Relevance of *In Vitro* Toxicology Studies in Risk Assessment

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Summary
In vitro toxicology used to determine the hazardous nature of a product is gaining wider acceptance in the scientific and regulatory community. Risk assessment involving whole animals has been the most accepted principle in toxicity investigation. In recent years, in vitro methods have been developed as potential alternatives to in vivo experiments. These in vitro methods have varying degrees of reliability and acceptance. Some of these may be directly employed as replacements for in vivo models; the other techniques are currently suitable only as screens or adjunct tests. Toxicologists have been working hard to develop new in vitro methods to be used in place of existing in vivo animal studies and thus secure a place in the regulatory battery of tests.

Zusammenfassung: Relevanz von *in vitro* Toxizitätsstudien bei der Risikobewertung
Die in vitro Toxikologie bei der Bewertung des Risikos eines Produkts gewinnt bei Wissenschaft und Behörden zunehmend an Akzeptanz. Die Risikobewertung unter Verwendung von Versuchstieren war bisher das meist akzeptierte Prinzip bei toxikologischen Untersuchungen. In den vergangenen Jahren wurden jedoch in vitro Methoden als potente Alternativen zu in vivo Methoden entwickelt. Diese in vitro Methoden variieren in ihrer Zuverlässigkeit und Akzeptanz. Einige von ihnen können direkt als Ersatz für Tiermodelle eingesetzt werden; andere sind momentan nur geeignet als Vorstudie oder Ergänzungsversuch verwendet zu werden. Die Toxikologen haben hart gearbeitet, um neue in vitro Methoden zu entwickeln und damit in vivo Tierversuche zu ersetzen. Sie haben ihnen damit einen sicheren Platz in der Batterie behördlich vorgeschriebener Tests verschafft.

Keywords: in vitro toxicology, risk assessment

1 Introduction

Russell and Burch (1959) proposed the framework of the 3Rs of refinement, reduction, and replacement more than 40 years ago. Since that landmark publication, significant progress has been made, especially in the arena of regulatory testing (Stephens et al., 2001). Several reviews of refinement and reduction alternatives have been written in recent years (Festing, 1999; Morton, 1995, 1998; Rowan, 1995). In both U.S. and European laboratories, scientists have vigorously pursued the development of *in vitro* methods to advance their science. During the last 20 years, the considerable and significant advances in tissue culture methodology, the use of chemically-defined cell and tissue culture media, and the availability of human cells have transformed *in vitro* methods from a new technology to a valuable research tool.

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The present review concentrates primarily on *in vitro* toxicology. Yet, all *in vitro* methods are alternatives to animal testing.

2 *In vivo* safety approach

The array of tests required for registration is determined by regulatory authorities. All testing of regulated chemicals must be done under good laboratory practice protocols that are standardised across the entire community. Most toxicity tests are conducted with laboratory animals. An extensive battery of toxicity studies is required to determine the nature and extent of the hazard posed by the various regulated chemicals. The required studies are designed to assess the possible adverse health effects on a variety of species that may result from single, multiple or lifetime exposure to a product via the skin, mouth, lungs or eyes. A variety of species are used to indicate whether the same effects are observed in different species, or if they are limited to a certain species. During the last 20 to 30 years it became obvious that traditional animal testing with the use of large numbers of animals has to undergo rigorous reassessment, and animal welfare concerns have been one of the driving forces for the development of *in vitro* alternative tests. The use of human end points is a further step in refining and improving toxicological tests.

3 Alternatives development is determined by three modules

Alternative methods described include tests already validated as well as those under development or already in use but awaiting final validation by ECVAM (European Centre for the Validation of Alternative Methods). First, the basic

biology of adverse responses to toxicants must be understood with sufficient mechanistic depth to support the selection of models and end points relevant to the process being studied. Second, *in vitro* methodology must be developed that is amenable to or can be adapted to toxicological applications. Third, the scientific basis and performance of assays in validation programmes must be sufficiently robust to convince the scientific and regulatory authorities that the proposed alternative assays can replace the traditional methods. Each of these three modules is rate limiting to the replacement of animal testing; however, new scientific advances coupled with streamlined review processes for alternative methods should accelerate the pace of new methods development.

4 *In vitro* safety approach (non-animal)

Clearly, in order to identify hazardous chemicals and control them as soon as possible, a radically different testing process is needed. The only approach that combines practicality with humanity uses rapid non-animal tests to characterise a large number of chemicals in a minimum amount of time. *In vitro* and computational methods can be combined in stepwise or decision-tree strategies customised to each type of toxicity. Stepwise testing has already been accepted by, and is used within, the OECD (Organisation for Economic Co-operation and Development), the USA and the European Union. In a stepwise strategy, using non-animal methods, testing progresses from quick and simple screening methods through tests specific to toxic mechanisms, to more sophisticated *in vitro* assays, where needed, which study target tissue effects. Some of the non-animal tests proposed here have been validated and accepted by regulatory authorities.

5 Application of *in vitro* toxicology

In vitro methods are routinely used by all industries, product development, drug discovery and regulatory bodies in toxicity testing, safety assessment and risk evaluation, and offer unique advantages. In the past, *in vitro* methodology was used as the last approach in product development to identify the underlying biology of undesired effects and, in some limited cases (such as receptor binding), to assist in product development. Only recently, *in vitro* methods have been used at earlier stages of chemical evaluations (Screening Existing TSCA Inventory Chemicals for Neurotoxicity, 1995).

6 Types of *in vitro* toxicology

6.1 Skin corrosion

Skin corrosion refers to the production of irreversible tissue damage in the skin following the application of a test material, as defined by the Globally Harmonised System for the Classification and Labelling of Chemical Substances and Mixtures (GHS) (OECD, 2001). Prevalidation studies were a first step towards defining alternative tests that could be used for skin corrosivity testing for regulatory purposes (Botham et al., 1995). Following this, a formal validation study of *in vitro* methods for assessing skin corrosion was conducted (Barratt et al., 1998; Fentem et al., 1998; OECD, 1996; Balls et al., 1995; ICCVAM, 1997). The outcome of these studies and other published literature led to the recommendation of two equivalent tests as replacements for the *in vivo* skin corrosivity test (ECVAM, 1998), i.e. the human skin model test (OECD Test Guideline 431) and the transcutaneous electrical resistance test (OECD Test Guideline 430).

6.2 Phototoxicity test

The reliability and relevance of the *in vitro* 3T3 NRU phototoxicity test was recently evaluated (Spielmann et al., 1994; Anon, 1998; Spielmann et al., 1998). The *in vitro* 3T3 NRU phototoxicity test was shown to be predictive for acute phototoxicity effects in animals and humans *in vivo*. The test is not designed to predict other adverse effects that may arise from the combined action of a chemical and light, i.e. it does not address photogenotoxicity, photoallergy or photocarcinogenicity, nor does it allow an assessment of phototoxic potency. In addition, the test has not been designed to address indirect mechanisms of phototoxicity, effects of metabolites of the test substance, or effects of mixtures.

6.3 Eye irritation

The Draize eye test earned more criticism than any other procedure used on animals, so, not surprisingly, scientists in the alternatives field have dedicated more time and attention to finding an alternative to this test than to any other method. Unfortunately, replacing the Draize test has proven to be more difficult than anticipated for several reasons. However, significant advances have been made in the development of *in vitro* alternatives for ocular safety testing (Frazier et al., 1987; Nardone and Bradlaw, 1983; Frazier, 1988; Wilcox and Bruner 1990). In spite of all scientific complexities, validated alternative assays are categorised as target organ/tissue assays, i.e. the bovine corneal opacity and permeability (BCOP) test, isolated rabbit eye (IRE) test and chicken enucleated eye test (CEET) or as organotypic models, i.e. the hen's egg test – chorioallantoic membrane (HET-CAM) assay and chorioallantoic membrane vascular assay (CAMVA).

6.4 Skin absorption

In vitro methods have been used for many years to measure skin absorption. There are a number of monographs that review this topic and provide detailed background on the use of an *in vitro* method (Bronaugh and Collier, 1991; Diembeck et al., 1999; Recommended Protocol for *In vitro* Percutaneous Absorption Rate Studies, 1996; Howes et al., 1996). In vitro methods measure the diffusion of chemicals into and across skin to a fluid reservoir and can utilise non-viable skin to measure diffusion only, or fresh, metabolically active skin to simultaneously measure diffusion and skin metabolism. Such methods have found particular use as a screen for comparing delivery of chemicals into and through skin from different formulations and can also provide useful models for the assessment of percutaneous absorption in humans. This method measures dermal absorption and delivery of a test substance using excised skin.

6.5 Skin sensitisation

The murine local lymph node assay (LLNA) is a test method developed to assess whether a chemical has the potential to induce allergic contact dermatitis (ACD) in humans. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 1999) has evaluated and accepted this alternative to currently practiced Guinea pig test methods for hazard identification of chemicals with the potential to produce acute contact dermatitis. LLNA offers animal welfare advantages compared to the use of the traditional method in that it provides for animal use refinement (i.e. elimination of distress and pain) and reduces the total number of animals required.

7 Systemic toxicity – new approaches

Acute oral toxicity testing is typically the first step in identifying and characterising the hazards associated with a particular chemical. The use of *in vitro* cell cultures as alternatives to predict acute lethality *in vivo* has been under study for almost 50 years (Smith et al., 1963).

The relationship found with the Registry of Cytotoxicity (RC) regression is used with in vitro data to predict starting doses for subsequent *in vivo* acute lethality assays (Spielmann et al., 1999). It was suggested that before initiating any *in vivo* lethality assay for a chemical, an *in vitro* cytotoxicity assay should be conducted to estimate the LD_{50} for that chemical. Using this estimate should make conducting *in vivo* assays much more efficient and result in reducing both the number of animals used and the amount of time required to obtain the final results. The workshop report includes a discussion of the potential number of animals saved, based on several currently available *in vivo* protocols (NIEHS, 2001), i.e. protocols that use new sequential dosing methods such as the Acute Toxic Class method (ATC, OECD TG 423; OECD, 1996) and the Up-and-Down Procedure (UDP, OECD TG 425; OECD, 1998b). The RC has made a major contribution to the knowledge of the correlation between *in vitro* cytotoxicity and *in vivo* lethality. The recommended approach takes advantage of the relationship between *in vitro* IC_{50}s and *in vivo* LD_{50}s derived from the RC for 347 chemicals (Halle, 1998).

8 Advantages of *in vitro* methods

Isolated cells, tissues and organs can be prepared and maintained in culture by methods that preserve properties characteristic of the same cells, tissues, and organs *in vivo*. Using such *in vitro* systems will permit data to be generated under controlled experimental conditions and in the absence of many complicating factors characteristic of experiments with whole animals. Once established, *in vitro* tests may provide toxicity information in a cost effective and time-saving manner. Information generated from *in vitro* test systems can be used to increase the efficiency of whole animal studies and decrease the number of animals used in toxicity testing. The relative simplicity and space-saving characteristics of *in vitro* methods are also viewed as advantages.

9 Limitations of *in vitro* methods

In vitro test systems are not available for all tissues and organs. In addition, normal systemic mechanisms of absorption, penetration, distribution and excretion are absent from *in vitro* test systems. *In vitro* systems lack the complex, interactive effects of the immune, blood, endocrine, nervous and reproductive systems and other integrated elements of the whole animal. Thus, *in vitro* tests cannot be used to study the complex nature of systemic toxicity like subchronic and chronic toxicity and carcinogenicity. Validation of new methods is time-consuming and expensive; acceptance of *in vitro* tests as alternatives to traditional toxicity testing in whole animals is expected to be slow (Frazier, 1990).

10 Conclusion

Reduction and refinement alternatives have significant potential to decrease the use and suffering of animals used in regulatory testing further. The pace of future progress in these areas will depend on how well several challenges are met, including increased interlaboratory collaboration and funding.

11 Recommendations

In order to take advantage of the scientific, economic, practical and ethical benefits of this non-animal strategy for the testing of existing and new chemicals, the European Commission must implement immediate priority action as follows:

1. All validated alternatives should be implemented by regulatory authorities.
2. Prospective alternatives method must be taken through fast-track interlaboratory validation and regulatory acceptance.
3. Alternatives should be funded in targeted areas, with scheduled processes for development, validation and acceptance.

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