

## Chapter 8: Target Organ and Target System Toxicity

### Introduction

The detection of specific target organ and target system toxicity is an important aspect of toxicological testing, and the conventional test procedures involve chronic, repeat-dose *in vivo* testing, often in a non-rodent species as well as in a rodent species. This kind of predictive testing represents an enormous challenge to those who, for a variety of scientific and ethical reasons, would like to see animal testing replaced by non-animal tests and testing strategies. It will be worthwhile putting considerable skill and effort into attempts to meet this challenge, not least because, as in the case of carcinogenicity testing (Chapter 9) and reproductive toxicity testing (Chapter 10), inter-species differences limit the usefulness of animal studies for predicting long-term target-organ and target-system effects in humans. It should also be borne in mind that strategies for assessing target organ and system toxicity need to take into account the biokinetic considerations of absorption, distribution, metabolism and excretion (ADME; Chapter 7).

During the past decade, several *in vitro* systems have been under development for detecting a number of target organ and target system effects (1). It is vital, if only for reasons of the costs incurred and the time required, that strategies are developed for the selective and sequential use of a widening range of optional and complementary approaches. In this chapter, preliminary approaches to establishing *in vitro* systems for long-term, repeat-dose testing are discussed, and examples are given of developments in relation to a target organ (the kidney) and to a target system (the nervous system).

### Current Status of Repeat-dose Toxicity Testing

Chronic toxicity is a consequence of the persistent or progressively deteriorating dysfunction of cells, organs or multiple organ systems, resulting from long-term exposure to a chemical. In animals (usually rodents), short-term, repeat-dose studies last 14–28 days (OECD Test Guideline [TG] 407 [oral toxicity], TG 410 [dermal toxicity] and TG 412 [inhalation toxicity]). The highest dose administered is designed to cause some toxicity, but not lethality. Upon completion of the test, a whole host of clinical and histological evaluations are recorded, including experimental observations and whole-body and individual-organ analyses. Other sub-chronic toxicity studies include the 90-day study

(OECD TG 408), and the combined repeat-dose toxicity and reproductive toxicity screening test.

During long-term exposure, some compounds can induce adaptive processes in their target cells. Changes in gene expression at the transcriptional, translational or post-translational levels can result from the induction or inhibition of enzyme systems, or by up-regulation or down-regulation of receptor–ligand interactions, thereby changing the functions of intracellular signal cascades. For instance, the increase of metallothionein expression following exposure to low doses of metals permits a higher toxic dose to be tolerated (2).

Another important parameter to take into consideration is the ability to recover from the toxic insult. Some *in vitro* studies on recovery and repeat-dose testing have been published; however, the relevance of measuring reversibility *in vitro* and the interpretation of the *in vitro* results in terms of the *in vivo* situation still needs to be established (3).

There have been a few attempts to study the long-term exposure of cells or tissue cultures to chemicals *in vitro*. In most of these studies, non-organ-specific parameters (for example, proliferation, protein synthesis and mitochondrial function) were used. Because the development of chronic diseases is almost always related to specific organs, the biological relevance of non-organ-specific effects in predicting the risk of the development of persistent damage may be questionable.

Table 8.1 summarises the currently available models for long-term testing in relation to three of the most important targets for toxicity and/or sites of detoxification (the liver, kidney and central nervous system), indicating the advantages and disadvantages of each approach (4–10). Liver toxicity is considered in Chapter 7.

### The ECVAM Workshop on *In Vitro* Methods for Long-term Toxicity Testing

An ECVAM workshop on novel advanced *in vitro* methods for long-term toxicity testing has been held, and the report and recommendations have recently been published (11). The main conclusions with regard to the development of *in vitro* repeat-dose toxicity tests are summarised in this section. The main recommendations of the workshop participants are included in the recommendations given below.

1. One of the problems with *in vitro* toxicity testing is the lack of a precise definition of “long-term”, as compared with *in vivo* approaches.

**Table 8.1. An overview of models for chronic toxicity testing**

<b>Models</b>	<b>Advantages</b>	<b>Limitations</b>
<b>Hepatotoxicity</b>		
Isolated perfused liver	The <i>in vitro</i> model closest to the <i>in vivo</i> situation	Short life-time (2–3 hours) Complicated and demanding set-up
Liver slices	Retains the <i>in vivo</i> tissue organisation	Short life-time (7 days)
Isolated hepatocytes	The most frequently used <i>in vitro</i> model for long-term hepatotoxicity testing Can produce a metabolite profile for a drug very similar to that found <i>in vivo</i>	Short life-time (24 hours) Loss of many liver-specific functions Availability of human cells very limited Non-human models are not always predictive of human <i>in vivo</i> situation
Collagen sandwich cultures	Structural and functional integrity retained for up to 15 days Normal cell shape is kept Intact structure, bile canaliculi are preserved ALAT and ASAT enzyme releases can be studied Successfully used in mimicking chronic treatment in the <i>in vivo</i> situation	Loss of several differentiated functions over time
Ito cells and Ito cell/hepatocyte co-cultures	Useful tools for studying liver fibrogenesis Life-time 96 hours	Not a well-established model
Genetically engineered cells expressing single human or animal P450 enzymes	Used as a tool to assess the involvement of certain enzymes in metabolism, metabolite formation, and metabolism-dependent toxicity Well-characterised, well-documented and easy to use	
Cell lines derived from human hepatoma	Expression of CYP1A1	Low expression of other CYPs
HepG2 cells grown under continuous medium supply	Permanent cell lines, well characterised	Laborious systems for routine use Only used in a few pilot studies
<b>Nephrotoxicity</b>		
Renal epithelial cell lines grown under continuous medium supply	Phenotypes with oxidative energy metabolism are available for tubular proximal cells Morphology very close to the <i>in vivo</i> parent cell type Culture periods of up to 6 weeks are possible Human-derived proximal tubular cell lines similar in function to parent cells are available Many functional parameters can be monitored, and a number of endpoints can easily be assessed	Laborious systems for routine use
<b>Neurotoxicity</b>		
Primary neuronal cell cultures (rat) and their reagggregates	Well characterised Identification of neurodegenerative compounds is possible Long-term exposure possible (7–14 days for monolayer; 3 months for reagggregates) Possibility to study oxidative stress and excitotoxicity Used in industry for screening pharmaceuticals and agrochemicals	Donor animals required More intensive than cell lines
Permanent neuronal cell lines	Useful for detection of delayed neurotoxicity caused by organophosphates	Only one model available

ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; CYP = cytochrome P450.

**Table 8.1. continued**

Models	Advantages	Limitations
<b>Neurotoxicity</b> Astrocytes	Culture system well developed Possibility to detect reactive astrocytes (cytokine production) Specific surface markers can be detected by cell imaging and FACS analysis after a toxic insult	Require long-term culture for differentiation
Oligodendrocytes	<i>In vitro</i> models for studying dysmyelination and demyelination are established Specific surface markers can be detected by cell imaging and FACS analysis after a toxic insult	Need long-term maintenance for maturation and/or testing of toxic compounds
Microglia	Rapid response to neuronal injury Cytokine production, morphology, phagocytosis and proliferation are useful endpoints Cell culture models and endpoints measurements are successfully developed Primary glial cell cultures are widely used in academia and in industry	Cultures require 1–2 weeks for the production of well-characterised microglia
Brain slices from hippocampus	Useful for detecting excitotoxic and/or convulsive properties of drugs (hardly detectable in rodents) Well-accepted for studying learning and memory deficits (difficult to detect in rodents) Used in agrochemical testing	Long-term culture conditions are required

FACS = fluorescence-activated cell sorting

“Long-term” in the *in vitro* context should be defined as meaning a minimum of 5 days, but the development of systems that can be used over several months is also necessary and feasible.

2. Relatively stable cultures are a prerequisite for long-term *in vitro* toxicity studies. Currently, systems such as brain-tissue slices, static cell cultures, transformed or immortalised cell lines, and genetically engineered cell lines, can meet this criterion.
3. The long-term maintenance of *in vitro* systems will require the use of new cell culture methods, which allow for the repeated or continuous administration of test compounds.
4. Because of the nature of long-term toxicity testing, and the need to maintain *in vitro* systems with physiological characteristics similar to those in the *in vivo* situation, it should be recognised that *in vitro* systems for chronic toxicity will be more complicated than *in vitro* systems for acute toxicity. This should not preclude the development of long-term *in vitro* systems, and their development should not be focused on their ability to permit high-throughput screening.
5. Attention needs to be paid to the definition, control and monitoring of cell cultures and culture conditions, taking into account organ-specific requirements.
6. Although IC<sub>50</sub> determinations are suitable for acute toxicity, they are not necessarily relevant for long-term studies. It can be assumed that compounds that are toxic over a short period will also be toxic over a long period. Therefore, long-term toxicity testing should be based on the determination of acute NOAELs.
7. For the development of relevant and reliable *in vitro* procedures for long-term testing, it will be necessary to identify and use good reference compounds for specific kinds of tissues and test items.

8. Measurements obtained with *in vitro* models should ideally be validated by comparison with human data. Because it is not usually possible to measure effects *in vivo* (at least not non-invasively, and not for subtle changes), methods for detecting effects in organs should be based on non-invasive imaging techniques, or on measurements in surrogate tissues (such as blood, urine or faeces).

### Repeat-dose Toxicity: Summary, Conclusions and Recommendations

A wide range of endpoints are investigated in *in vivo* chronic toxicity studies, so an integrated approach to chronic toxicity testing, based on the use of alternative methods with complementary endpoints, will need to be developed, if the current reliance on chronic animal tests is to be reduced. At present, a variety of *in vitro* systems, derived mainly from the liver, kidney and brain, are being developed. Considerable investment at the research level is needed to maintain progress in this area.

#### Long-term prospects

The readiness of *in vitro* models for long-term effects to undergo prevalidation and validation will depend on progress made at the research and test development levels, for which recommendations are given below.

#### Recommendations for research and development

1. Initial efforts should focus on identifying compounds with well-established effects, for use as reference standards, and on tackling specific problems relevant to particular kinds of tissues and test items (ideally, based on a knowledge of effects in humans), rather than on trying to replace chronic testing *in vivo* as a whole.
2. Attempts should be made to identify relevant biomarkers of exposure and effect. The endpoints selected should cover general cytotoxic mechanisms and cell type-specific mechanisms of toxicity.
3. Wherever possible, human-based *in vitro* cell systems should be used.
4. Test development should be focused on the use of:
  - a) non-invasive imaging techniques for detecting long-term effects *in vivo*;
  - b) simplified test parameters, such as electrophysiological applications for neurotoxicological long-term models, and the use of specific dyes or other markers, which permit changes in cell function to be monitored;
  - c) perfusion culture systems, with an emphasis on miniaturisation and practicability, to provide effective, technically simple and sensitive systems for assessing the effects of test compounds with biologically relevant endpoints;
  - d) metabolically competent, genetically engineered cell lines, grown on microporous supports and continuously perfused with conventional culture medium, as these systems represent promising models for evaluating the effects of continuous low doses and long-term exposure in the liver, kidney and neuronal tissue;
  - e) methods employing human-based hepatic, renal and neuronal cell lines expressing a wide range of drug- and xenobiotic-metabolising enzymes and transport molecules, as an alternative to primary cultures;
  - f) long-term culture methods (several weeks to months) for hepatic and renal epithelial and endothelial cells, as well as for neurons and glial cells;
  - g) co-culture systems: i) neurons with glial cells; ii) hepatocytes with monocytic, Ito cells and/or endothelial cells; iii) renal epithelial cells with renal microvascular endothelial cells; and iv) renal glomerular mesangial cells with glomerular endothelial and/or glomerular epithelial cells;
  - h) liquid-gas phase organotypic models for culturing lung epithelial cells; and
  - i) systems for predicting toxicotolerance.
5. It is also recommended that the following activities are carried out:
  - a) an assessment of the immunological basis of target organ toxicity; and
  - b) an assessment of the usefulness and application of genomics and proteomics in repeat-dose toxicity testing.

#### Current Status of Nephrotoxicity Testing

The kidney is a highly complex organ, composed of many different types of cell, including epithelial,

endothelial and interstitial cells. Its main function is the regulation of both the volume and the composition of the extracellular fluid by regulating the excretion of solutes and water. The kidneys receive about 20% of the resting cardiac output, they are metabolically active organs, and they reabsorb, metabolise and secrete many compounds. They are therefore highly susceptible to the effects of chemical toxicants. Toxic compounds affect mainly the cells lining the vasculature, and the epithelial cells of the proximal tubules of the nephrons, which represent 60% of the total renal cell mass. Tubular damage and loss of epithelial integrity result in a decrease in the re-absorptive capacity of the kidney (12, 13).

The use of cell culture techniques to study nephrotoxicity *in vitro* has recently gained in importance, because of improvements in methods used for growing homogeneous cultures of renal cells. Several *in vitro* methods are being investigated as models for nephrotoxicity (for example, 14). Table 8.2 provides a summary of the methods available, with their advantages and disadvantages.

Primary cultures of glomerular mesangial and epithelial cells from various sites along the nephron, and permanent renal epithelial cell lines, can be employed. Primary cultures are difficult to obtain, because of the large number of cell types present in the kidney. These cells tend to dedifferentiate within hours, sometimes because of a lack of suitably specific culture media. In addition, there is not enough knowledge with respect to markers that can be used to monitor the maintenance of differentiation *in situ*. Despite all these problems, primary cultures maintain characteristics closer to the *in vivo* situation than do cell lines. Nevertheless, renal epithelial cell lines have proved to be a very powerful tool in studying important aspects of nephrotoxicity *in vitro*. They retain a number of differentiated functions of their *in vivo* ancestor cells, have an unlimited life-span, and do not require time-consuming isolation procedures (15).

A number of *in vitro* endpoints are being investigated. For example, the measurement of barrier function is a new criterion for assessing cell injury in epithelial monolayers that separate fluid compartments (see Chapter 7; and 16, 17). An ECVAM study on the use of trans-epithelial resistance and inulin permeability as nephrotoxicity endpoints in LLC-PK1 and MDCK cells is in progress. The aim of the study is to establish whether trans-epithelial resistance and trans-epithelial marker permeability can be used as general predictors of nephrotoxicity in intact renal epithelia *in vitro*, by investigating the actions of a range of chemicals that have been identified as nephrotoxic *in vivo*.

## Nephrotoxicity: Summary, Conclusions and Recommendations

Renal trans-epithelial resistance and paracellular permeability are reproducible endpoints for assessing barrier function.

### Short-term and medium-term prospects

1. Investigations on the use of renal tissue slices as a model for studying the mechanisms of nephrotoxicity.
2. The immortalisation of primary proximal tubular cell cultures, and further characterisation of the new cell lines, to establish whether they can retain the characteristics of their *in vivo* precursor cells through several passages.
3. The further development of the use of the HK-2 (human proximal tubular epithelial) and LLC-PK1 cells (porcine proximal tubular epithelial) cell lines, grown under long-term conditions, and assessments of their usefulness for transport studies.
4. Investigations into the use of molecular biological techniques to re-express lost functions (for example, specific transporters, enzymes or receptors).

### Long-term prospects

1. The further development of the available renal co-culture systems (such as MDCK cells with metabolically competent cells), and the development of human renal systems, as epithelial and endothelial cells can be successfully grown in combination under static culture conditions and under continuous medium perfusion.
2. An assessment of the extent to which loss of barrier function can account for kidney damage.

## Current Status of Neurotoxicity Testing

Neurotoxicity is a major form of target-organ toxicity that can result in lethality. The current OECD TGs 418 (18), 419 (19) and 424 (20) for assessing the neurotoxic effects of chemicals are based on *in vivo* studies. There are a number of deficiencies in the current whole-animal approach for evaluating the safety of chemicals, including escalating costs, slow throughput of compounds, and an increase in animal usage, as a result of the increasing number of chemicals being developed and commercialised (21, 22). In addi-

**Table 8.2: The current status of models for nephrotoxicity testing**

Models	Advantages	Limitations
<b>Higher order systems</b>		
Rat or rabbit isolated perfused kidney	Tubulovascular integrity is preserved	Not useful for routine studies Renal function is maintained only for a short period
Isolated perfused nephrons or nephron segments	Allows functional characterisation (transport, electrical properties) of nephron segments Useful to localise enzyme systems, metabolic pathways, and receptor distribution	Not suitable for routine <i>in vitro</i> studies Short life-span
<b>In Vitro</b>		
Renal tissue slices	Allows the study of transport and toxicity Good results with human kidney slices Interesting tools to study nephrotoxicity, their mechanisms and prevention	Contain heterogeneous cell population Cells and exposed surfaces damaged by slicing Short life-span
Isolated glomeruli, tubular fragments, and renal cells	Assessment of acute effects of chemicals is possible Retention of <i>in vivo</i> architecture of the epithelium Useful for studying unspecific but sensitive endpoints of toxicity testing Study of nephrotoxins in metabolism and transport is possible	Life-span limited to a few hours
Primary cultures of glomerular and epithelial cells	Close to <i>in vivo</i> situation Use of microporous supports to grow the cells improves differentiated state during prolonged culture times	Limited life-span Isolation procedure is difficult Rat proximal tubules are very difficult to grow
<b>In vitro permanent cell lines</b>		
LLC-PK1 (proximal tubule)	Unlimited life-span Possibility of long-term exposure under controlled conditions	Dedifferentiation occurs in culture Possible transdifferentiation occurs
OK (proximal nephron)	Rapid isolation is possible	
JTC-12 (proximal tubule)	Appropriate culture conditions available for continuous culture medium perfusion, well-defined extracellular matrix, and new technologies can be used to induce re-expression of lost functions	
MDCK (collecting duct)		
A6 (distal tubule/collecting duct)	Gluconeogenic strains of OK and LLC-PK1 cells have been obtained	

MDCK = Madin–Darby canine kidney

tion, the tests incorporated into the current guidelines do not always generate the mechanistic data required for a scientifically based human risk assessment (21). Therefore, the need to develop mechanistically relevant alternatives to conventional animal toxicity testing is widely recognised (21–23). The best way forward will be to design and assess the use of tiered strategies based on batteries of tests that encompass the most important neurotoxic endpoints. To date, no single *in vitro* method for neurotoxicity has been validated, and no battery or testing strategy has been independently evaluated. A major challenge is to identify a short-list of appropriate neurospecific endpoints to be incorporated into a testing strategy.

### Testing Strategies for *In Vitro* Neurotoxicity

A tiered approach is widely considered to give an optimum balance between the accurate detection of possible neurotoxins and ease, speed and the efficient use of resources (21). Although consensus has not been reached on the specific composition and design of an optimal testing strategy, a commonly recommended approach is to employ at least two tiers of tests. Typically, a first tier should enable specific neurotoxicants to be distinguished from general cytotoxicants, by using combinations of non-specific and neurospecific

endpoints in neural and non-neural cell lines. A second tier should enable different classes of neurotoxicants to be distinguished, and should address more-specific mechanistic questions. The second tier is therefore likely to involve the use of specific neural endpoints, such as the activities of acetylcholinesterase (AChE) and other enzymes involved in neural function. A number of testing strategies have been proposed (21, 22, 24–28). Many of these schemes are complex, but they make useful suggestions for future development.

To provide a means of linking the external dose of a chemical to the target-organ concentrations that produce toxicity, a generic testing strategy for neurotoxicity has been based on the integrated use of biokinetic and biodynamic parameters (29). The proposed scheme is based on a measurement of basal cytotoxicity, combined with the use of biokinetic models to predict the dose of a chemical which could result in sufficiently high concentrations to cause non-specific effects in, for example, the central nervous system (CNS) or peripheral nervous system (PNS). In addition, it is suggested that the chemical should be tested by using a battery of neuron-specific *in vitro* tests. If a high ratio of general cytotoxicity to specific cytotoxicity is observed in these *in vitro* systems, and if biokinetic considerations indicate an accumulation of the chemical at the target site in the nervous system, the chemical of concern could be regarded as an acute or chronic neurotoxicant.

### Tests for general neurotoxicity

Tests used for screening purposes should be simple and designed to detect a broad array of chemical insults to the nervous system. They should be able to detect compounds that may be toxic either to the adult or to the developing CNS and PNS.

Although many different approaches have been reported (for example, 21, 30, 31), the measurement of cytotoxic endpoints in human cell lines, such as inhibition of neutral red uptake (NRU) and MTT reduction, is generally proposed. Some preliminary feasibility studies have already been carried out. For example, Williams *et al.* (27, 32) studied a total of 43 chemicals, by using a battery of neuroblastomas, primary neural and astrocyte cultures, and fibroblasts as non-neural cells. The endpoints analysed were MTT reduction, NRU and lactate dehydrogenase (LDH) release. The results indicated that a battery composed of human IMR-32 neuroblastoma cells, fibroblast cultures and primary astrocytes did not permit discrimination between neurotoxicants, gliotoxins, and general cytotoxicants. However, the use of human IMR-32 neuroblastoma cells, with NRU as an endpoint, emerged as a suitable model

for inclusion in a more-comprehensive first-tier screen for neurotoxicants.

Weiss & Sawyer (33) tested 50 MEIC reference chemicals in primary cultures of chick embryo fore-brain neurons, by using the MTT and NRU assays. The NRU assay again appeared to be more sensitive to chemical toxicity; however, both assays were equally predictive when the data they provided were compared with *in vivo* toxicity data obtained from the Registry of Toxic Effects of Chemical Substances (RTECS).

Finally, Xie & Harvey (34), tested the first nine MEIC chemicals in the NG108-15 neuroblastoma cell line, by using the MTT assay to measure cell viability, and by monitoring changes in cell resting membrane potential (RMP) as a neurospecific marker. The authors concluded that the MTT assay could be a better approach than RMP measurement for detecting neurotoxic effects *in vitro*, because even simple electrophysiological techniques are not suitable for rapid screening, and because there was a strong correlation between the rank order of potencies of the chemicals in the MTT and RMP assays (28).

### Tests for mechanism-specific neurotoxicity

Mechanism-specific neurotoxicity tests are useful when general *in vitro* screening tests are not capable of detecting the effects of specific chemical classes (23). In a series of papers by Veronesi and co-workers (for a review, see 28), the use of neuroblastoma cells as effective test models for organophosphate neuropathy was demonstrated. It was found that the human SH-SY5Y cell line could identify active esterase inhibitors among the organophosphates tested, and could also distinguish between organophosphates that caused delayed neuropathy and those that did not. The authors therefore concluded that there was a distinct possibility that a human cell line could be used for the screening of organophosphates, and that neuropathy target esterase (NTE) inhibition could be identified without using laboratory animals.

In another study, Henschler *et al.* (35) were able to establish an almost perfect correlation between *in vitro* and *in vivo* data for a series of organophosphorus compounds. The *in vivo* data were obtained with the standard *in vivo* hen test, whereas the *in vitro* data were obtained by observing and quantifying the development of neurite-like processes in neuroblastoma (N-18) and glioma (C6) cell lines.

Forsby *et al.* (36) analysed specific neurotoxicity on synaptic events, by using  $\text{Ca}^{2+}$  homeostasis as an endpoint with human neuroblastoma cells (SH-SY5Y). In their recent review, Balls & Walum (28) concluded that although perturbation of calcium

homeostasis may be a common denominator in many neurotoxic conditions, the determination of intracellular calcium concentrations in resting and activated cells is not sufficient for the identification of a broad range of neurotoxic compounds. *In vitro* neurotoxicity tests for assessing synaptic activity should therefore be based on, for example, determination of the activities of ligand-operated ion channels, adenylate cyclase-coupled receptors, and neurotransmitter transporters, and on measurements of transmitter release, oxidative stress and stress gene activation.

Among the various *in vitro* models proposed as second-tier tests for the assessment of neurotoxicity (Table 8.3), reaggregating brain cell cultures appear to be among the most promising models. These systems consist of three-dimensional, integrated populations of neurons and glial cells, derived from fetal rat telencephalons. The advantages of the three-dimensional models for neurotoxicity testing are: a) they are robust; b) they can be used in both acute and chronic exposure studies; c) they permit testing in both immature and differentiated cells; d) they reproduce most of the cell–cell interactions that occur *in vivo*, including morphogenetic steps involving proliferation, synaptogenesis and myelination; and e) they can be used for studying a wide range of specific neurotoxic endpoints, including both microglial and astroglial activation, which are early markers of neurotoxicity (37, 38). Reproducible data show that adverse effects observed in aggregating brain-cell cultures occur at similar concentrations to those observed *in vivo* (39–41).

One drawback of re-aggregating brain cell cultures is the need to work with primary cells. However, it appears that logistical difficulties associated with the availability and distribution of reaggregating cultures can be overcome, since preliminary results on the possible cryopreservation of the aggregate cultures are very promising. Other limitations are the incomplete reconstitution of the *in vivo* cytoarchitecture, and the need for specialised equipment.

In the long term, human embryonic cell lines are likely to be promising *in vitro* models for a wide range of purposes. Such systems are advantageous, in that they avoid the difficulty of extrapolating between species, and they also avoid the use of animals. A human embryonic cell line has been shown to be capable of differentiation, under controlled conditions, into the various classes of neural cells, neurons, astrocytes, oligodendrocytes and microglia (42). Such systems may therefore reach a level of biological complexity comparable to aggregate cultures. However, they need to be further developed and assessed for their relevance to the *in vivo* situation.

Another model that holds promise for the detection of mechanistic pathways of neurotoxicity is

the genetically modified pheochromocytoma 12 (PC12) cell line (43). Two classes of genetically modified PC12 cell lines have been patented by ECVAM, which differ in terms of their sensitivity to P53-mediated and Bcl2-mediated cell death (44). The usefulness of these cells as a part of a testing strategy for neurotoxicity is now being assessed by ECVAM.

## Neurotoxicity: Summary, Conclusions and Recommendations

Many *in vitro* systems are being developed to investigate the wide variety of endpoints associated with neurotoxicity. This is an area where a reduction in animal use is likely to result from the integrated use of alternative tests with complementary endpoints. An important question in relation to the EU Chemicals Policy is the extent to which all mechanisms need to be assessed, since, from a strategic viewpoint, it is possible that an assessment of barrier function (see Chapter 7, section on the BBB), combined with assessments of basal cytotoxicity (Chapter 2) and energy metabolism, might be sufficient to identify substances of concern.

### Short-term prospects

1. The prevalidation of the complementary use of re-aggregating brain cell cultures and the SH-SY5Y human neuroblastoma cell line.
2. The prevalidation of the complementary use of primary glial and neuronal cell cultures for assessing neurotoxicity.
3. An evaluation of the usefulness of genetically modified PC12 cell lines.

### Recommendations for research and development

1. The development and evaluation of a tiered testing strategy for neurotoxicity, taking into account the results obtained in previous studies (21, 22, 25, 27, 28, 32, 33).
2. The further development of *in vitro* models for evaluating the mechanisms of neurotoxicity, such as genetically engineered cell lines, and re-aggregating cultures of human embryonic stem cell lines.
3. The evaluation of genomics, proteomics and new electrophysiological and biochemical profiling methods for neurotoxicity testing.

**Table 8.3: An overview of alternative test batteries proposed for neurotoxicity testing**

Strategy	Test systems	Endpoints
Balls & Walum (28)	<i>First tier</i> Structure-activity relationships Pharmacokinetic models	
	<i>Second tier</i> Human cell lines	<i>Second tier</i> Cell viability tests (necrosis and apoptosis)
	<i>Third tier</i> Human cell lines	<i>Third tier</i> Class-specific (for example, organophosphate) toxicity Axonal degeneration Cell signalling alterations Neurosecretory function Markers of neuroimmunotoxicity
	<i>Fourth tier</i> CNS and PNS models of animal origin, including: brain spheroids, dorsal root ganglia, explants, dissociated neuronal and glial primary cultures, and microglial/mast cells	<i>Fourth tier</i> Glial fibrillary acidic protein (GFAP) Myelin basic protein (MBP) Neurofilament protein (NFP) Markers of excitotoxicity Markers of oxidative stress Morphological and neurochemical markers of synaptic damage Markers of neuroimmunotoxicity
Atterwill et al. (21)	<i>First tier</i> Primary culture enriched in neurons Primary culture enriched in astrocytes Primary culture of dorsal root ganglia (for PNS toxicity) Clonal cell lines of neural origin	<i>First tier</i> MTT reduction NRU Fluorescein diacetate hydrolysis Cell-specific markers such as GFAP, NFP Cell growth, proliferation, motility, adhesion and process formation
	<i>Second tier</i> Whole-brain reaggregate cultures Clonal cell lines of neural origin	<i>Second tier</i> GFAP NFP MBP Malondialdehyde assay Calcium levels
	<i>Third tier</i> New models	<i>Third tier</i> New endpoints
Williams et al. (32)	<i>First tier</i> Human neuroblastoma (IMR-32 or SHSY-5Y) Pure rat astrocyte cultures or glioma cells Fibroblasts (i.e. non-neural cells)	<i>First tier</i> NRU MTT reduction Trypan blue exclusion Total protein content AChE activity NTE activity
	<i>Second tier</i> Rat embryo whole-brain reaggregate cultures	<i>Second tier</i> Diameter Total protein content LDH release Neuron-specific enolase GFAP Neurotransmitter markers

AChE = acetylcholinesterase; CNS = central nervous system; GABA =  $\gamma$ aminobutyric acid; LDH = lactate dehydrogenase; NTE = neuropathy target esterase; NRU = neutral red uptake; PNS = peripheral nervous system.

**Table 8.3: An overview of alternative test batteries proposed for neurotoxicity testing**

Strategy	Test systems	Endpoints
	<i>Third tier</i> Pure neurons Pure astrocytes Human cell model	<i>Third tier</i> Cytotoxicity 2-Deoxyglucose uptake/release GFAP/NFP
Costa (22)	Neuronal and glial cell lines A non-neuronal cell line Rat primary micromass or reaggregate cultures	<i>Cytotoxic endpoints</i> Cell death Membrane permeability Mitochondrial function Cell growth and division Energy regulation Synthesis of macromolecules  <i>Neurospecific endpoints</i> Neurotransmitter-synthesising enzymes (glutamic acid decarboxylate, dopamine hydroxylase, choline acetyltransferase, tyrosine hydroxylase) Acetylcholinesterase Neuron-specific enolase Glutamine synthetase Neuronal receptors Neurite extension
Abdulla & Campbell (26)	<i>First tier</i> Cell lines (neuroblastomas/gliomas) Primary monolayer cultures (for example, chick DRG) Rat or chick midbrain micromass cultures Microglia PC12 cells  <i>Second tier</i> Reaggregate culture (for example, chick embryo) Retinal cultures Organotypic culture/explant culture Primary cultures of individual neural cell types	<i>First tier</i> Membrane integrity (for example, LDH release) Mitochondrial enzyme function Differentiation effects CR3 expression (microglia to macrophages) Neurite outgrowth Gene expression (for example, <i>c-fos/c-jun</i> )  <i>Second tier</i> Aggregation (cell–cell interactions, gap junction formation) Growth Differentiation Neurochemistry Neurotransmitter synthesis (for example, acetylcholine) Receptor expression (for example, GABA receptors) Electrophysiology on <i>ex vivo</i> insect neurons
Veronesi (24)	Neuroblastoma cell lines (human SY5Y and murine NIE 115) MCL-5 and AHH-1 TK+/- lymphoblastoids Neuroblastoma cells co-cultured with muscle cells	<i>Neurotoxic endpoints</i> Acetylcholinesterase activity NTE activity Histochemistry Receptor status Neurite formation  <i>Cytotoxic endpoints</i> Organelle viability Intercellular communication LDH release

*AChE* = acetylcholinesterase; *CNS* = central nervous system; *GABA* =  $\gamma$ aminobutyric acid; *LDH* = lactate dehydrogenase; *NTE* = neuropathy target esterase; *NRU* = neutral red uptake; *PNS* = peripheral nervous system.

## References

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