Acute Oral Toxicity

Erik Walum

Cell and Molecular Biology, Research Department, Pharmacia & Upjohn, Stockholm, Sweden

The purposes of acute toxicity testing are to obtain information on the biologic activity of a chemical and gain insight into its mechanism of action. The information on acute systemic toxicity generated by the test is used in hazard identification and risk management in the context of production, handling, and use of chemicals. The LD_{50} value, defined as the statistically derived dose that, when administered in an acute toxicity test, is expected to cause death in 50% of the treated animals in a given period, is currently the basis for toxicologic classification of chemicals. For a classical LD_{50} study, laboratory mice and rats are the species typically selected. Often both sexes must be used for regulatory purposes. When oral administration is combined with parenteral, information on the bioavailability of the tested compound is obtained. The result of the extensive discussions on the significance of the LD_{50} value and the concomitant development of alternative procedures is that authorities today do not usually demand classical LD_{50} tests involving a large number of animals. The limit test, the fixed-dose procedure, the toxic class method, and the up-and-down methods all represent simplified alternatives using only a few animals. Efforts have also been made to develop *in vitro* systems; e.g., it has been suggested that acute systemic toxicity can be broken down into a number of biokinetic, cellular, and molecular elements, each of which can be identified and quantified in appropriate models. The various elements may then be used in different combinations to model large numbers of toxic events to predict hazard and classify compounds. — *Environ Health Perspect* 106(Suppl 2): 497–503 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-2/497-503walum/abstract.html*

Key Words: acute toxicity, LD_{50}, alternatives, limit test, fixed dose procedure, toxic class method, up-and-down method, human toxicity, cytotoxicity, MEIC program

Introduction

This paper gives a short review of methods for acute toxicity testing with the emphasis on the median lethal dose (LD_{50}) test and alternative procedures that fulfill the requirement of reducing, refining, or replacing the use of animals in toxicity testing (the 3R principle). Furthermore, this review mirrors the current discussion on the usefulness of different procedures for acute toxicity testing and surveys the conclusions of various panels, commissions, and groups.

Acute toxicity is usually defined as the adverse change(s) occurring immediately or a short time following a single or short period of exposure to a substance or substances or as adverse effects occurring within a short time of administration of a single dose of a substance or multiple doses given within 24 hr. An adverse effect is "any effect that results in functional impairment and/or biochemical lesions that may affect the performance of the whole organism or that reduce the organ's ability to respond to an additional challenge" (*1*). Consequently, a chemical that enters the organism via the oral route during a restricted time and produces any adverse effect with little delay is orally and acutely toxic. However, the term acute oral toxicity is most often used in connection to lethality and LD_{50} determinations.

Studies of acute systemic toxicity attempt to determine the dose-dependent adverse effect that may occur and various appropriate data may be collected when determining the comprehensive acute toxicity profile of a substance. This may include the incidence of lethality. It has been claimed that when properly performed and closely observed, an acute toxicity test can give more information about the biologic properties of a chemical compound than any other single test, and even if the incidence of lethality were never computed as a consequence of such a test, one would only have lost a small proportion of the available information (*2*). If the dose-dependent lethality incidence is determined in a precise manner, it is usually expressed as an LD_{50}. This is defined as the statistically derived dose that, when administered in an acute toxicity test, is expected to cause death in 50% of the treated animals in a given period (*3*). For a classical LD_{50} study, laboratory mice and rats are species typically selected. Often the use of both sexes and a route of exposure anticipated to be the most probable route of exposure for humans are necessary for regulatory purposes. When oral administration is combined with parenteral, information on the bioavailability of the tested compound is obtained.

The purposes of acute toxicity testing are to obtain information on the biologic activity of a chemical and gain insight into its mechanism of action. Long-term studies usually start with a dose-finding exercise under acute conditions. Furthermore, the information on acute systemic toxicity generated by the test is used in hazard identification and risk management in the context of production, handling, and use of chemicals. The LD_{50} value (precise or approximate) is currently the basis for toxicologic classification of chemicals and is thus required by government authorities in different situations. The dosed animals are closely observed during the first 24 hr and then day by day for as long as 2 weeks and changes in appearance and behavior are noted. A large number of clinical signs can be used to characterize acute systemic toxicity and describe its progression (*1*). There is some question concerning the use of extensive pathologic assessment as part of an acute study. However, gross necropsies are the minimum requested by most governmental regulatory bodies, as are weight determinations prior to dosing and after 1 and 2 weeks. Determination of a precise LD_{50} value within an acute toxicity study is motivated mainly by the authorities' different requirements for classification of chemicals. In the past the LD_{50} has been used for industrial chemicals as the basis for the various toxicity classification systems that are or have been in operation

This paper was prepared as background for the 13th Meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC): Alternative Testing Methodologies held 26–31 January 1997 in Ispra, Italy. Manuscript received at *EHP* 9 May 1997; accepted 9 December 1997.

Address correspondence to Prof. E. Walum, Cell and Molecular Biology, Research Department, Pharmacia & Upjohn, SP12/5, S-112 87 Stockholm, Sweden. Telephone: 46 8 695 72 54. Fax: 46 8 695 40 87. E-mail: erik.walum@eu.pnu.com

Abbreviations used: ECVAM, the European Centre for the Validation of Alternative Methods; IC_{50}, median inhibitory concentration; LC_{50}, median lethal concentration; LD_{50}, median lethal dose; MEIC, Multicenter Evaluation of *In Vitro* Cytotoxicity; OECD, Organisation for Economic Co-operation and Development; PLS, partial least square modeling with latent variables.

throughout the world (*3*). At present the following chemical labeling and classification of acute systemic toxicity based on oral LD_{50} values are recommended by the Organisation for Economic Co-operation and Development (OECD): very toxic, ≤5 mg/kg body weight; toxic, >5≤50 mg/kg; harmful, >50≤500 mg/kg; and no label, >500≤2000 mg/kg.

The absolute LD_{50} value for a compound varies among different laboratories, and these variations have been attributed to differences in e.g., protocol details, animal strains, caging, and test-chemical source. LD_{50} and alternative methods for testing of acute toxicity have been discussed extensively in different international forums during the last two decades (*1,3–7*). In particular, the necessity to determine a high statistical accuracy in the LD_{50} value has been questioned. Oliver (*3*) has summarized the two major points of criticism clearly.

First, he states that the median lethal dose is not an absolute value but is an inherently variable biologic parameter that cannot be compared to constants such as molecular weight or melting point. This means that an LD_{50} cannot be described in terms of accuracy, only of precision. In addition, the precision is relevant only for the experiment from which the LD_{50} was derived and does not increase the probability that in subsequent experiments the LD_{50} will be identical or even similar (*3*).

Oliver's second point is that the value refers only to mortality and is illustrative of no other clinical expression of toxicity. The representation of a compound's acute systemic toxicity as its LD_{50} is considered a descriptive limitation that outweighs the otherwise obvious attraction of converting a sometimes complex picture into a simple numerical index that can be subsequently utilized by those who have a limited understanding of toxicology (*3*).

The result of the extensive discussions on the significance of the LD_{50} value and the concomitant development of alternative procedures is that authorities today do not usually demand classical LD_{50} tests involving a large number of animals, i.e., 10 animals per group, 10 doses, and both sexes—a total of 200 animals. On the contrary, OECD guidelines 401 (*8*), 420 (*9*), and 423 (*10*) describe well established, validated alternative methods that reduce animal suffering and/or use much fewer animals than the classical method (*4*). Using one of these tests, the number of animals required for determining an LD_{50} value can be reduced by one order of magnitude. However, these methods can be considered adaptations to the shortcomings of LD_{50} determinations rather than attempts to improve the scientific value of quantitative acute systemic toxicity studies. Efforts have also been made to combine a more scientific view on acute toxicity testing with the development of alternative methods. One example is the idea that acute systemic toxicity can be broken down into a number of biokinetic, cellular, and molecular elements, each of which can be identified and quantified in appropriate model systems. The various elements can then be used in different combinations to model large numbers of toxic events. Whereas the standard animal model is fixed, the integrated *in vitro* system can be optimized for a certain class of compounds, a specific mechanism of action, or a particular target organism. The programs of the Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC) (*11–13*) and the integrated toxicity testing scheme of the European Research Group for Alternatives in Toxicity Testing/Swedish National Board for Laboratory Animals (*14*) address these theories, and the use of *in vitro* methods for the classification and labeling of chemicals has been proposed by Seibert et al. (*15*) and further reviewed by Seibert et al. (*7*). In summary, the following scheme is proposed (Figure 1): as a first step, a test for general cytotoxicity activity would be conducted. The results would be converted to an equivalent effective body dose by means of a prediction model based on physicochemical data and basic assumptions about toxicokinetic parameters *in vivo* and *in vitro*. If the results were positive, that is, if they indicated that the compound should be classified as very toxic, no further testing would be needed. If the results were negative, Stage 2 testing would be performed. Again, if the results were positive (that is, classification in the highest toxicity class was indicated), testing would be stopped at this stage. If not, Stage 3 testing would be performed. Finally, the chemical would be classified as very toxic, toxic, harmful, or no label, according to the lowest median effective concentration value determined at any of the three testing levels. If the results indicated that the chemical should be assigned to the lowest toxicity class, a limited *in vivo* study might be needed.

Limit Test

The limit test, described in OECD guideline 401 (*8*), is a restricted version of an acute toxicity test and is used after a range-finding study or a literature search has made it likely that the chemical is of low toxicity. Several measures have been taken to reduce the number of animals needed for

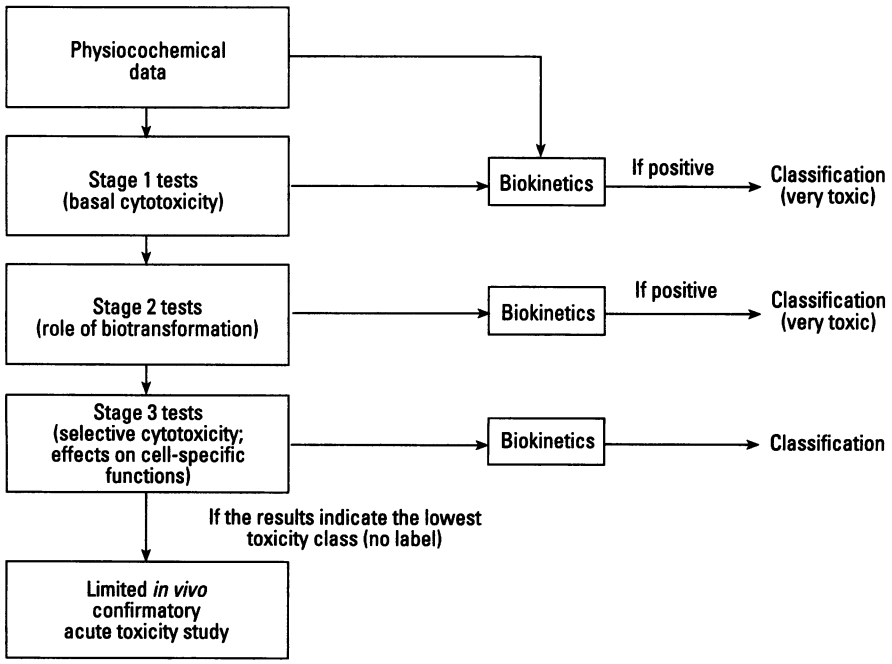

Figure 1. A three-stage tiered scheme for *in vitro* testing for acute systemic toxicity and for the classification and labeling of chemicals [adapted from Seibert et al. (7)].

a complete study. The limit test ideally involves the use of three groups (three dose levels; the highest dose limit is set to 2 g/kg and is the initial starting dose) of five animals of a single sex, with assessment of toxicity in the second sex in a separate study. In this second study five animals are used at a single dose level to establish the absence of any significant sex difference in toxicity.

Fixed-Dose Procedure

The fixed-dose procedure was first proposed by the British Toxicology Society in 1984 (*16*). After an international validation study involving 20 reference chemicals tested in 31 laboratories from 11 different countries (*17*), the procedure was incorporated into the OECD guidelines (guideline 420) in 1992 (*9*). The results of the validation study showed a remarkable consistency between laboratories and it was concluded that the data generated could be used both for risk assessment and ranking chemicals for classification. Further evaluation of the method has proven its usefulness (*18–21*).

The test substance is given at one of the four fixed-dose levels (5, 50, 500, and 2000 mg/kg) to five male and five female rats. The objective is to identify a dose that produces clear signs of toxicity but no mortality (*22*). Depending on the results of the first test, either no further testing is needed or a higher or lower dose is tested: If mortality occurs, retesting at a lower dose level is necessary (except if the original dose chosen is 5 mg/kg). If no signs of toxicity occur at the initial dose, it is necessary to retest at a higher dose level. The results are thus interpreted in relation to animal survival and evident toxicity (*5*) and it becomes possible to assign the chemical to one of the OECD classification categories.

Toxic Class Method

The toxic class method has been validated both nationally (*23*) and internationally (*24*). The latter study included 20 test substances and nine laboratories in five countries. The method is described in OECD guideline 423 (*10*) and is based on the assessment of lethality. In principle three animals are dosed with one of three fixed levels corresponding to the oral LD_{50} classification limits. The purpose of the procedure is to identify the lowest dose level that causes two or three animals to die. Three animals of one sex are dosed at the middle level. If two or three animals die, retesting is done at the lower level. When fewer than two die, the test is repeated at the same level, but with the other sex. If two or three animals die in this step, the test is repeated at the lower level, and if fewer than two die, the test is repeated at the higher level. Several studies have evaluated the method and found it a valuable alternative test for classification of chemicals (*20,25*).

Up-and-Down Method

In this type of procedure a single animal (or sometimes two to four animals) is exposed with subsequent doses adjusted up or down by a constant factor depending on the outcome of the previous dose (*26*). If an animal dies during the initial step of the test, another animal is given a dose reduced by a factor of, e.g., 1.3. If this exposure does not result in toxicity, the dose is elevated by an equivalent constant factor until five animals have been dosed or the limit dose is reached. Although time consuming, the up-and-down procedure can give good results with the use of as few as six to nine animals (*18,21*). Proposals are being circulated for acceptance of the up-and-down method into OECD guidelines (*27*).

Predictive Power of Animal Tests for Human Toxicity

The problem of extrapolation of animal data to humans is well recognized (*28*). According to Garattini (*29*) there are three main points to address: *a*) different animal species dispose of chemicals in different ways; therefore, the concept of doses (e.g., milligram/kilogram body weight) should be gradually replaced by the use of concentrations (mol/blood or tissue volume); *b*) biotransformation of chemicals may lead to metabolites with biologic activity; hence we need to know more precisely what chemical species are responsible for which toxicologic effect; *c*) equal concentrations of chemicals do not mean equal effects across animal species because the sensitivity of organs, cells, enzymes, or receptors may be different in different species. These three aspects must be considered in relation to other factors capable of modulating toxic effects, e.g., strain, sex, age, concomitant pathology, hereditary defects, and previous or concomitant exposure to other chemicals.

The MEIC program was instigated by the Scandinavian Society of Cell Toxicology to investigate the relevance of *in vitro* cytotoxicity for the prediction of human acute and sublethal toxicity, subacute toxicity, organ-specific toxicity, and local irritance. The first 50 reference chemicals (Table 1) were chosen by the Swedish Poison Information Centre because they have known lethal doses and blood concentrations in humans (clinical and/or autopsy data) plus good animal (rat and mouse) LD_{50} data. One of the first priorities of the MEIC program was to use this information to investigate the predictivity of rat and mouse lethal doses for human lethal dosage to be used as reference for the later *in vitro* prediction of human toxicity (*11*). A preliminary calculation involving 30 MEIC chemicals showed a good correlation between animal and human data and indicated that human acute lethal doses were better predicted by the mouse than the rat data (*13*). The final MEIC evaluation, performed on all 50 chemicals, confirmed these results: rat LD_{50} values predicted human acute doses less well ($r^2 = 0.61$; Figure 2A), whereas mouse LD_{50} values correlated somewhat better ($r^2 = 0.65$; Figure 2B) (*30,31*).

The relationship between body weight, body surface area, specific surface area, and/or basal metabolic rate and oral LD_{50} has been investigated in several studies. It is evident that the predictive power of animal LD_{50} determinations for human toxicity can be improved if these parameters are accounted for in the extrapolation model [reviewed by Rhodes et al. (*1*)].

Cytotoxicity Determinations

In many investigations attempts have been made to find a correlation between simple cytotoxicity determinations *in vitro* and animal LD_{50} for many chemicals.

Despite the fact that such a relationship seems unlikely, many reports claim to have established a good correlation (e.g., *32–35*). Furthermore, these data cannot be dismissed as merely the result of good (or bad) luck, poor statistical methods, or the use of too few reference chemicals: a firm relationship between cytotoxicity *in vitro* and systemic toxicity *in vivo* does exist. A hypothesis has been presented (*12*) that could explain the existence of such a connection. The concept of basal cytotoxicity states that the mechanisms of action of most toxic chemicals are related to biochemical processes expressed in all cells. Consequently, toxic concentrations may be determined *in vitro* as well as *in vivo*.

It is often claimed that because there are so many different possible targets for a toxic compound in the whole organism, it is difficult to decide which model system to choose as an *in vitro* test. According to the notion of basal cytotoxicity, most toxic chemicals exert their ultimate action by interference with basic cellular functions.

Table 1. Oral LD_{50} doses for rat and mouse and mean oral lethal dose for humans.

Chemical	LD_{50} rat, μmol/kg	LD_{50} mouse, μmol/kg	Average dose human, μmol/kg
Paracetamol	15,899	2235	1795.16
Acetylsalicylic acid	1110	1387	2140.48
Ferrous sulfate	2100	4477	3581.02
Diazepam	1236	159	250.80
Amitriptyline	1154	505	133.80
Digoxin	36	23	0.17
Ethylene glycol	75,684	88,567	25,304.81
Methanol	175,327	227,414	48,954.16
Ethanol	153,145	74,837	102,262.16
Isopropanol	83,927	59,884	42,785.83
1,1,1,-Trichlorethane	71,964	44,978	53,544.86
Phenol	3369	2869	1669.96
Sodium chloride	51,370	68,493	39,138.94
Sodium fluoride	1238	1357	2210.88
Malathion	878	575	2248.36
2,4-Dichlorophenoxyacetic acid	1697	1570	1745.31
Xylene	40,490	19,953	8474.58
Nicotine	308	21	4.40
Potassium cyanide	77	131	43.89
Lithium sulfate	5578	10,828	9691.84
Theophylline	1354	1304	872.05
Propoxyphene HCL	223	678	65.35
Proparnolol HCL	1575	1082	241.71
Phenobarbital	697	590	479.68
Paraquat	537	644	214.71
Arsenic trioxide	74	159	20.94
Cupric sulfate	1880	2012	1163.62
Mercuric chloride	4	22	94.71
Thioridazine HCL	2445	946	168.48
Thallium sulfate	32	47	27.73
Warfarin	5	10	347.42
Lindane	261	151	835.13
Chloroform	7605	302	8375.21
Carbon tetrachloride	15,280	53,726	8545.42
Isoniazid	9117	970	1250.39
Dichloromethane	18,846	10,280	16,321.72
Barium nitrate	1358	1016	142.09
Hexachlorophene	138	165	526.63
Pentachlorophenol	101	105	107.25
Verapamil HCL	220	331	249.76
Chloroquine phosphate	1208	969	163.38
Orphenadrine HCL	834	327	163.51
Quinidine sulfate	610	676	187.42
Diphenylhydantoin	6480	595	1189.06
Chloramphenicol	7735	4641	884.02
Sodium oxalate	83,284	38,019	2665.25
Amphetamine sulfate	149	65	54.27
Caffeine	989	654	698.84
Altropine sulfate	864	674	2.47
Potassium chloride	34,853	20,107	3829.95

These functions are fundamental to all cells, regardless of whether they are in *in vivo* or *in vitro* situations. If the assumption is correct, chemically unrelated compounds with a wide range of toxic mechanisms can be tested for their acute toxicities in relatively simple cell culture systems. Support for this thought has been obtained through the interlaboratory validation programs of the Foundation for the Replacement of Animals in Medical Experiments and the MEIC, which have shown that cytotoxicity ranking is largely independent of the cell type and the end point used in the particular test. The idea of basal cytotoxicity also explains why there is not a perfect match between *in vitro* and *in vivo* data: Because the biochemical targets for toxicity are not expressed equally in all cells, the toxic concentration of compounds with a very specific action will differ between organs as well as between species. An important function of cells *in vivo* is their ability to metabolize chemicals to more- or less-toxic compounds. This function is usually expressed to a small extent in cultured cells, which results in limited activation or deactivation of test chemicals or in the *in vitro* accumulation of intermediates that do not occur *in vivo*. This fact, together with the lack of other toxicokinetic functions in the cell culture system, further adds to the restrained *in vivo–in vitro* correlation.

In traditional toxicology the organism tends to be regarded as the primary unit for the expression of toxic effects. Consequently, the onset and duration of effects, as well as the effects themselves (e.g., changes in behavior and alterations in blood flow, renal functions, and metabolic parameters), become signs of impaired homeostasis and are inevitably bound to the intact organism. In *in vitro* toxicology the cell is considered the toxicologic unit. Whole-animal reactions are thus transformed into perturbations of cellular functions. Chemicals exert their actions on different levels of cellular organization. Basal cytotoxicity represents the most fundamental and most common form of cellular toxicity. Chemicals can also interfere with differentiated functions and thereby cause organ-specific effects. Finally, at the highest level of integration, intercellular functions are impaired.

The importance of toxicokinetic considerations in the extrapolation of animal data to the human situation has already been emphasized. In the simplest case, the oral acute toxicity of a chemical is tested on one species, with the recording of symptoms; the determined lethal dose is a function of the absorption, distribution, biotransformation, excretion, and critical target organ concentration of the chemical. The value of the prediction of acute human toxicity based on this information will depend on the similarity between the test species and humans in all the events involved. The death of the animal because of one critical effect, which may or may not be relevant to humans, will prevent an evaluation of the other important effects. The modeling of quantitative toxicity *in vitro* is more scientific and less of a gamble. It involves a multiple analysis of many parameters, which can be studied separately or in combination—a procedure that can be designed to fit the questions asked. Predictions are modeled on primary data and can easily be connected directly with mechanistic studies in the same *in vitro* systems. Each piece of data can be evaluated in terms of its relevance for the corresponding human event. This results in an ability to control uncertainty of each element, and

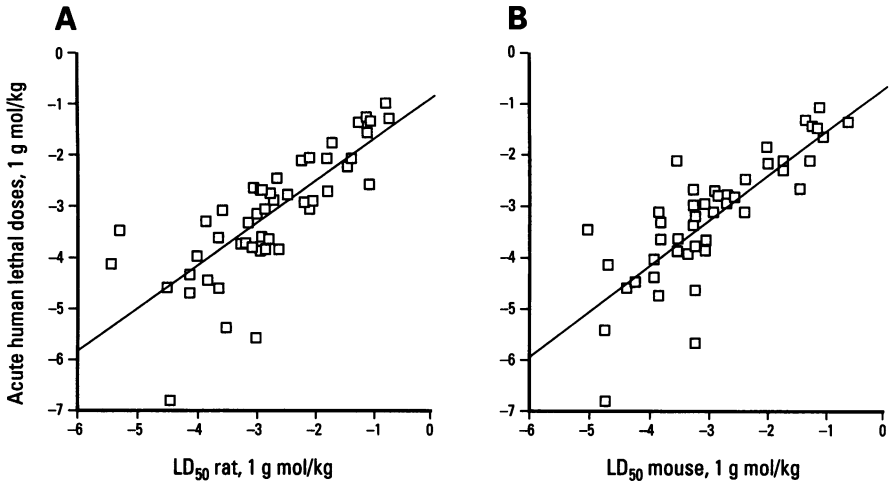

Figure 2. Linear regression analysis of the prediction of human acute toxicity by (*A*) rat and (*B*) mouse LD_{50} values [adapted from Ekwall et al. (*31*)]. *A*) $y = 0.829 \times 0.877$; $r^2 = 0.607$. *B*) $y = 0.902 \times 0.551$; $r^2 = 0.653$.

thereby the whole model. Whereas the animal model can only be improved in a limited sense, i.e., by the adoption of more sophisticated end point measurements and the alterations of single genes or the introduction of new genetic material, the *in vitro* approach can be continuously developed from primitive methods that give restricted predictions to sophisticated, more reliable models with a mechanistic basis.

Predictive Power of Cytotoxicity Tests for Human Toxicity

As part of the MEIC program and as its prime effort, approximately 30 laboratories have tested the 50 MEIC reference chemicals (Table 1) in a total of 68 *in vitro* cytotoxicity tests, resulting in median inhibitory concentrations (IC_{50} values) for different exposure and observation times, cell types, and toxicity criteria (*33*). These results have recently been analyzed (*31*) according to principles agreed on and published at the start of the program (*11*). All 68 sets of IC_{50} values were compared by multivariate partial least squares (PLS) analysis to acute lethal blood concentrations in humans. The human data were obtained from both clinical and forensic medicine handbooks. Values for peak concentrations from median lethal concentration (LC_{50}) curves over time were included. Furthermore, average values from the most predictive tests have been preliminarily compared to handbook lethal concentrations as well as to peaks and 24- and 48-hr values from the LC_{50} curves by linear regression analysis. The latter analysis also tested a previous hypothesis (*36*) on a probable extra sensitivity of the brain to basal cytotoxicity.

The following results were obtained from this first preliminary analysis: the PLS multivariate analysis indicated that most of the 68 tests provided the same information with a good total, including all tests, prediction of human handbook lethal concentrations ($r^2 = 0.73$), and peak concentrations from LC_{50} curves ($r^2 = 0.78$). Tests with human cells were the most predictive; average IC_{50} values for all ten 24-hr cytotoxicity tests with human cell lines predicted human handbook lethal concentrations reasonably well (clinical, $r^2 = 0.69$; forensic, $r^2 = 0.70$) and peaks from human LC_{50} curves similarly ($r^2 = 0.74$). When IC_{50} values for the 32 chemicals in the study, known to pass the blood–brain barrier freely, were compensated for brain sensitivity by a 10× reduction, the average human cell line cytotoxicity for all 50 chemicals predicted peak values from LC_{50} curves much better ($r^2 = 0.84$).

It may be concluded, therefore, that cytotoxicity tests with human cell lines predict human lethal blood concentrations in parity with mouse and rat LD_{50} predictions of human acute doses for the 50 reference chemicals. Moreover, the human cell line prediction may be improved by simple toxicokinetic knowledge, i.e., data on blood–brain barrier penetration, to predict human toxicity even better than the standard animal test.

Conclusions

With three well established alternative methods to the classical LD_{50} test it should be possible to abandon the latter in favor of the more humane fixed-dose procedure, acute toxicity class method, and up-and-down method (*27*). *In vitro* methods do not yet represent a realistic alternative to *in vivo* methods for regulatory acute oral toxicity determinations. The problem of translating *in vitro* toxic concentrations into *in vivo* doses requires further research and development (*7,12,15,28,29,31*). However, in cases in which acute toxicity testing is performed for reasons other than those requested by government or medical authorities, e.g., screening as part of chemical and drug development, it may be possible to replace *in vivo* studies with cytotoxicity measurements because a good picture of the significance of *in vitro* acute toxicity studies for *in vivo* toxicity is emerging.

The conclusions and recommendations of the Workshop on Acute Toxicity Testing, sponsored by the European Centre for the Validation of Alternative Methods (ECVAM) (*7*) are summarized here because they outline the state of the art. The recommendations are as follows:

- *In vitro* toxicity tests could be used in a tiered testing scheme to reduce the number of animals used and to reduce animal suffering. Such an approach is a natural progression of recent attempts to refine *in vivo* acute toxicity tests by the use of sequential dosing methods, such as the toxic class and up-and-down procedures. Furthermore, *in vitro* tests could be used in conjunction with alternative *in vivo* tests to optimize the choice of the initial dose: in these *in vivo* tests, use of the lowest number of animals possible depends on the correct choice of starting dose. This choice could be optimized by conducting appropriate *in vitro* tests prior to any animal tests that were then considered to be necessary.
- Many studies have shown good correlations between *in vitro* cytotoxicity data obtained with undifferentiated cell lines and LD_{50} data. However, acute systemic toxicity can be caused by a variety of mechanisms. Therefore, basal cytotoxicity tests are not sufficient to cover all possible mechanisms of acute toxicity and must be supplemented by more sophisticated approaches.
- It is essential in any testing scheme that a quantitative comparison be made of the concentrations of test materials that exert basal cytotoxicity as opposed to selective cytotoxic or cell-specific function effects.
- Ways must be found to take toxicokinetic parameters into account when predictions of *in vivo* data are based on *in vitro* data.

- The specific characteristics of the *in vitro* system (e.g., protein content, cell concentration, ratio of cell/membrane volumes) in use in relation to the tested chemical (e.g., pKa, lipophilicity, volatility) must be considered.
- The detection of selective toxicity requires a comparison of the toxicities of the same chemical in different cell types. A simple undifferentiated cell line is not likely to give a toxicity value representative for an effect mediated by the interference of the chemical with a very specific mechanism, nor is a highly differentiated cell, which is not representative of the cells of the target organ.
- A three-stage tiered scheme for *in vitro* testing of acute systemic toxicity can be used for the classification and labeling of chemicals (Figure 1).

The neurotoxicity of compounds that penetrate the blood–brain barrier is an important determinant of the final acute toxicity. A number of *in vitro* models for determining the ability of chemicals to penetrate the blood–brain barrier are available (*37*). Furthermore, the conclusions and recommendations from the ECVAM Workshop on *In Vitro* Neurotoxicity Testing (*38*) should be considered in the development of alternative methods for testing of acute toxicity. The main suggestions in this report (*38*) are:

- To develop and evaluate alternative experimental models and end points relevant to human neurotoxicity.
- To foster collaboration among the various European Union neuropharmacotoxicologic research initiatives to develop new and specific biomarkers, end points, and models for *in vitro* neurotoxicologic studies.
- To devise and (pre)validate a three-tiered *in vitro* model encompassing basal cytotoxicologic, cell physiologic, and neuronal cell-specific end points.
- To validate such a tiered testing scheme on a multicenter basis under the auspices of ECVAM.
- To use toxicokinetic data to modify *in vitro* neurotoxic critical concentrations in the final comparison with critical *in vivo* concentrations.

In summary, it is obvious that significant progress is being made toward the understanding of the different components that constitute the complex events designated acute systemic toxicity. This understanding is required for the appropriate modeling of the different toxicologic elements and therefore is a prerequisite for the development of alternative methods that do not involve laboratory animals. It is also recognized that a single *in vitro* test cannot replace the classical LD_{50} test or its animal-based alternatives. Tiered testing schemes seem to represent the solution to the problem and such schemes should therefore be developed, refined, optimized, and validated; when (or if) found to perform as necessary, they should be implemented in regulatory work.

REFERENCES AND NOTES

1. Rhodes C, Thomas M, Athis J. Principles of testing for acute toxic effects. In: General and Applied Toxicology. Vol 1 (Ballantyne B, Marrs T, Turner P, eds). New York:Stockton Press, 1993;49–87.
2. Paget E. The LD_{50} test. Acta Pharmacol Toxicol 52(Suppl II:6–19 (1983).
3. Oliver JA. Opportunities for using fewer animals in acute toxicity studies. In: Chemicals Testing and Animal Welfare. Solna, Sweden:The National Chemicals Inspectorate, 1986;119–142.
4. Clark DG, Fielder R, Joseph C, Gardner J, Smith M. The future for acute oral toxicity testing. In: Animals and Alternatives in Toxicology (Balls M, Bridges J, Southee J, eds). London:Macmillan Academic and Professional Ltd., 1991;1–21.
5. Fielder RJ. The fixed dose procedure as an alternative to the classical LD_{50} test: acceptance by the EEC and OECD. In: Alternative Methods in Toxicology and the Life Sciences. Vol 11 (Goldberg AM, van Zutphen LFM, eds). New York:Mary Ann Liebert, 1995;269–274.
6. Lindgren P, Thelestam M, Lindquist NG, eds. First CFN symposium on LD_{50} and possible alternatives. Acta Pharmacol Toxicol 52 (Suppl II) (1983).
7. Seibert H, Balls M, Fentem JH, Bianchi V, Clothier RH, Dierickx PJ, Ekwall B, Garle MJ, Gómez-Lechón MJ, Gribaldo L et al. Acute toxicity testing *in vitro* and the classification and labelling of chemicals. ATLA 24:499–510 (1996).
8. OECD. OECD Guideline for Testing of Chemicals, No 401: Acute Oral Toxicity. Paris:Organisation for Economic Co-operation and Development, 1987.
9. OECD. OECD Guideline for Testing of Chemicals, No 420: Acute Oral Toxicity-Fixed Dose Method. Paris:Organisation for Economic Co-operation and Development, 1992.
10. OECD. OECD Guideline for Testing of Chemicals, No 423: Acute Oral Toxicity-Acute Toxic Class Method. Paris:Organisation for Economic Co-operation and Development, 1996.
11. Bondesson I, Ekwall B, Hellberg S, Romert L, Stenberg K, Walum E. MEIC-a new international multicenter project to evaluate the relevance to human toxicity of *in vitro* cytotoxicity tests. Cell Biol Toxicol 5:331–348 (1989).
12. Ekwall B. Validation of *in vitro* cytotoxicity tests. In: *In Vitro* Alternatives to Animal Pharmaco-Toxicology (Castell JV, Gómez-Lechón MJ, eds). Madrid:Farmaindustria, 1992;361–390.
13. Walum E, Nilsson M, Clemedson C, Ekwall B. The MEIC program and its implications for the prediction of acute human systemic toxicity. In: Alternative Methods in Toxicology and the Life Sciences. Vol 11 (Goldberg AM, van Zutphen LFM, eds). New York:Mary Ann Liebert, 1995;275–282.
14. Walum E, Forsby A, Clemedson C, Ekwall B. Dynamic qualities of validation and the evolution of new *in vitro* toxicological tests. ATLA 24:333–338 (1996).
15. Seibert H, Gulden M, Voss J-U. An *in vitro* toxicity testing strategy for the classification and labelling of chemicals according to their potential acute lethal potency. Toxicol In Vitro 8:847–850 (1994).
16. British Toxicology Society Working Party on Toxicity. A new approach to the classification of substances and preparations on the basis of their acute toxicity. Hum Toxicol 3:85–92 (1984).
17. van den Heuvel MJ, Clark DG, Fielder RJ, Koundakjian PP, Oliver GJ, Pelling D, Tomlinson NJ, Walker AP. The international validation of a fixed-dose procedure as an alternative to the classical LD_{50} test. Food Chem Toxicol 28:469–482 (1990).
18. Lipnick RL, Cotruo JA, Hill RN, Bruce RD, Stitzel KA, Walker AP, Chu I, Goddard M, Segal L, Springer JA. Comparison of the up-and-down, conventional LD_{50}, and fixed-dose acute toxicity procedures. Food Chem Toxicol 33:223–231 (1995).
19. Stallard N, Whitehead A. Reducing animal numbers in the fixed-dose procedure. Hum Exp Toxicol 14:315–323 (1995).
20. Stallard N, Whitehead A. The fixed dose procedure and the acute toxic class method: a mathematical comparison. Hum Exp Toxicol 14:974–990 (1995).

21. Yam J, Reer PJ, Bruce RD. Comparison of the up-and-down method and the fixed-dose procedure for acute oral toxicity testing. Food Chem Toxicol 29:259–263 (1991).
22. Tamborini P, Sigg H, Zbinden G. Acute toxicity testing in the non lethal dose range: a new approach. Regulatory Toxicol Pharmacol 12:69–87 (1990).
23. Schlede E, Mischke U, Roll R, Kayser D. A national validation study of the acute-toxic class method; an alternative to the LD_{50} test. Arch Toxicol 66:455–470 (1992).
24. Schlede E, Mischke U, Diener W, Kayser D. The international validation study of the acute toxic class method (oral). Arch Toxicol 69:659–670 (1995).
25. Diener W, Siccha L, Mischke U, Kayser D, Schelde E. The biometric evaluation of the acute toxic class method (oral). Arch Toxicol 68:599–610 (1994).
26. Bruce RD. An up-and-down procedure for acute toxicity testing. Fundam Appl Toxicol 6:151–157 (1985).
27. Fielder R. Acceptance of acute toxicity alternatives: the challenges ahead [Abstract]. ATLA 24(Special Issue):89 (1996).
28. Garattini S. Notes on xenobiotic metabolism. Ann New York Acad Sci 407:1–25 (1983).
29. Garattini S. Toxic effects of chemicals: difficulties in extrapolating data from animals to man. CRC Crit Rev Toxicol 16:1–29 (1985).
30. Ekwall B, Clemedson C, Crafoord B, Ekwall B, Hallander S, Walum E, Bondesson I. MEIC evaluation of acute systemic toxicity. Part V: Rodent and human toxicity data for the 50 reference chemicals. ATLA (in press).
31. Ekwall B, Walum E, Clemedson C, Barile FA, Castano A, Clothier RH, Dierickx P, Ekwall B, Ferro M, Fiskesjö G et al. MEIC evaluation of acute systemic toxicity. Part VI: Prediction of human toxicity by rodent LD_{50} values and results from 61 *in vitro* tests. ATLA (in press).
32. Halle W, Göres E. Register der Zytotoxicität (IC_{50}) in der Zellkultur und Möglichkeiten zu Abschäzung der akuten Toxizität (LD_{50}) Beiträge zur Wirkstofforschung, heft nr. 32. Berlin:Akademie-Industrie-Komplex, Arzneimittelfoschung, 1988.
33. Clemedson C, McFarlane-Abdulla E, Andersson M, Barile FA, Calleja MC, Chesné C, Clothier RH, Cottin M, Curren R, Dierickx P et al. MEIC-evaluation of acute systemic toxicity. Part II: *In vitro* results from 68 toxicity assays used to test the first 30 reference chemicals and a comparative cytotoxicity analysis. ATLA 24(Suppl 1):273–311 (1996).
34. Walum E, Peterson A. On the application of cultured neuroblastoma cells in chemical toxicity screening. J Toxicol Environ Health 13:511–520 (1984).
35. Clothier RH, Hulme LM, Smith M, Balls M. Comparison of the *in vitro* cytotoxicities and acute *in vivo* toxicities of 59 chemicals. Mol Toxicol 1:571–577 (1987).
36. Ekwall B. Preliminary studies on the validity of *in vitro* measurement of drug toxicity using HeLa cells. III: Lethal action to man of 43 drugs related to the HeLa cell toxicity of lethal drug concentrations. Toxicol Lett 5:319–331 (1980).
37. Leahy DE. Pharmacokinetics in early drug development. ATLA 25:17–31 (1997).
38. Atterwill CK, Bruinink A, Drejer J, Duarte E, McFarlane Abdulla E, Meridith C, Nicotera P, Regan C, Rodrigues-Farré E et al. *In vitro* neurotoxicity testing. ATLA 22:350–362 (1994).