

FOCUS ON RESEARCH

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A position paper by IVTIP, the In Vitro Testing Industrial Platform

To make the European industry competitive in a global market we need in vitro test systems that support business needs by being:

- mechanism based
- high throughput
- relevant to man

Major obstacles which stand in our way are our lack of understanding of the basic cellular mechanisms involved in toxicity and a shortage of suitable cell lines and cryopreserved tissues. There is also a clear need for high-throughput screens for detecting toxicity.

These limitations could be overcome by encouraging EU-funding into basic research, the provision of phenotypically-stable, immortalised cell lines, and access to well-characterised cryopreserved primary cells and tissues (including human and non-human primate).

The development of high throughput screens for detecting toxicity are an absolute necessity for all areas of toxicity testing. The development of such screens is seen as a long term goal, which requires substantial fundamental research into the mechanism of toxicity. Screens should be designed in such a way that they can be performed using a few molecules only of the test compounds.

In addition, the development of in vitro tests might be one of the tools to reduce, refine or replace the number of animal tests commonly used in research or required by regulatory authorities. Industry would be pleased if the near future would bring a 'toolbox', or a set of methods and approaches, which is acceptable from a scientific and regulatory point of view.

Whatever the rationale to develop in vitro tests, they should be based on sound scientific principles and have a high predictive value.

Recommendations

Novel approach

Within projects to be included in the 5th Framework Programme, we wish to encourage a novel approach to in vitro test system development. Emphasis should be on an understanding of the fundamental mechanisms that these systems are intended to model. Only with such an understanding we might be able to develop the predictive tests that have, so far, eluded us using an empirical approach.

In this, we hope to achieve an increasing mechanistic understanding in model cell types, later extrapolated and applied to specific cells, rather than the generation of data in a broad range of cells. It is recommended that this research builds on and further expands current research efforts in related scientific fields and makes optimal use of new technologies, such as molecular biology.

General industrial criteria

In all research projects, creativity of contractors should not be hindered by the constraints of industrial application. However, the following general criteria should be considered when developing in vitro test systems for industrial and regulatory acceptance.

Test systems should:

- be relevant to industrial use and applicable to biotechnology-derived materials, and pharmaceutical, cosmetic and industrial chemicals;
- have maximum human relevance;
- be validated, robust and simple to perform. In this, we would rather see a focus on reliability and extrapolation of a limited number of standardized test systems than on the creation of a large

- number of new, specific tests;
- have the potential to refine, reduce or replace the in vivo tests currently required by regulatory authorities.

Quality control

Cells and tissues used should be from species used in toxicological research and man, and their stability and inherent variation established. Cells and cell lines used in standard protocols should be characterized and checked regularly for stability of karyotype and metabolic profile. To facilitate this approach, IVTIP encourages the establishment of cell/tissue banks. We also support a more efficient use of non-human tissue, for example the establishment of an information system alerting researchers to the impending availability of such material. To stimulate further standardization, facilities containing compounds to be tested by contractors could be set up.

Areas for toxicity research

To further expand on current knowledge and the initiatives contained in the 4th Framework Programme, we have identified several areas for future toxicity research in which the recommendations described above for in vitro tests in general could be accommodated:

- local toxicity (skin and eye irritation, corrosivity, skin sensitization); basal and target
- organ toxicity;
- immunotoxicity;
- reproductive toxicity;
- neurotoxicity.

On the following pages we will describe in more detail what type of toxicity projects IVTIP would like to see addressed in the 5th Framework Programme.

In Vitro basal and target organ toxicity research strategy

IVTIP recommends that emphasis should be placed on the development and subsequent validation of standardised protocols for tests using well characterised cells which examine the molecular basis of cell damage/death and effects on specific metabolic pathways. Special emphasis should be paid on toxicological relevance using cells of main toxicological targets and cell lines, and on common mechanisms of acute and, if possible, subacute toxicity.

Research programmes should focus on the following steps:

1. Basic research on mechanisms of cell death

For studies on mechanisms of cell damage/death a range of chemicals of different classes which have known effects in vivo and comprehensive and accurate existing data-bases should be used. The biochemical, structural and functional effects of both high single (acute) or low repeated (sub-acute) doses are to be examined and the results should be compared with those obtained using the broader end-points of cell death such as neutral red uptake etc. Suitability and limitations of cell lines, primary cells, co-culture systems and tissue slices have to be examined with regard to toxicological relevance/good quality in vivo results. In this work, attention should be paid to research results generated in projects addressing apoptosis.

2. Intra- and inter-species variation

The stability and inherent variation in any tissue culture system used for toxicity evaluation should be clearly established. Cells and tissues of main targets of preferentially used toxicological animals species and of man should be utilised. The cellular properties of each system should be defined as far as possible (e.g. specific cellular functions, Phase I and Phase II metabolism, cell surface receptor activity etc.). Cells and cell lines used in standard protocols must be characterised and regularly checked for stability of karyotype and metabolic profile and need of exposure controls should be examined. Improvement of cell/tissue banks for human and animals tissues and cryopreservation techniques should be encouraged to assist standardization.

3. Hazard assessment from in vitro data

Prediction of hazard from in vitro cytotoxicity data requires consideration of additional data such as SAR and physico-chemical properties and in vivo toxicity data taken from reliable and reproducible animal studies. Results obtained from standardised in vitro biotransformation and bioavailability tests can be also included and prediction by computer-based scientific models or from validated 'expert systems' may be of value.

Research strategy on immunotoxicity

Although the primary evaluation of immunotoxicity cannot yet be accurately modelled in vitro, various ex vivo techniques provide very important information about the immunobiological mechanisms. 'Immunotoxicity' covers both the stimulation and the depression of the immune system, either as in allergic reactions or in autoimmune diseases or secondarily by leading to increased susceptibility to infection/cancer.

Our needs

Batteries of immunotoxicological tests that have been validated with mild/moderate immunomodulatory substances are highly needed. We also need more information about the normal functional reserve and how we extrapolate from experiments to the findings in animals studies and the situation in humans. Due to the highly specific nature of the human immune response to various agents, only tests which reflect the human response are relevant for safety evaluation for man.

Commercially available validated kits and specific, high-quality antibodies are absolutely necessary in order to give the toxicologist the necessary tools to detect the relevant mediators, immunoglobulins etc. Immunologists tend to work mainly on man and mice, while toxicologists mainly deal with other kinds of animal species. Therefore, the established immunological systems have to be validated and/or adapted for the needs of toxicology. In this respect, the in vivo immunological history is vital in interpreting the results adequately.

Areas of research

Focus on the following areas of research should lead to the development of screening assays for the assessment of the immunomodulatory potential of drugs and chemicals. The relevance for the cosmetic industry should be addressed.

- The analysis of the toxicity often depends on read outs of an immune status, which has been established in vivo. It is a major scientific challenge to develop alternative in vitro immunization protocols. In vitro sensitization and restimulation of antigen specific immune responses should have high priority.
- The generation of new in vitro tests should be aimed at predicting human immuno toxicity responses and immunomodulatory effects of agents.
- Basic research into mechanisms:
Put further emphasis on analysis of the expression and activity of key immunoregulatory cytokines and mediators. This could be done in selected cells, tissues and/or fluids following in vivo exposure or only in vitro incubation - if possible in combination with the analysis of specific mRNA cytokine expression to find out how the individual cell is responding to stimuli. The subsequent increased understanding of the nature of the immunological events which may provoke contact, respiratory or drug allergy will facilitate the design of novel ex vivo methods for the identification of allergic potential. Basic research into immunoregulatory factors generating non-specific responses is also important.
- Establish and implement in vitro immune function tests to substitute the host-resistance studies which are designed to detect increased susceptibility to infections and/or malignancies and which mostly require death or severe disease as the endpoint.
- Support the development of in vitro models for potency evaluation of vaccines: Invest into basic research into the right class of protective antibodies and components of the cellular immune system and characterize the relevant epitopes and their presentation. The development of tests to assess reactogenicity of adjuvants would be welcome.

Research strategy on reproductive toxicity

Reproductive toxicity can be divided broadly into effects during pregnancy (developmental toxicity) and effects on fertility. Tests capable of detecting potent developmental toxicants (FETAX, modified CHEST, micromass, and rat whole embryo culture) already exist and currently are being considered for further validation by ECVAM. These 'first generation' test systems, however, are in need of refinement. Whole embryos and primary cell cultures need replacing with propagated cell lines (e.g. embryonic stem cells), and more specific (and predictive) end-points need to be developed to improve sensitivity and practicality.

In contrast to developmental toxicity, the development of in vitro systems for detecting effects on fertility, has largely been neglected. Recently, however, reports of an apparent decrease in human semen quality over the last 50 years, and the suggestion that this has been caused by environmental chemicals, has resulted in a number of initiatives to develop such tests. At the fore-front of these has been the inclusion, at the request of IVTIP, of a number of relevant projects into the Developmental Pharmacology component of the 4th Framework of the EU Biotechnology programme.

Recommendations

To build on current knowledge and the initiatives of the 4th Framework Programme we have now identified projects for the 5th Framework Programme. Within these projects we wish to encourage a novel approach to test system development. The emphasis should be on understanding the cellular mechanisms of toxicity so that we are able to develop the highly predictive in vitro screens that have so far eluded us using an empirical approach. To this end, we recommend the application of advances in related scientific fields and the use of new technologies, in particular molecular biology, which we believe offers great opportunities for progress.

Projects to be considered include the development of:

- test systems to determine the oestrogenic and/or anti-androgenic potential of chemicals, preferably with some functional relevance to effects on male reproductive organs;
- testicular cell culture systems capable of sustaining spermatogenesis, with specific markers for toxicological damage. There is already a considerable amount of work in this area using immortalised somatic cell lines and germ cells from transgenic mice;
- the characterisation of normal sperm function (motility, capacitation, acrosome reaction, sperm-egg binding etc.), and the relationship between effects of chemicals on these events and effects on fertility and subsequent development of embryos;
- teratogenicity screens using propagated cell lines with end-points (transgenic markers, FACS etc.) identified from research into the molecular basis of cell proliferation, migration and differentiation and their modulation by teratogens. Such test systems could be used for hazard labelling and high throughput screening, and are rapidly becoming technically feasible.

Basic research is also required in the following areas to enable the future development of alternative test systems in reproductive toxicology:

- oogenesis;
- epididymal sperm maturation;
- the mechanism of implantation and the way in which chemicals can prevent it from occurring.

Research strategy in neurotoxicology

In neurotoxicology, sensitive methods for scientific or regulatory purposes are needed to replace complex and sometimes painful in vivo studies in the field of pharmaceuticals, pest control and industrial compounds. The existing systems are mostly highly specific and difficult to handle. A battery of in vitro tests implemented in a tiered testing approach is recommended.

Tiered testing approach

In a tiered testing programme, a single compound or a chemical class is characterized step by step. The first step of such a programme consists of documenting the cytotoxic profile using basic toxicological investigations in reliable cell cultures (primary or secondary) of the nervous system. If the compound shows a distinct 'inherent' toxicity in these test systems, the next step is

recommended.

In the second step, specific assays are required, which investigate specific neuronal endpoints like the neurotransmitter system, development and differentiation of the neuronal and glial cells and the cytoskeleton. These endpoints should also be determined in reliable cell or slice cultures.

The third step, which includes specific investigations in the field of electrophysiology and receptor and channel characterization, should only be recommended if a specific screening is needed or when a mechanistic approach is required.

To implement such a programme, existing tests need to be optimized and new tests for specific neurotoxicological endpoints need to be developed.

Optimization existing cell culture systems

The current battery of in vitro tests comprises:

- cell cultures of neuronal and glial cells from rodents or avians;
- brain slices or reaggregation cultures;
- permanent neuronal cells, available in international (maybe central) cell banks.

All these test system have their advantages and disadvantages, because these systems were developed for scientific research and have not been optimized for general purposes in neurotoxicology.

Before recommending the optimization of these tests, agreement is needed which tests are required and appropriate to provide reliable results. Requirements are that these systems must be reliable, give data comparable to existing in vivo data from different animal species and can be used in step one and two experiments of a tiered testing programme.

Specific methods

The battery of tests for specific neurotoxicological endpoints as required for step two research in the tiered testing programme is endless and may comprise:

- the neurotransmitter system (determination of various neurotransmitters intracellular and extracellular; determination of the anabolic and catabolic enzymes);
- development and differentiation of the main cell types (neuronal, glial);
- the cytoskeleton, axonal transport;
- energy management.

Recommendations

It is recommended to develop a battery of in vitro tests which examine different functional and structural aspects of the nervous system. This battery should comprise optimized existing systems as well as newly developed tests. These tests should be implemented in a tiered testing approach which determines step-wise the risk for human health. A tiered programme allows a case by case decision in characterizing distinct compounds or a chemical group in the field of neurotoxicology by using more and more complex and specific assays.