

Chapter 4: Acute Lethal Toxicity

Introduction

Acute systemic toxicity testing is conducted to determine the hazard potential of a single exposure to a chemical or product (by the oral, dermal or respiratory routes). The assessment of lethality, which forms part of acute systemic toxicity testing, has conventionally relied on the *in vivo* measurement of the median lethal dose (LD50), i.e. the dose of a test chemical that kills 50% of the animals in the experimental group. During acute toxicity testing, non-lethal endpoints can also be assessed, to identify specific target-organ and target-system toxicities.

The LD50 value is used as a reference value to classify chemicals on the basis of acute toxic hazard, and to define appropriate doses for use in other *in vivo* tests. The classical LD50 test attracted much criticism for both scientific and animal-welfare reasons (1), and was modified to become an approximate LD50 test or a limit test. More-recently, reduction and refinement alternatives have been accepted at the OECD level (2–4).

The main question being addressed in the development and validation of alternatives to acute lethality testing is whether QSAR and/or *in vitro* methods can provide a reliable basis for acute hazard classification and/or a valid estimate of appropriate starting doses for non-lethal *in vivo* studies.

In Vitro Methods for Acute Lethal Toxicity

Acute systemic toxicity *in vivo* can result from toxicity at the cellular level, i.e. cytotoxicity, which in turn results from interference with structures and/or properties essential for cell survival, proliferation and/or function. These effects can involve, for example: the integrity of membranes and the cytoskeleton; metabolism; the synthesis and degradation or release of cellular constituents or products; ion regulation; and cell division. It is useful to distinguish between three types of cytotoxicity (5). *Basal (or general) cytotoxicity* involves one or more of the above-mentioned structures or processes, when all of the cell types studied show similar sensitivities. *Selective (or cell-specific) cytotoxicity* occurs when some types of differentiated cells are more sensitive to the effects of a particular toxicant than others; for example, as a result of biotransformation, binding to specific receptors, or uptake by specific mechanisms. *Cell-specific function toxicity* occurs when the toxicant affects structures or processes that may not be critical for the affected cells them-

selves, but which are critical for the organism as a whole. For example, such toxicity can involve effects on cell–cell communication, through the synthesis, release, binding and degradation of cytokines, hormones and transmitters, or on specific transport processes.

A large number of *in vitro* cytotoxicity tests have been developed, employing a variety of cell lines and endpoint measurements (Table 4.1).

For example the FRAME cytotoxicity (BCL-D1 kenacid blue) assay went through a blind trial involving four laboratories and 50 chemicals (6). Subsequently, the same chemicals were put through the 3T3 kenacid blue assay (7). These studies demonstrated the reproducibility of the cytotoxicity assays concerned.

The focus of this chapter is on the assessment of basal cytotoxicity and its association with acute lethal toxicity. The rationale for relating basal cytotoxicity to acute lethal toxicity is that the latter often arises from critical organ failure, which results from loss of cell viability and function. No basal cytotoxicity tests have been through a formal validation study for the specific purpose of replacing the rodent LD50 test, although a number of *in vitro–in vivo* correlation studies have indicated that IC50 values can reliably be used to predict LD50 values with a reasonable degree of precision (Table 4.2). An example of such a study on 59 chemicals is provided by Clothier *et al.* (8).

In Vitro Cytotoxicity Databases

Huge amounts of *in vitro* cytotoxicity data exist, some of which have been incorporated into the following databases, which are therefore useful resources for test development and validation activities.

1. The Halle and Gores Registry of Cytotoxicity, which contains 1912 individual IC50 values for 347 chemicals. The IC50 values were obtained from multiple reports in the literature, averaged for each chemical, and then paired with acute oral LD50 values for the rat, and with intraperitoneal (i.p.) LD50 values for the mouse, obtained from the National Institute for Occupational Safety and Health (NIOSH) Registry of Toxic Effects of Chemical Substances (RTECS). The details of this registry were originally published in German (9), but have been translated into English under the auspices of ECVAM and ZEBET, and will shortly be published in *ATLA*.

Table 4.1: An overview of *in vitro* assays for cytotoxicity/acute lethal toxicity

Endpoint	Measurement	Cell line(s)	Study
Cell growth/ viability	ATP content or leakage	ELD cells (mouse); erythrocytes (mouse); LS-L929 cells (mouse); hepatocytes (rat); spermatozoa (bovine); HL-60 cells (human)	Clemedson <i>et al.</i> (11)
	Cell morphology	C9 cells (rat); hepatocytes (rat); L2 cells (rat); MDBK cells (bovine); Chang liver cells (human); HeLa cells (human); McCoy cells (human); WI-1003/Hep G2 cells (human)	Clemedson <i>et al.</i> (11)
	Chromium release	LS-L929 cells (mouse)	Clemedson <i>et al.</i> (11)
	Creatine kinase activity	Muscle cells (rat)	Clemedson <i>et al.</i> (11)
	Haemolysis	Erythrocytes (human)	Clemedson <i>et al.</i> (11)
	Killing index (sic)	SQ-5 cells (human)	Clemedson <i>et al.</i> (11)
	LDH release	3T3 cells (mouse); hepatocytes (rat, human); Hep 2 cells (human); Hep G2 cells (human); lymphocytes (human); SQ-5 cells (human); corneal cells (human)	Clemedson <i>et al.</i> (11)
	Neutral red uptake	3T3 cells (mouse); L929 cells (mouse); V79 cells; NB41-A3 cells (mouse); BHK cells (hamster); hepatocytes (rat, human); HeLa cells (human); Hep 2 cells (human); keratinocytes (human)	Clemedson <i>et al.</i> (11) Start <i>et al.</i> (22), Riddell <i>et al.</i> (23, 24), Clothier <i>et al.</i> (25), Spielmann <i>et al.</i> (26)
	Neutral red release	Rabbit corneal fibroblasts or mouse embryonic fibroblasts or normal human epidermal keratinocytes	Reader <i>et al.</i> (27)
	Plating efficiency	HeLa cells (human)	Clemedson <i>et al.</i> (11)
	Viable cell count	LS-L929 cells (mouse); polymorphonuclear leukocytes (human)	Clemedson <i>et al.</i> (11)
	Cell cycle distribution	Daudi cells (human); RERF-LC-AI cells (human)	Clemedson <i>et al.</i> (11)
	Glucose consumption	Muscle cells (rat)	Clemedson <i>et al.</i> (11)
	Macromolecule content	HTC cells (rat); Hep G2 cells (human)	Clemedson <i>et al.</i> (11)
	MTT metabolism	3T3 cells (mouse); L929 cells (mouse); NG108-15 cells (mouse, rat); V79 cells (hamster); hepatocytes (rat, human); Detroit 155, DET dermal fibroblast (human); FaO cells (human); HFL1 cells (human); 3D Skin ² , Dermal Model ZK1100 keratinocytes (human); lymphocytes (human); RERF-LC-AI cells (human); WS1 cells (human)	Clemedson <i>et al.</i> (11)

LDH = lactate dehydrogenase; MDBK = Madin–Darby bovine kidney; NK = natural killer.

Table 4.1: continued

Endpoint	Measurement	Cell line(s)	Study
Cell growth	pH change	L2 cells (rat); Chang liver cells (human); HeLa cells (human); WI-1003/Hep G2 cells (human)	
	Total protein content	3T3 or 3T3-L1 cells (mouse); Hepa-1c1c7 (mouse); L929 cells (mouse); V79 cells (hamster); hepatocytes (rat); PC12h cells (rat); LLC-PK1 cells (pig); HeLa cells (human); MRC-5 cells (human); NB-1 cells (human); Chinese hamster V79 cells	Clemedson <i>et al.</i> (11) Spielmann <i>et al.</i> (26) Reader <i>et al.</i> (27) Hulme <i>et al.</i> (28) Fry <i>et al.</i> (29)
	Tritiated-proline uptake	L2 cells (rat)	Clemedson <i>et al.</i> (11)
	Tritiated-thymidine incorporation	Peripheral lymphocytes (human)	Clemedson <i>et al.</i> (11) Spielmann <i>et al.</i> (26)
Specialised function effects	Cell resting membrane potential	NG108-15 (mouse, rat)	Clemedson <i>et al.</i> (11)
	Chemotactic peptide-stimulated chemotaxis/locomotion	Polymorphonuclear leukocytes (human)	Clemedson <i>et al.</i> (11)
	Ethoxycoumarin deethylase (EOD) activity	Hepatocytes (rat)	Clemedson <i>et al.</i> (11)
	Inhibition of NK cell-mediated cytotoxicity	Natural killer cells (human)	Clemedson <i>et al.</i> (11)
	Intracellular glycogen content	Hepatocytes (rat)	Clemedson <i>et al.</i> (11)
	Motility or velocity	Spermatozoa (bovine)	Clemedson <i>et al.</i> (11)
	Spontaneous contractility	Muscle cells (rat)	Clemedson <i>et al.</i> (11)

LDH = lactate dehydrogenase; NK = natural killer.

- The FRAME database, which contains IC20, IC50 and IC80 values for over 250 chemicals tested on mouse 3T3 cells with the kenacid blue assay for total protein content. Fifty-nine IC50 values have been compared with mouse i.p. and rat oral LD50 data.
- The Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) database, which contains data for 50 chemicals and 69 different assay methods (10). In the MEIC study, the data were compared with human lethal blood concentrations (11, 12).
- The MEMO (the MEIC monographs on time-related human lethal blood concentrations) database, which supplements the MEIC database with data for an additional 25 chemicals.

The MEIC and EDIT projects

The results of the MEIC study demonstrated that human basal cytotoxicity tests are relevant for predicting the human acute toxicity of chemicals. In addition, the results show that other important toxic mechanisms exist, which might only be measured by

supplementary *in vitro* toxicity tests, and that modelling of human toxicity is improved by the additional use of toxicokinetic data.

The EDIT (Evaluation-guided Development of New *In Vitro* Test Batteries) project was initiated as a follow-up to the MEIC study in 1998 (13). The EDIT project was established as a 6-year effort by international cytotoxicology laboratories, with the aim of developing new *in vitro* tests for toxicity and toxicokinetics, for incorporation into optimised test batteries for acute and chronic systemic toxicity.

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

The status of several major international *in vitro* initiatives directed toward reducing the use of laboratory animals for acute toxicity testing was reviewed in October 2000, at the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity (14). The workshop participants concluded that none of the proposed *in vitro* models had been validated for reliability and relevance in any formal studies. The participants made recommendations for future research and development, and for validation efforts in the field of *in vitro* systemic toxicity testing.

One of the workshop's recommendations for reducing and refining the use of animals for lethal-

ity assays in the near-term, was the publication of guidance for using *in vitro* cytotoxicity assays to estimate starting doses for acute oral lethality assays (15). The use of such assays could reduce animal testing by obviating the need to perform dose range-finding experiments in animals.

As a follow-up to the international workshop, there is to be a validation study on the use of murine 3T3 cells and human keratinocytes for predicting acute systemic toxicity. The study will initially lead to the definition of a standardised protocol (including positive and negative controls), and will define limiting parameters for establishing the reliability of the method (16, 17). The study will be used to evaluate the capacity of the basal cytotoxicity assays to predict: a) rat oral and/or mouse i.p. LD50 values; and b) human lethal blood concentrations. To meet these goals, this collaborative validation study is currently being sponsored and managed by ICCVAM and ECVAM.

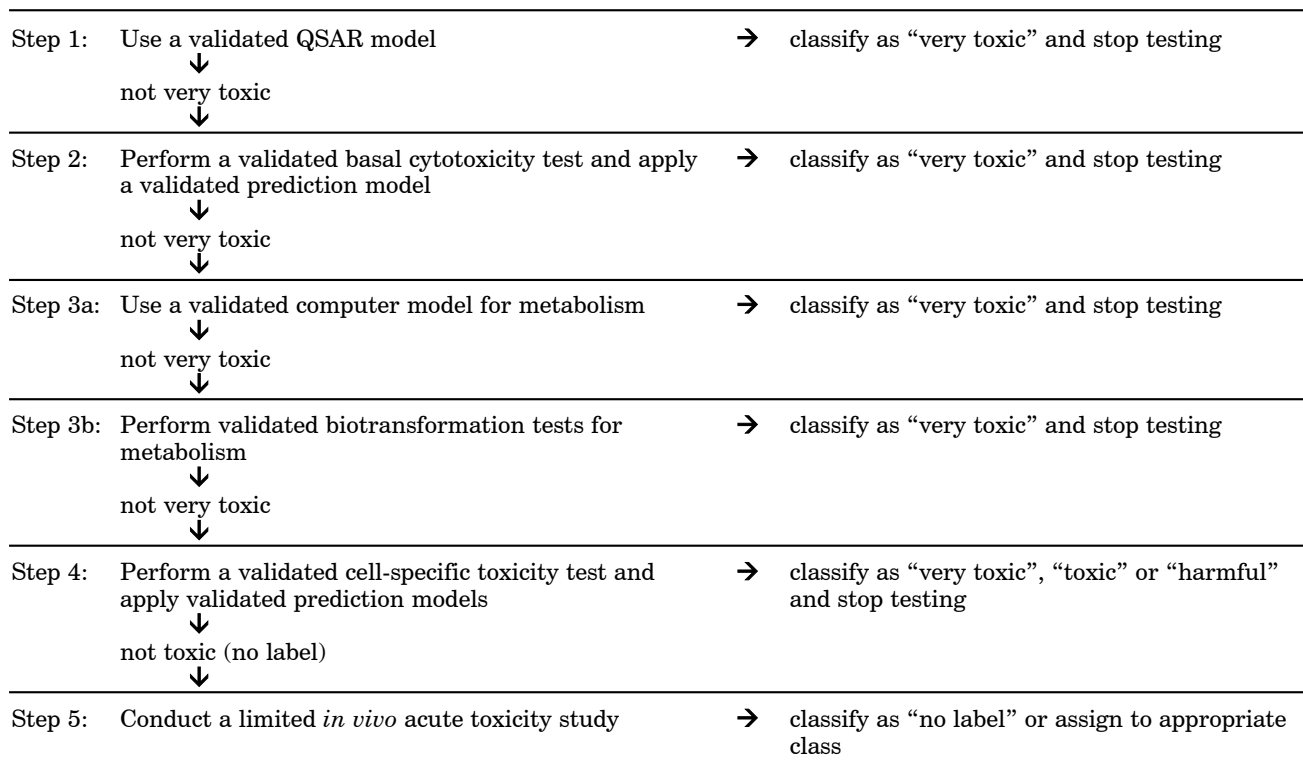
Structure-Activity Relationships for Acute Lethal Toxicity

QSARs for acute lethal toxicity to rodents have been reviewed by Cronin & Dearden (18). More recently, Johnson & Jurs (19) used regression analysis and neural networks to predict the rodent

Table 4.2: Acute systemic toxicity: examples of *in vitro/in vivo* studies and/or approaches

	References
Studies conducted by FRAME and partners	Knox <i>et al.</i> (6) Clothier <i>et al.</i> (7, 8) Riddell <i>et al.</i> (23, 24) Hulme <i>et al.</i> (28) Fry <i>et al.</i> (29) Balls & Clothier (30) Fentem <i>et al.</i> (31)
The MEIC (Multicentre Evaluation of <i>In Vitro</i> Cytotoxicity) study	Clemedson & Ekwall (32) Ekwall <i>et al.</i> (33)
The ECITTS (ERGATT/CFN Integrated Toxicity Testing Strategy) study	De Jongh <i>et al.</i> (34)
Japanese Society of Alternatives to Animal Experiments (JSSAE) activities	Ohno <i>et al.</i> (35–37)
Testing framework proposed under the auspices of SGOMSEC	Curren <i>et al.</i> (38)
General form of a testing strategy proposed during an ECVAM workshop	Seibert <i>et al.</i> (5)
The ZEBET approach for predicting starting doses for acute <i>in vivo</i> testing	Halle & Goeres (9) Spielmann <i>et al.</i> (26) Halle <i>et al.</i> (39)

SGOMSEC = Scientific Group Methodologies for the safety Evaluation of Chemicals

Figure 4.1: A tiered testing strategy for acute lethal toxicity

To interpret the results of step 3 (biotransformation assessment) in terms of an LD50 estimate, it would be necessary to apply a suitable prediction model or biokinetic model.

Animal testing (step 5) should not be conducted as a matter of routine, but only in situations where a satisfactory classification could not be derived by the application of steps 1–4.

QSAR = quantitative structure–activity relationship.

toxicities of a diverse set of 115 anilines. In a study by Wang *et al.* (20), the LD50 values of over 46,000 single organic substances taken from the RTECS database were analysed, on the basis of which a number of structural alerts (i.e. fragments associated with toxicity) were identified. In a study by Calleja *et al.* (21), QSARs for acute lethal toxicity to humans were investigated by using data for 38 structurally diverse chemicals, selected from the 50 priority chemicals identified in the MEIC programme (see below). The most predictive physico-chemical properties were the logarithmic octanol–water partition coefficient (log P) and the heat of formation, along with additional properties that describe molecular size and electronic properties. The results of such studies are promising, but more work will be needed to assess the extent to which QSAR models can be used to replace and/or reduce *in vivo* testing. One possibility is that QSAR models could be used to prioritise the *in vitro* testing of a number of chemicals, or to decide on the need to conduct *in vitro* tests for a particular chemical.

A Tiered Testing Strategy for Acute Lethal Toxicity

The outline of a testing strategy for the classification and labelling of chemicals according to their acute systemic toxicity was proposed in ECVAM workshop report 16 (5), and is developed further in Figure 4.1. The first step is based on the use of QSARs to predict acute toxicity. If the result of this step were to indicate that the compound should be classified as “very toxic”, no testing would need to be performed. The second step is based on a basal cytotoxicity test, such as the 3T3 neutral red uptake (NRU) test. If the result of the second step were to indicate that the compound should be classified as “very toxic”, no further testing would need to be considered. If not, the third step, based on the assessment of biotransformation, would have to be performed. Again, if the result were positive, testing would be stopped at this stage. If not, the third step, based on the use of cell-specific toxicity tests, would have to be performed. Finally, the chemical would be classified as “very toxic”, “toxic”, “harmful” or “no label”, according to

the lowest EC50 value determined at any of the three testing levels. If all of the results indicated that the chemicals should be assigned to the lowest toxicity class (i.e. "no label"), a limited *in vivo* study might need to be carried out, to confirm the absence of significant acute lethal potential.

Acute Lethal Toxicity: Summary, Conclusions and Recommendations

Standardised basal cytotoxicity tests, such as the 3T3 NRU assay, are already widely used for non-regulatory purposes, and could be used immediately for priority setting among chemicals, and for establishing the starting dose for *in vivo* acute toxicity testing.

Future activities in this area should aim to: a) reduce and replace the use of the rodent test for determining LD50 values, through the development and validation of QSARs and cytotoxicity tests; b) ensure the widest possible use of QSARs and cytotoxicity tests as a means of estimating LD50 values; and c) promote the development and validation of a tiered testing strategy for acute systemic toxicity.

The prospects outlined below focus on the use of QSARs and basal cytotoxicity tests for predicting acute lethal toxicity. The uses of alternative methods for predicting metabolism (Step 3 in Figure 4.1) and organ-specific toxicities (Step 4 in Figure 4.1) are addressed in Chapters 7 and 8, respectively.

Short-term prospects

1. Development of a comprehensive database containing *in vitro* results (for example, IC50 values) and corresponding *in vivo* LD50 values (rat oral or mouse i.p., and/or human lethal blood levels), to allow *in vitro*-*in vivo* comparisons to be made. ECVAM has initiated the development of such a database.
2. Validation of basal cytotoxicity assays for predicting: a) rat oral and/or mouse i.p. LD50 values; and b) human lethal blood concentrations. Such a study has been initiated under the auspices of ICCVAM and ECVAM.
3. Development of QSAR models for predicting *in vitro* cytotoxicity.

Medium-term prospects

1. Validation of QSAR models for predicting *in vitro* cytotoxicity.
2. Replacement of the need for *in vivo* testing for classifying chemicals on the basis of acute toxic

potential, as well as for prioritising and dose-setting for other kinds of studies.

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