The Integrated Use of Alternative Methods in Toxicological Risk Evaluation

ECVAM Integrated Testing Strategies Task Force Report 1

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Summary — The ECVAM Task Force on Integrated Testing Strategies was established in December 1996, with the remit of assessing the current status of integrated toxicity testing, and of making proposals regarding the design and implementation of integrated testing strategies. The first step in an integrated testing strategy is usually to determine the chemical functionality of a substance, on the basis of its structure and physicochemical properties. The biokinetic and dynamic behaviours of the chemical in various in vitro systems are then assessed. The various elements are then integrated, in either a parallel or a stepwise fashion, to make predictions of the local or systemic toxicity of the chemical of interest. In this report, a generic scheme for local/systemic toxicity, and a specific scheme for target organ toxicity, are proposed. The scope and limitations of the approaches are discussed. The task force hopes that its proposals will stimulate a discussion on the feasibility of this type of approach and it welcomes any feedback. It is planned that the discussion points will be elaborated in a second task force report.

Key words: chemical, toxicity, toxicological, assessment, testing strategy, integrated, parallel, stepwise, hierarchical.

Introduction

From a number of ECVAM workshop reports (1–4), it is clear that the full potential of alternative approaches in toxicological risk assessment has yet to be fully realised. For instance, the application of a new chemical to cultured cells derived from a particular tissue permits the determination of the concentration of that chemical at which a certain effect occurs in that cell type. This sometimes leads to a knowledge of the fundamental mechanisms underlying the toxic effects, but it does not yield the profile of toxic effects caused by the chemical in the intact organism. Alternatively, a number of studies have attempted to combine the use of a variety of methods (5–7). Typically, the methods and data are combined in parallel or in series, and are applied before any animal tests are carried out. Studies such as these have given rise to the concept of an “integrated testing strategy”, which can be defined as follows:

"An integrated testing strategy is any approach to the evaluation of toxicity which serves to reduce, refine or replace an existing animal procedure, and which is based on the use of two or more of the following: physicochemical data, in vitro data, human data (for
example, epidemiological, clinical case reports), animal data (where unavoidable), computational methods (such as quantitative structure-activity relationships [QSAR]) and biokinetic models.”

The ECVAM Task Force on Integrated Testing Strategies was established in December 1996 under the chairmanship of Bas Blaauuboer (Research Institute of Toxicology, Utrecht University, The Netherlands). The remit given to the task force was to assess the current status of integrated approaches to toxicity testing, and to make proposals with regard to the design and implementation of integrated testing strategies. In this report, examples of both the parallel approach and the tiered approach are presented, and, in accordance with the remit given to the task force, two integrated testing schemes are proposed. The task force hopes that these proposals will encourage a discussion on the feasibility of this type of approach and welcomes any points of discussion. It is planned that these discussion points will be elaborated in a second task force report.

Background

There have been a number of important stages in the development of the concept of integrated testing strategies. First, the application of QSAR techniques by using physicochemical parameters led to the concept of the base-line toxicity of chemicals in the aquatic environment (8). More recently, the use of multivariate analysis (for example, principal components analysis [PCA] and neural networks) has demonstrated the validity of the QSAR approach for modelling specific types of toxicity, such as the skin corrosivity of organic acids, bases and phenols (9). In addition, it is now appreciated that QSAR models do not need to be restricted to the analysis of physicochemical data, but can also be applied to data generated by the wide range of available in vitro methods. A useful resource for developing these QSARs is the database of in vitro and human clinical data developed as part of the Multicentre Evaluation of In Vitro Cytotoxicity (MEIC) programme (10–13). The human data are already available on the Cytotoxicology Laboratory Uppsala (CTLU) Web site (http://www.ctlu.se); the in vitro data will be added later.

The parallel approach to integrated testing

Probably the best-documented example of the parallel approach is the European Research Group for Alternatives in Toxicity Testing/Swedish Board of Laboratory Animals (ERGATT/CFN) Integrated Toxicity Testing Scheme (ECITTS; 14, 15), which is being developed as part of the ECITTS programme, a multicentre project established in 1991 between members of ERGATT with the financial support of the CFN. The use of “elementary analysis” is considered to be a more-efficient approach for assessing chemical toxicity than the use of animal-based investigations. It is based on the assumption that the mode of action can be broken down into a number of biokinetic and cellular “elements” or events, each of which can be identified and quantified in an appropriate model system. The use of test batteries for predicting various types of local and systemic toxicity in combination with biokinetic modelling provides a comprehensive toxicity profile for chemicals of interest. At present eight compounds are being investigated in ECITTS, with an emphasis on the prediction of neurotoxicity (16). The building blocks of the scheme are presented in Table I, and the inter-relationships between them are illustrated in Figure 1.

Tiered approaches to integrated testing

There are many examples of the use of non-animal methods as screens in the determination of toxic potential. Perhaps the best known example is the Ames test for determining the mutagenic potential of chemicals. Another example is the measurement of unscheduled DNA synthesis in hepatocyte cultures. In general, knowledge of the potential of a chemical to cause a particular toxic effect is used to take decisions with regard to further testing.

More-elaborate tiered testing schemes have been proposed for eye irritancy (17), neurotoxicity (18), immunotoxicity (19) and phototoxicity (20). Phototoxicity is of particular interest, since it is the first testing scheme that has been successful in a formal validation study (21). It consists of three steps.

2. In vitro cytotoxicity testing in the absence and presence of light.
Table I: Building blocks of the ERGATT/CFN Integrated Toxicity Testing Scheme (ECITTS)

<table>
<thead>
<tr>
<th>Type of building block</th>
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| Experimental           | Collection of *in vitro* data for:  
1. Biokinetic parameters, including biotransformation.  
2. Cytotoxicity and neurotoxicity data (in different *in vitro* systems). |
| Modelling              | 1. Incorporation of *in vitro* data in a physiologically based biokinetic model.  
2. Determination of target tissue concentration.  
| Validation             | 1. Validation of the model against *in vivo* kinetics.  
2. Validation of the model against *in vivo* toxicity |

Figure 1: Building blocks of the ERGATT/CFN Integrated Toxicity Testing Scheme (ECITTS)

\[ EC20 = \text{concentration of test chemical which causes 20\% of the maximum observed response;} \]
\[ \text{NOEL} = \text{no observed effect level; LOEL = lowest observed effect level.} \]

Another interesting example is the scheme proposed for skin sensitisation (6), which also involves three steps.

1. Screening the chemical structure for structural alerts by using the Deductive Estimation of Risk from Existing Knowledge (DEREK) expert system (22, 23), to assess the potential of a chemical to react with skin proteins either directly or after metabolism.

2. Calculating skin permeability and/or partition coefficients by using QSAR methods (24) to assess whether the chemical can reach the relevant biological compartment for reaction or biotransformation.

3. Testing in the local lymph node assay, an animal procedure which can be considered to be a reduction and a refinement when compared with the procedures currently preferred by regulatory bodies (for example, the guinea-pig maximisation test).

A Generic Scheme for Evaluating Chemical Toxicity

In this section, an integrated decision-making strategy for toxicological evaluation is proposed (Figure 2), which takes into account earlier proposals for parallel and tiered testing approaches. It incorporates existing knowledge (including physicochemical data), biokinetic considerations and toxicological test systems ranging from the determination of basal cytotoxicity to highly specific target tissue assays. A prerequisite for the success of this scheme is the availability of appropriate, well-validated non-animal methods.

A key feature of the scheme is the presence of decision points, i.e. stages at which decisions are made with regard to further testing. In addition, the scheme permits expert judgement to be made at different stages of the scheme for the purposes of hazard assessment (including classification and labelling). The proposed scheme consists of four stages.

1. Assessments based on physicochemical properties and chemical functionality.

2. Biokinetic modelling, including the modelling of biotransformation, tissue partition and transport, together with basal cytotoxicity testing.

3. Selection from a battery of specific cytotoxicity and cell-specific function toxicity tests in the case of high tissue concentrations.

4. Selection from a battery of specific in vitro tests, if indicated by historical data for related chemical structures, regulatory requirements, or the intended use of the chemical.

On the basis of the outcome of each stage, expert judgement would result in identification of toxic hazard and/or progression to the next stage of the scheme. Thus, the scheme can be used both for screening purposes and for generating more-comprehensive toxicological profiles.

Stage I

In the first stage, chemical structure and any relevant physicochemical data (for example, melting point) are taken into account. Some physicochemical parameters (for example, logP) and chemical functionality (for example, electrophilicity) can be determined from the structure. These data are then used to predict toxicological properties by using appropriate QSAR models. Currently available models include those for the prediction of skin irritation and corrosivity (5, 9, 25), eye irritation (26), skin sensitisation (27) and mutagenicity (28), for specific chemical classes.

Stage II

Central to Stage II is a biokinetic model, which describes the time-course of the concentration of a chemical in various tissues of the body. Such models can be constructed for any animal species of interest, provided that the appropriate physiological parameters are available (for example, blood flow, tissue size and composition). A comprehensive compilation of these parameters is provided by Brown et al. (29). Compound-specific parameters, such as tissue partition coefficients, can be calculated from physicochemical properties, whereas parameters such as the rates of biotransformation and transport need to be determined with appropriate in vitro systems (30). When bioactivation is identified as
Figure 2: A generic scheme for evaluating chemical toxicity

**Structure/available data**

- Calculate/measure physiochemical properties **and** decide chemical functionalities

- Quantitative structure-activity relationship models – enough data?
  - Yes → In vitro data → Classify
  - No → Biokinetics

**Biokinetics**
- Metabolites
- Basal cytotoxicity
- Bioactivation relevant?
  - Yes → Prediction models for toxicodynamics → Classify
  - No → Other considerations

**Other considerations**
- Low
- High
- Battery of relevant tests
  - Limited in vivo testing

**Ratio**
- EC50(basal)/EC50 (specific)

**Battery of tissue-specific in vitro tests**

- Enough data?
  - Yes → Classify; risk evaluation
  - No → Limited in vivo testing

- For: acute or chronic toxicity; systemic toxicity (NOEL)

**Corrosivity, irritation, sensitisation, mutagenicity, etc.**

**EC50 = concentration of test chemical which causes 50% of the maximum observed response; NOEL = no observed effect level.**
an important process, the biokinetics of any relevant metabolites also need to be incorporated. The integration of physicochemical properties and in vitro data into physiologically based biokinetic models is illustrated by DeJongh et al. (31) and Houston & Carlile (32, 33), respectively.

Also important at this stage is knowledge about the basal cytotoxicity of a chemical and any relevant metabolites, i.e. toxicity toward processes which are common to all cell types (3). This can be determined with a number of basal cytotoxicity tests, such as the neutral red uptake (NRU) assay.

The output of biokinetic models is the prediction of concentration/time-courses in different tissues, and the identification of tissues which concentrate the chemical. This information can be combined with the basal cytotoxicity data to make a prediction of the acute systemic toxicity of the chemical, so that chemicals which are potentially hazardous to specific tissues can be identified. It might also be possible to predict non-specific effects, such as the accumulation of non-reactive lipophilic chemicals in biological membranes. This is referred to as base-line toxicity, and can manifest itself as a general “narcotic” effect in the nervous system (34).

Stage III

If the outcome of Stage II indicates the presence of high concentrations of a chemical in a particular tissue, the toxicity of the chemical in that tissue needs to be determined. Two forms of specific toxicity are recognised: a) selective toxicity, in which some cell types are more sensitive than others; and b) cell-specific function toxicity, in which the chemical affects events or structures which are unique to, or particularly dominant in, a specific cell type. This could include effects which are critical to the organism as a whole, such as the inhibition of albumin production in hepatocytes, cytokine production or hormone production (3).

The outcome of the experiments carried out at this stage can be expressed as the ratio of the effective concentration for basal cytotoxicity to the effective concentration for the cell-specific toxicity. Ratios considerably greater than one would indicate specific target organ toxicity, and a requirement to determine a no observed effect level (NOEL). The choice of cell types is based mainly on biokinetic considerations or information of a physicochemical nature for chemicals with similar characteristics.

Stage IV

Even when a chemical does not appear to accumulate in any specific tissue, or when the ratio of basal cytotoxicity to specific toxicity is not particularly high, there can still be other reasons for investigating more closely the tissue-specific effects of the chemical, for example, to comply with particular regulatory requirements related to the expected use of the chemical. In such cases, test systems could include tissue-specific tests similar to those used in Stage III, but they could also include tests for other toxicological endpoints, such as developmental toxicity and carcinogenicity. The outcome of this stage will be similar to the outcome of Stage III, i.e. the classification and prediction of systemic toxicity.

If a chemical cannot be evaluated toxicologically by applying these four stages, it might be necessary to conduct limited in vivo studies. However, the information gained in previous stages will enable such studies to be more focused than they would otherwise have been, so they will be limited. Thus, these in vivo studies can be regarded as true refinements in comparison with animal procedures based on current testing guidelines.

A Specific Scheme for Evaluating Target Tissue Toxicity

If a specific target organ or tissue is of particular interest for a particular chemical, the scheme can be simplified, although the key elements of the procedure would remain the same. Again, the starting point of the process would be a consideration of chemical structure, physicochemical properties and the corresponding chemical functionalities. It might be possible to construct (Q)SARs capable of predicting organ-specific or tissue-specific biological activities for specific groups of chemicals from this information. As an example, a specific scheme for neurotoxicity is proposed in Figure 3.

However, for the majority of chemicals, it would be necessary to take biokinetic considerations into account. In combination with a measurement of basal cytotoxicity, biokinetic models could be used to predict the dose of a chemical which could result in
Figure 3: A specific scheme for evaluating neurotoxicity

Neurotoxicity: structure/available data

Calculate/measure physicochemical properties and decide chemical functionalities

Quantitative structure-activity relationship models – enough data?

Yes → Classify

No → Biokinetics: tissue partition, biotransformation transport

Basal cytotoxicity: for example, neutral red uptake

Bioactivation relevant?

Yes → Prediction models for toxidynamics

No → Metabolites

Classification: potential neurotoxicity, for example, non-specific basal toxicity (narcosis)

Battery of neuronal-specific in vitro tests

Ratio

EC50(basal) - EC50 (specific)

Low → Limited in vivo testing

High → Classify; risk evaluation

For: acute or chronic toxicity; systemic toxicity (NOEL)

EC50 = concentration of test chemical which causes 50% of the maximum observed response; NOEL = no observed effect level.
sufficiently high concentrations to cause non-specific effects, for example, in the brain or peripheral nervous system. If such a prediction was not feasible, the chemical could be tested by using a battery of neuronal system-based in vitro tests. If a high ratio of general cytotoxicity to specific cytotoxicity is observed in these neuronal in vitro systems, and if biokinetic considerations indicate an accumulation of the chemical in the nervous system, a hazard evaluation could be performed for the acute or chronic neurotoxic activity of the chemical of interest. If this were not possible, limited in vivo testing could be carried out.

Conclusions and Recommendations

Integrated testing strategies combine the use of toxicodynamic and biokinetic parameters, since the toxicity of chemicals is determined by two main sets of characteristics: a) the mode of action and the resulting effects on the organism (dynamics); and b) the changes in concentration of the chemical with time, in various parts of the organism (biokinetics).

A high priority should be placed on the evaluation and development of testing strategies for the prediction of acute and chronic systemic toxicity. A prerequisite for these activities is the availability of a comprehensive data set containing existing in vivo data and the corresponding data obtained by alternative methods (i.e. the predictions made by computational prediction techniques and in vitro methods). Therefore, a project should be initiated to collate these data, and to identify deficiencies.

Research should be carried out into the application of new and existing in vitro techniques and the development of computational methods for the accurate and rapid determination of biokinetic parameters. We believe that the latter is a feasible option for parameters such as partition coefficients and protein binding affinities, while the further development of in vitro technologies should be encouraged for assessment of the more complex and ill-defined parameters describing biotransformation and transport processes.

The schemes for evaluating chemical toxicity proposed in this report should be evaluated by comparing their outcomes with “classical” toxicity data for groups of compounds. A promising basis for this exercise would be the results of studies obtained in the ECITTS programme.

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References


Integrated testing strategies


