out alternative method. In general, however, the testing programs of industry tend to follow the most efficient track (i.e., use the standard *in vivo* test methods that regulatory agencies assuredly accept).

Industry is also concerned about how *in vitro* cytotoxicity test data might be interpreted by regulators. The group noted their considerable comfort with the regulatory interpretation of data from current *in vivo* methods.

10.2 Submission of *In Vitro* and *In Vivo* Data to ICCVAM

Because they understood the value of parallel testing with *in vivo* and *in vitro* methods, the group indicated their willingness to provide such data to ICCVAM in order to advance the development and validation of more predictive *in vitro* test methods. However, they explicitly stated that certain guarantees (e.g., assurance that unfavorable *in vitro* data in the presence of favorable *in vivo* data would not be used in any regulatory action) and incentives (e.g., grants for development of methods, tax incentives, expedited regulatory review) would likely be necessary to encourage industry to share data. It is not hard to visualize that a more sensitive endpoint might give rise to more stringent regulation. Many private companies would require "safe harbor" agreements, which prevent regulatory actions based on the submitted data. Only those entities with a long history of conventional testing and a full understanding of their products' potential hazards might participate in the absence of such prior agreements.

It should also be recognized that companies are likely to consider any mechanistic information to be proprietary. Toxicologists increasingly favor mechanistic studies and their interpretation. Efforts might be in place to use any mechanism-related cage-side observations and measurements to assist in the development of more humane endpoints in systemic toxicity testing. Findings during cage-side observations are often similar for different modes of toxic action and would therefore not help in discerning possible mechanisms of action. In addition, any such observations made by a contract laboratory would be the property of the test sponsor, who might be less inclined to share data than the staff conducting the test. In

routine acute toxicity tests, cage-side observations are not particularly thorough. More thorough observations might be performed if prior information indicated that there could be significant toxicity issues for the chemical under study.

During the discussion of sharing data with ICCVAM or regulatory agencies, the group noted that currently the cost-benefit ratio does not justify using the validated *in vitro* methods to set starting doses for acute oral toxicity tests because the number of animals used is already at a minimum. They also reiterated the concerns about the possibility of more stringent regulation. Data "call-ins" are not often helpful because the findings are based on protocols that are not completely comparable. Acute toxicity data constitute valuable proprietary information that companies are not likely to share. Participants also noted that the national and international requirements for data submission differ among the regulatory agencies because of their specific statutory mandates.

There was, however, enthusiasm for creation of a public/private consortium that would facilitate data collection and submission. Such a consortium could set priorities, define the level of detail necessary for data submissions, work to standardize protocols, emphasize the value of better science in providing more confident regulatory decisions, and possibly do some cost sharing in the process. One example is a biomarkers consortium (Predictive Safety Testing Consortium[43]) formed under the FDA Critical Path Initiative, which consists of representatives from the FDA, government research groups, academia, and industry. The group suggested that the International Life Sciences Institute (ILSI) could assist with an effort like this. In short, setting up a consortium benefits business. The costs of research, development, and validation are significant (e.g., ECVAM commits $60-80 million per year to development of alternatives).

[43] http://www.fda.gov/oc/initiatives/criticalpath/projectsummary/consortium.html

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11.0 Workshop Outcomes

Workshop participants recommended the identification and characterization of the following key pathways for the manifestation of acute systemic toxicity: general cellular function, aerobic metabolism, xenobiotic metabolism, sodium/potassium ATP-ase pump, oxidative stress, neuronal transmission, cardiac conduction, receptor activity, and immune response. Workshop participants recommended the development and use of noninvasive data collection devices/procedures to obtain detailed physiological data from acute animal toxicity tests. These methods will help identify the modes/ mechanisms of acute systemic toxicity for various types of chemicals. Such methods will also facilitate the development of biomarkers for pathophysiological effects. Sample collection and processing procedures, as well as biomarker detection and quantification, should be standardized in order to facilitate consistent interpretation of biomarker measurements for the key toxicity pathways.

In order to model key toxicity pathways, a broad array of *in vitro* test methods must be developed to screen chemicals for various modes/mechanisms of acute chemical action. Alternative *in vitro* test systems to model key pathways for multiple levels of organization, whole animal, whole organs, and cellular pathways should be developed. Mechanistic information from *in vivo* studies and *in vitro* models can be used to establish computational approaches to predict *in vivo* acute toxicity. For example, tools for determining *in vitro* and *in vivo* toxicokinetics must be developed for dose-response assessments and various associated extrapolations (e.g., *in vivo* to *in vitro*, interspecies). Expert panels should be convened to address the issues of design and use of appropriate biomarkers to identify/assess acute systemic toxicity, development of cell lines for mechanistic *in vitro* test methods, and implementation of the appropriate test methods and data analysis procedures.

Identifying the mechanisms of acute chemical toxicity may also lead to the development of earlier, more humane endpoints for acute systemic toxicity tests. Workshop participants recommended the FDP as the preferred acute oral toxicity test method because the primary endpoint, evident toxicity, is more humane than death, the primary endpoint of other acute systemic toxicity test methods. However, the FDP is not used widely because it does not meet the needs of some regulatory agencies and because there is a lack of clear guidance on the clinical signs that constitute *evident toxicity*. To encourage wider use of the FDP, objective criteria to characterize evident toxicity should be developed. Biomarkers such as behavioral observations, body temperature, body weight, and feed and water consumption were suggested as measurements that should be considered in order to determine which measurements are sufficiently predictive of evident toxicity.

Industry should be encouraged to use *in vitro* toxicity test methods and submit alternative and standard toxicity test data to government agencies to further the development of *in vitro* test methods. To facilitate such data sharing, “safe harbor” agreements, which prevent regulatory actions based on the submitted data, should be arranged with companies willing to share data. Additionally, the creation of a public/private consortium that would facilitate data collection and submission should be considered.

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